

# Evolution in Action: Plants Resistant to Herbicides

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## Key Words

herbicide resistance, resistance mechanism, resistance mutation, cytochrome P450, herbicide translocation

## Abstract

Modern herbicides make major contributions to global food production by easily removing weeds and substituting for destructive soil cultivation. However, persistent herbicide selection of huge weed numbers across vast areas can result in the rapid evolution of herbicide resistance. Herbicides target specific enzymes, and mutations are selected that confer resistance-endowing amino acid substitutions, decreasing herbicide binding. Where herbicides bind within an enzyme catalytic site very few mutations give resistance while conserving enzyme functionality. Where herbicides bind away from a catalytic site many resistance-endowing mutations may evolve. Increasingly, resistance evolves due to mechanisms limiting herbicide reaching target sites. Especially threatening are herbicide-degrading cytochrome P450 enzymes able to detoxify existing, new, and even herbicides yet to be discovered. Global weed species are accumulating resistance mechanisms, displaying multiple resistance across many herbicides and posing a great challenge to herbicide sustainability in world agriculture. Fascinating genetic issues associated with resistance evolution remain to be investigated, especially the possibility of herbicide stress unleashing epigenetic gene expression. Understanding resistance and building sustainable solutions to herbicide resistance evolution are necessary and worthy challenges.

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## INTRODUCTION

For at least 10,000 years a critical human endeavor has been the cultivation of plants for food and fiber, and now, the world's great crops sustain more than 6 billion people. Any threats to crop productivity have grave consequences. Every year a major threat comes from infestations of wild plant species (weeds). Since

the dawn of agriculture, humans have battled to control weeds threatening crop survival and productivity. Over the past 40 years, modern herbicides have largely replaced human, animal, and mechanical weed control, and they make a significant contribution to the high productivity of global agriculture. Despite their success, herbicides have not resulted in the extinction of weeds, just as insecticides have not removed pests nor have antibiotics eliminated human disease pathogens. Indeed, the weed challenge in world crops remains more or less stable. Evolutionary forces acting on genetic diversity in large populations explain how biological organisms survive catastrophic natural events. Commencing with the brilliant insights of the nineteenth-century natural scientists Darwin, Lamarck, Mendel, Wallace, and those following them, there has developed an understanding that natural selection acting on genetic diversity enables persistence of life under changing circumstances. While many environmental changes are long-term, there are also sudden, catastrophic events that cause high mortality (e.g., herbicides applied to huge weed populations). For targeted plants, herbicides are a rapid, extreme stress event, but there are some initially very rare individuals with genes enabling survival and reproduction, and where herbicide selection is relentless, resistance evolves.

Herbicide resistance is an evolutionary process, and its dynamics and impact are dependent upon the factors summarized in **Table 1** (scope and space constraints mean that here we focus only on the more fundamental aspects of evolved resistance mechanisms/genes). There can be a diversity of resistance genes and specific herbicidal, operational, and biological factors can determine which resistance genes are enriched (**Table 1**). A key point is that pollen exchange means cross-pollinated plants can rapidly share and accumulate resistance genes. Herbicide properties and dose strongly influence the types of resistance genes that can be enriched (**Table 1**). For reviews of genetic and other factors influencing herbicide resistance evolution, see References 18, 25, 52, 73 and 150.

**Table 1** Factors influencing herbicide resistance evolution in weed populations

**Genetic**

1. Frequency of resistance genes
2. Number of resistance genes
3. Dominance of resistance genes
4. Fitness cost of resistance genes

**Biology of weed species**

1. Cross-pollination versus self-pollination
2. Seed production capacity
3. Seed longevity in soil seedbank
4. Seed/pollen movement capacity

**Herbicide**

1. Chemical structure
2. Site of action
3. Residual activity

**Operational**

1. Herbicide dose
2. Skills of the operator (treatment machinery, timing, environmental conditions, etc.)
3. Agro-ecosystem factors (nonherbicide weed control practices, crop rotation, agronomy, etc.)

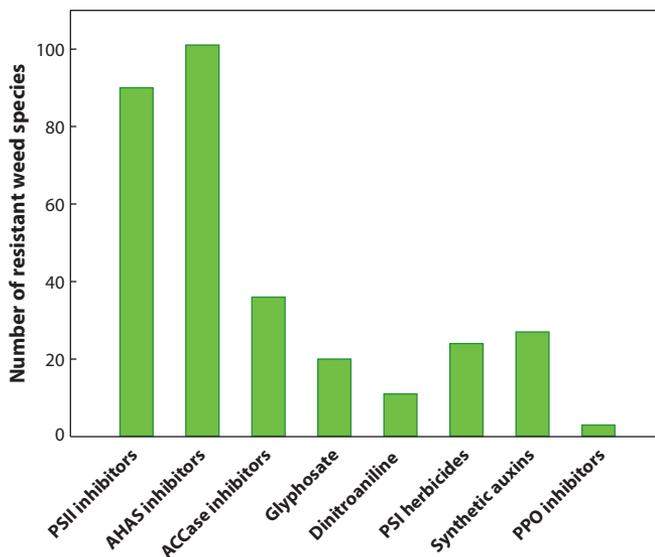
A global list of herbicide-resistant weeds is comprehensively collated (67) at the web-site <http://www.weedscience.org> (Figure 1). Herbicide resistance in general is reviewed in several books (15, 35, 54, 83, 131, 134).

Effective herbicides have chemical properties enabling them to enter the plant, be translocated, and reach their target site at a lethal dose. The great majority of herbicides inhibit specific plant enzymes (target site) that are essential in plant metabolism. In reviewing evolved herbicide resistance we consider target-site versus non-target-site resistance. Evolved target-site resistance exists when herbicide(s) reach the target site at a lethal dose but there are changes at the target site that limit herbicide impact (see section below on Target-Site Herbicide Resistance). Evolved non-target-site resistance involves mechanisms that minimize the amount of active herbicide reaching the target site (see section below on Non-Target-Site Herbicide Resistance).

**TARGET-SITE HERBICIDE RESISTANCE**

Evolved target-site resistance can occur by gene mutation conferring amino acid change

in a target enzyme that prevents herbicide binding. Alternatively, target-site resistance can be conferred by overexpression of a target enzyme (gene amplification or changes in a gene promoter). If a resistance-endowing mutation impairs enzyme functionality and/or plant



**Figure 1**

Number of weed species that have evolved resistance to major herbicide modes of action (to July 2009, data from Reference 67).

performance a resistance fitness cost may result (173).

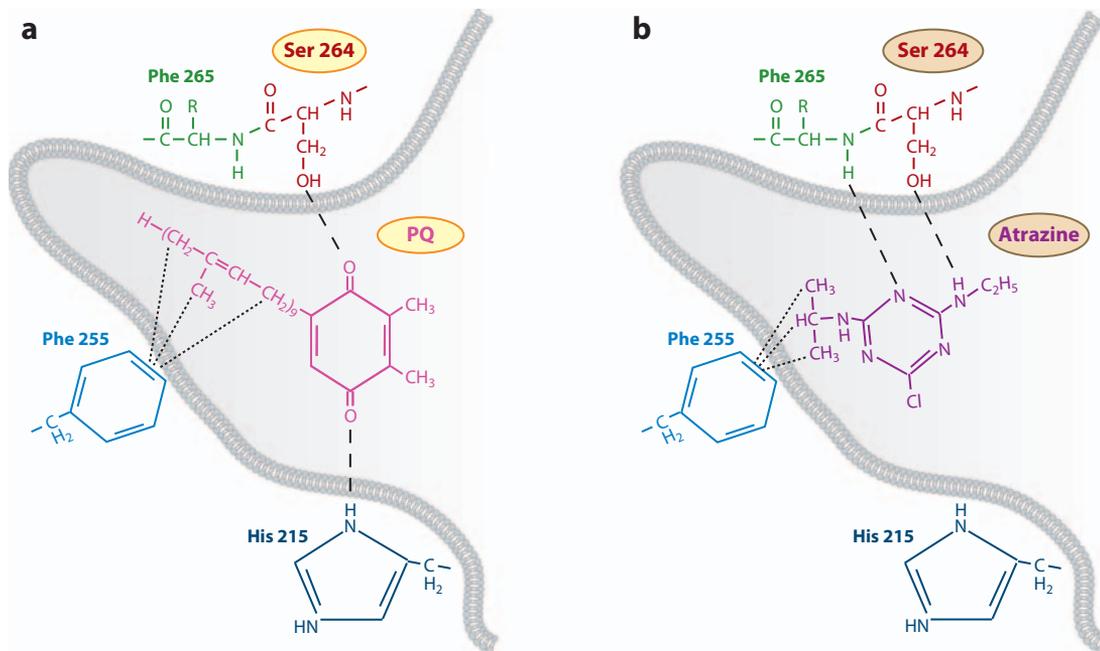
### Resistance to PSII-Inhibiting Triazine Herbicides: One *psbA* Gene Mutation Independently Evolving Worldwide

Photosynthesis involves biophysical capture and transduction of sunlight energy to drive electron transport to produce NADPH and ATP for the carbon reduction cycle. Many herbicides across several structurally diverse chemical groups (e.g., triazines, triazinones, ureas, uracils, biscarbamates) inhibit photosynthesis through the same mechanism of action. They compete with plastoquinone (PQ) at the PQ binding site on the D1 protein within the photosystem two (PSII) complex. PSII electron transport inhibition stops NADPH and ATP production and the carbon reduction cycle, leading to carbohydrate starvation and oxidative stress. From the 1950s onwards, the triazine herbicides became widely adopted in the maize-growing regions of the world and their persistent use on huge, genetically diverse weed populations has led to resistance evolution. Since the landmark first publication (151), triazine resistance has globally evolved in 68 weed species (**Figure 1**) (67). Arntzen et al. (5) and Gronwald (57) have expertly reviewed the literature on PSII triazine resistance. The striking feature of PSII triazine target-site resistance is that a single resistance mutation has independently globally evolved. A point mutation in the maternally inherited chloroplastic *psbA* gene encoding the D1 protein causes a Ser-264-Gly amino acid substitution in the PQ binding site (53, 68). With a few exceptions (see section on Non-Target-Site Herbicide Resistance), virtually all evolved triazine-resistant weed species have this mutation.

**Molecular interactions between the PSII D1 protein and triazine herbicides.** Building on the knowledge that triazines compete with PQ at the PQ binding site and the Nobel prize-winning achievement of the crystal structure of the PSII-like reaction center of

photosynthetic purple bacteria (104), there is an exquisite understanding of how PQ and triazines bind to the D1 protein (**Figure 2**) and thus how the Ser-264-Gly mutation confers triazine resistance. As triazines and PQ directly compete for the PQ binding site, Ser-264-Gly is one of very few options for amino acid changes that prevent triazine binding while still enabling PQ binding. Molecular structure and modeling of PSII show that at the D1 protein PQ binding site, Ser-264 provides a hydrogen bond that is important for PQ or triazine binding (**Figure 2**). Substitution with glycine removes this hydrogen bond, preventing triazine binding (see References 79, 113, 138 for reviews). However, while providing high-level triazine resistance, this mutation also compromises PQ binding and therefore comes at the cost of reduced photosynthesis (see Reference 57 for a review).

**Other resistance-endowing *psbA* gene mutations.** The Ser-264-Gly mutation prevents triazine binding but there is normal activity of nontriazine PSII herbicides that have different chemistry and binding (see References 113, 138 for reviews). Therefore, target-site resistance to nontriazine PSII herbicides requires different mutations, and indeed five such mutations have been reported in a small number of weed species. A weed biotype selected by both triazine and urea PSII-inhibiting herbicides has a Ser-264-Thr mutation in the D1 protein conferring resistance to both herbicide chemistries (96). This mutation either partially blocks triazine and urea herbicide entry into the PQ binding site or interferes with herbicide interaction with Phe-255. From selection with nontriazine PSII herbicides, a Val-219-Ile mutation has evolved in two weed species (102, 103), and an Asn-266-Thr (120), an Ala-251-Val (100) and a Phe-255-Ile (125) mutation has evolved separately in three weed species. These mutations confer resistance to certain nontriazine PSII herbicides for which the widely evolved Ser-264-Gly mutation does not confer resistance.



**Figure 2**

The interaction of plastoquinone (PQ) and atrazine with amino acids within the PQ binding site of the PSII D1 protein (modified from Reference 49). (a) PQ at the PQ binding site is hydrogen-bonded with His-215 and Ser-264. (b) Atrazine at the PQ binding site is hydrogen-bonded with Phe-265 and Ser-264 (this prevents PQ binding). Substitution of the Ser-264 with Gly removes the hydrogen bond, preventing atrazine binding. PQ binding affinity is also reduced, but the protein is still functional. Dashed lines indicate hydrogen bonds and dotted lines indicate hydrophobic interactions.

***psbA* gene mutations: effect on PSII functionality and plant fitness.** Many studies demonstrate that the Ser-264-Gly mutation significantly reduces plant fitness. This mutation reduces PQ binding (**Figure 2**) and therefore photosynthesis, explaining in part the fitness cost. However, there are other adverse pleiotropic effects of this mutation, dependent on environmental conditions (especially temperature and light). The wealth of literature showing a fitness cost for the Ser-264-Gly mutation has been extensively reviewed (57, 70, 173) and needs no further consideration here. Little is known about the fitness cost of other PSII herbicide resistance mutations of the *psbA* gene, although limited studies indicate that the Ser-264-Thr or Asn-266-Thr mutations also levy a fitness cost (see Reference 173 for a review). In conclusion, it is clear that target-site PSII triazine herbicide resistance is nearly

always due to the *psbA* Ser-264-Gly mutation. The fitness cost associated with this mutation has reduced its adverse impact on world agriculture. The independent global evolution of the Ser-264-Gly mutation most likely reflects the overwhelming use of triazine compared to other PSII herbicides. A note of caution is that as the Ser-264-Gly mutation evolved early and often, researchers have examined for and found this mutation and have not then searched for other *psbA* gene mutations (165) or the coexistence of other resistance mechanisms such as non-target-site resistance. Other *psbA* mutations have evolved and, as well, non-target-site resistance to PSII herbicides has evolved by two different metabolism-based enzyme systems (see section on Non-Target-Site Herbicide Resistance). We emphasize that herbicide selection of billions of genetically diverse plants means that resistant weed populations

are likely to reflect diversity in resistance genes and researchers should examine for all possible resistance mechanisms, both target-site and non-target-site.

### Resistance to AHAS-Inhibiting Herbicides: Many Resistance Mutations

Acetohydroxyacid synthase (AHAS, EC2.2.1.6), also referred to as acetolactate synthase (ALS), is the first enzyme in the biosynthetic pathway for the branched-chain amino acids valine, leucine, and isoleucine. AHAS catalyzes the formation of both aceto-hydroxybutyrate and acetolactate and is the target site for a large number of herbicides across the dissimilar sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine, pyrimidinyl-thiobenzoates, and sulfonyl-aminocarbonyl-triazolinone herbicide chemistries. These herbicides are all potent inhibitors of AHAS, thereby stopping synthesis of the branched-chain amino acids, with subsequent plant death. AHAS-inhibiting herbicides control many weed species, have low mammalian toxicity, and are selective in major world crops. These favorable qualities ensured their global, intensive use in many different crops over huge areas. The evolution of herbicide-resistant weeds (101 species to date) rapidly followed (**Figure 1**) (67). The extensive AHAS-inhibiting herbicide resistance literature has been thoroughly reviewed (153, 168), so here we focus on recent developments.

It was quickly established that AHAS herbicide-resistant plants could have a mutant-resistant AHAS enzyme (92, 152), and reports of resistant AHAS in many weeds followed. When reviewed in 1994 (153), it was known that AHAS Pro-197 could be substituted with either His or Thr to encode a resistant AHAS (59). Since 1994, an amazing number of resistance-endowing mutations have been identified. When reviewed in 2002, 13 resistance amino acid substitutions at five sites within AHAS had been identified in weeds (168). Now, in 2009, there are 22 resistance substitutions at

seven sites across AHAS (**Table 2**) (167, 168). Remarkably, at Pro-197, 11 amino acid substitutions can endow AHAS herbicide resistance. Pro-197 mutations are by far the most often observed (**Table 2**). At Pro-197, substitution with Ser is a particularly common mutation, relative to the many other possible Pro-197 resistance-endowing substitutions. As discussed below, the Pro-197-Ser substitution most likely achieves AHAS herbicide resistance without any major adverse impact on AHAS functionality. Also, the Pro-197-Ser substitution requires only one nucleotide mutation, whereas some of the Pro-197 amino acid substitutions (e.g., Ile, Lys, Met, Trp) require two nucleotide changes and thus will be slower to evolve. Especially in cross-pollinated species, several AHAS mutations can be present in an individual plant (e.g., 123, 180, 192, 196).

### Molecular interactions between AHAS and herbicides.

AHAS has both a catalytic and a regulatory subunit. The regulatory subunit stimulates activity of the catalytic subunit and confers sensitivity to feedback inhibition by branched-chain amino acids (see Reference 40 for a review). Major advances have been made in the elucidation of the crystal structure of the yeast (98, 117, 118) and then the plant (*Arabidopsis thaliana*) catalytic subunit (99) in complex with various AHAS herbicides. These achievements enable precise identification of AHAS herbicide binding sites and provide an exquisite understanding of the detailed molecular interactions between AHAS, cofactors, and herbicides (40, 97). This work has revealed that the AHAS catalytic site is deep within a channel and that, crucially, AHAS herbicides do not bind within the catalytic site. Rather, they bind across an herbicide binding domain that straddles the channel entry, thereby blocking substrate access to the catalytic site (**Figure 3**). Across this domain, 18 amino acid residues are involved in herbicide binding (99). Structurally different AHAS herbicides orientate differently in the herbicide binding domain, with partial overlap (**Figure 3**). Thus, a particular amino acid substitution within the herbicide binding

**Table 2 Resistance-endowing acetohydroxyacid synthase amino acid substitutions in field-evolved resistant weed species**

Amino acid and position <sup>a</sup>	Resistance substitution	Resistance spectrum <sup>b</sup>		Number of species in which mutation detected	References <sup>c</sup>
		SU	IMI		
Ala-122	Thr	S	R	5	S. Friesen & S. Powles, unpublished data
	Tyr	r	R	1	
Pro-197	His	R	S/r	4	180 180 180
	Thr	R	S/r	6	
	Arg	R	S	3	
	Leu	R	R/r/S	8	
	Gln	R	S	4	
	Ser	R	S	14	
	Ala	R	S	6	
	Ile	R	r	1	
	Met	R	–	1	
	Lys	R	–	1	
	Trp	R	–	1	
Ala-205	Val	r/S	R/r	4	
Asp-376	Glu	R/r	R	4	Also see 71
Trp-574	Leu	R	R	16	180
	Arg <sup>d</sup>	R	R	1	
Ser-653	Thr	S/r	R	3	80
	Asn	S/r	R	2	
	Ile	r	R	1	
Gly-654	Glu	–	R	1	154
	Asp	S	R	1	80

<sup>a</sup>Amino acid numbering refers to the *A. thaliana* acetohydroxyacid synthase (AHAS) gene.

<sup>b</sup>SU: sulfonylurea; IMI: imidazolinone; S: susceptible; R: resistant; r: low to moderately resistant; dash: not determined. For resistance spectrum to other AHAS herbicide chemistries, see Reference 167.

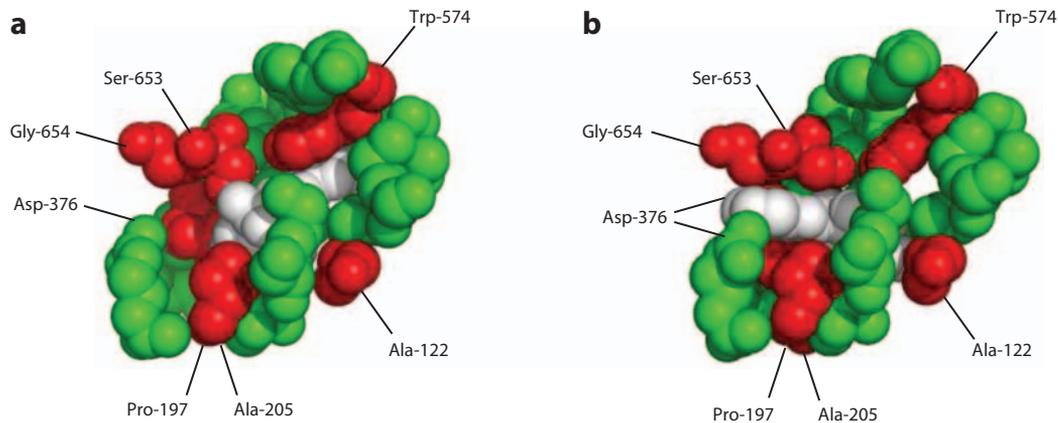
<sup>c</sup>Unless otherwise specified, references and data are from Reference 167.

<sup>d</sup>Only heterozygous resistant individuals were found.

domain can confer resistance to some but not to other AHAS herbicides (Table 2). There is a broad spectrum of resistance conferred by the Trp-574-Leu mutation (Table 2) (167) because Trp-574 is important not only for defining the shape of the active-site channel but also for anchoring both SU and IMI herbicides to AHAS (99). This molecular and structural knowledge of the AHAS herbicide binding domain, the effect of resistance mutations, and the fact of the separate catalytic site provide the explanation for the many AHAS resistance mutations that have evolved (Table 2). As the AHAS herbicides bind away from the catalytic site, there can be many mutations that prevent herbicide binding without adversely affecting the catalytic

site (Figure 3). This also helps explain why there is a high frequency of AHAS-resistant plants present in a susceptible population before AHAS herbicide selection (139).

**AHAS gene mutations: effect on AHAS functionality and plant fitness.** Of the many AHAS resistance mutations that have evolved in weeds worldwide (Table 2) only a few have been properly investigated for fitness cost. Early work indicated that some resistance mutations showed no or negligible fitness cost (see References 70, 168 for reviews), whereas the Trp-574-Leu substitution can have a substantial fitness cost (164). Vila-Aiub et al. (173) have recently reviewed AHAS herbicide resistance



**Figure 3**

Simulation model of *Arabidopsis* AHAS structure in complex with the SU herbicide chlorsulfuron (*a*) or the IMI herbicide imazaquin (*b*). The herbicides are colored white; the residues that have evolved resistance substitutions are colored red. Note that the SU herbicide is bound deeper and closer to and has more contact with the catalytic site than does the IMI herbicide. The perspective of the images is that the atoms of the herbicides at the bottom left are those that are at the entrance of the channel, and those at the top right are inside the channel, leading to the catalytic site. (S. Friesen & S. Powles, unpublished data.)

and fitness costs, so here we focus on the effect of resistance mutations on AHAS functionality. As AHAS herbicides do not resemble the normal AHAS substrate and bind at an herbicide binding domain separate from the catalytic site, it is likely that some resistance mutations have a negligible effect on AHAS functionality, whereas others will alter AHAS functionality and/or have other (adverse) pleiotropic effects on the plant. Therefore, unsurprisingly, studies with particular resistant biotypes show reduced (6, 42, 43), increased (13, 194, 196), or unchanged (13, 142) AHAS activity. In general, it has been found that resistance mutations do not drastically change AHAS substrate affinity ( $K_m$ ), but rather that they change AHAS sensitivity to branched-chain amino acid feedback inhibition, resulting in accumulation of these amino acids (e.g., 6, 42, 43, 142). To know the precise effect of each of the 22 resistance mutations (**Table 2**), studies need to be conducted with known genotypes for each of these mutations. Recently, we have generated purified homozygous *Lolium rigidum* plants for each of the Pro-197-Ala, Pro-197-Arg, Pro-197-Gln, Pro-197-Ser, and Trp-574-Leu mutations, and

determined AHAS kinetics for each of these individual mutations. We have found that the very common Pro-197-Ser mutation (**Table 2**) has no effect on AHAS kinetics, relative to wild-type AHAS or some of the other amino acid substitutions at Pro-197 (Q. Yu, H. Han, S. Powles, manuscript in preparation). This helps to explain why this mutation is so common. We believe that when this work is completed for a range of AHAS resistance mutations, it will reveal that some mutations have no adverse effects on AHAS functionality, while other mutations have clear adverse impacts. It remains to be established whether and to what extent the effect of these AHAS mutations impacts plant fitness. In conclusion, there is now good understanding of how plants so easily evolve target site AHAS herbicide resistance. Elegant molecular structural and modeling work reveals that AHAS herbicide binding and catalytic sites are spatially separate, and many resistance mutations have a negligible impact on AHAS functionality. This explains how there can be so many AHAS resistance gene mutations. Note, however, that resistance evolution to AHAS herbicides is not restricted to AHAS gene mutations.

Non-target-site, metabolism-based resistance to AHAS herbicides is also a powerful and widely occurring resistance mechanism (see section below on Cytochrome P450 Monooxygenases and Evolved Herbicide Resistance).

### Resistance to ACCase-Inhibiting Herbicides: Eight Resistance Mutations

Acetyl-coenzyme A carboxylase (ACCase, EC.6.4.1.2) is a key enzyme in lipid biosynthesis that catalyzes the formation of malonyl-CoA from the carboxylation of acetyl-CoA. Two types of ACCase have been recognized: The heteromeric prokaryotic ACCase is composed of multiple subunits, whereas the homomeric eukaryotic ACCase is a large multidomain protein. Plants have both cytosolic and plastidic ACCase. In grasses the plastidic ACCase is homomeric and is the target site for three herbicide classes. Importantly, in most dicots the plastidic ACCase is multimeric and is not sensitive to herbicides. Thus most dicot species tolerate ACCase-inhibiting herbicides well, but most grass species are susceptible, meaning that ACCase herbicides control only grass weed species. ACCase herbicides, introduced since 1978, have become widely used for grass weed control in world agriculture. There are many ACCase herbicides across the aryloxyphenoxypropionate (APP), cyclohexanedione (CHD) and phenylpyrazoline (PPZ) chemical groups. In response to global and often intensive ACCase herbicide selection, many grass weeds (36 species, **Figure 1**) (67) have evolved resistance since the first report in *L. rigidum* (65). For example, most populations of *L. rigidum* across a huge Australian grain-growing region (90, 14, 114) and a considerable proportion of *Alopecurus myosuroides* in northwestern Europe are now ACCase herbicide resistant (29, 108). There are substantial areas of ACCase herbicide-resistant grass weeds in various parts of the world. The evolution of ACCase herbicide resistance has been comprehensively reviewed (34, 36), so here we focus on recent developments.

It was first established that ACCase herbicide resistance could be target-site based due to a resistant ACCase (121), and then three laboratories independently identified a Leu-1781-Ile resistance-endowing mutation in the CT (carboxyl transferase) domain of plastidic ACCase of resistant grass weeds (32, 199, 204). Progressively since then, seven other resistance substitutions have been identified in various grass species (**Table 3**). Of these mutations, Leu-1781-Ile is the most common. Given the large number of ACCase herbicides across three chemically distinct groups, the ACCase herbicide resistance spectrum endowed by the eight resistance mutations has been elucidated only for some ACCase herbicide groups in some grass weeds (**Table 3**). It is increasingly recognized that the level and spectrum of target-site ACCase herbicide resistance are determined by the particular resistance mutation, homozygosity/heterozygosity of the plants for the mutation, and, importantly, the herbicide and dose used for evaluation (28, 75, 190). To obtain precise information it is necessary to have well-characterized genotypes with known mutations and to carefully select herbicide and dose (28, 33, 126, 190). For example, the Asp-2078-Gly mutation confers high-level resistance to many (APP and CHD) ACCase herbicides but very low-level resistance to the (CHD) herbicide clethodim (28, 190). Thus it is erroneous to assume that all target-site resistance mutations endow high-level resistance. The reality is that on a case-by-case basis particular target-site mutations give from high-level to quite low-level resistance (28, 75, 190). Generalizations should not be made and indeed, in general, the importance of herbicide dose and homozygosity/heterozygosity for the resistance mutation(s) in evaluating a resistance mutation is underappreciated in herbicide resistance research.

**Molecular interactions between ACCase and herbicides.** In comparison to AHAS (discussed above), for ACCase less is known as to herbicide binding and influence on the catalytic site, and how mutations confer resistance.

**Table 3** Resistance-endowing plastidic ACCase CT domain amino acid substitutions in field-evolved resistant grass weed species

Amino acid substitution <sup>a</sup>	Grass weed species	Resistance spectrum <sup>b</sup>			References <sup>c</sup>
		APP	CHD	PPZ	
Ile-1781-Leu	<i>Alopecurus myosuroides</i>	R	R	R	Also see 126
	<i>Avena fatua</i>	R	R	r	Also see 17
	<i>A. sterilis</i>	R	R	–	89
	<i>Lolium multiflorum</i>	–	R	–	182
	<i>L. rigidum</i>	R	R	R	Also see 190, 205
	<i>Setaria viridis</i>	R	R	–	
Trp-1999-Cys	<i>A. sterilis</i>	R <sup>d</sup> /S	S	–	89
Trp-2027-Cys	<i>A. myosuroides</i>	R	S	R	Also see 126
	<i>A. sterilis</i>	R/r	r	–	89
	<i>L. rigidum</i>	–	r	–	190
Ile-2041-Asn	<i>A. myosuroides</i>	R	S	r	Also see 126
	<i>A. sterilis</i>	R	r	–	89
	<i>Phalaris paradoxa</i>	–	–	–	69
	<i>L. rigidum</i>	R	r/S	–	Also see 190, 206
Ile-2041-Val	<i>L. rigidum</i>	S/R	S	–	
Asp-2078-Gly	<i>A. myosuroides</i>	R	R	R	Also see 126
	<i>A. sterilis</i>	R	R	–	89
	<i>L. multiflorum</i>	R	R	R	75
	<i>L. rigidum</i>	R	R	R	190
	<i>P. paradoxa</i>	R	R	R	69
Cys-2088-Arg	<i>L. rigidum</i>	R	R	R	190
Gly-2096-Ala	<i>A. myosuroides</i>	R	r/S	S	Also see 126

Abbreviations: ACCase: Acetyl-coenzyme A carboxylase; AT: carboxyl transferase.

<sup>a</sup>Amino acid positions correspond to the full-length plastidic ACCase in *A. myosuroides*.

<sup>b</sup>APP: aryloxyphenoxypropionates; CHD: cyclohexanediones; PPZ: phenylpyrazolines; R: resistant; S: susceptible; r: low to moderately resistant; dash: not determined.

<sup>c</sup>Unless otherwise specified, see 28, 34 for references.

<sup>d</sup>Resistant only to Fenoxaprop.

However, knowledge is accumulating, commencing with evidence that the inhibition of ACCase by ACCase herbicides is nearly competitive with the substrate acetyl-CoA (see Reference 36 for a review). A pivotal advance has been the achievement of the crystal structure of the yeast ACCase CT domain, which revealed that the ACCase catalytic site is situated in a cavity at the interface of the dimer (202, 203). There is evidence that ACCase (APP group) herbicides bind within a domain close to and partially overlapping the catalytic site (202). However, the precise binding details of the many ACCase herbicides across three chemical groups (APP, CHD, PPZ) remain unknown. Some resistance amino acid substitutions

(Table 3) may prevent herbicide binding without having an effect on substrate acetyl-CoA binding at the catalytic site, whereas other mutations that overlap the catalytic site may adversely impact acetyl-CoA binding and therefore ACCase functionality (33). Further insight has come from three-dimensional modeling, which indicates that resistance amino acid substitutions do occur within the catalytic cavity and change the shape of the cavity, hampering herbicide access to the binding site (33). Definitive proof awaits achievement of the crystal structure of plant ACCase in the presence and absence of bound herbicides. Note that for target-site ACCase resistance, notwithstanding the presence of

resistance mutations (**Table 3**), a few ACCase herbicides continue to be effective on many resistant populations. Such ACCase herbicides have a particular chemical structure that enables them to bind and inhibit ACCase, despite the presence of resistance mutations.

**ACCase gene mutations: effect on ACCase functionality and plant fitness.** Some of the eight different resistance-endowing ACCase gene mutations (**Table 3**) have a fitness cost, but others do not (see Reference 173 for a review). In *A. myosuroides*, using segregating F2 families with careful genotyping to obtain homozygous and heterozygous individuals, no fitness cost was found for the Ile-1781-Leu or Ile-2041-Asn mutations (101). Similarly, there was no fitness cost in a *L. rigidum* biotype with the Ile-1781-Leu mutation (171, 174). Indeed, a study with a resistant genotype of *Setaria italica* with the Ile-1781-Leu mutation displays a fitness advantage for this mutation (178). The 1781-Leu allele is the wild type in the grass species *Poa annua*, *Festuca rubra*, and *F. bromoides* (31, 191). However, some ACCase resistance mutations do impose a fitness cost. *A. myosuroides* homozygous for the Asp-2078-Gly mutation has a fitness cost (101). Equally, *L. rigidum* genotypes homozygous for the Asp-2078-Gly or the Cys-2088-Arg mutation also exhibit a fitness cost (M. Vila-Aiub & S. Powles, unpublished data). Our work with *L. rigidum* populations homozygous for ACCase target-site mutations showed that 1781 mutation does not change ACCase activity, whereas 2078 or 2088 mutation significantly reduces ACCase activity (190). As yet there are no published studies on fitness cost for the other ACCase resistance mutations identified in **Table 3**, and work on this is needed. In conclusion, widespread and persistent ACCase herbicide selection has resulted in the evolution of many resistant grass weeds. Eight amino acid substitutions in the CT domain of ACCase have evolved. Especially in cross-pollinated weed species, individuals may be heterozygous for several mutations, and exhibit complex patterns of cross-resistance across ACCase herbicides. In addition, it must be emphasized

that non-target-site-based ACCase herbicide resistance is also widespread. For example, the contribution of target-site- versus non-target-site-based resistance was evaluated using 243 ACCase herbicide-resistant *A. myosuroides* French populations, which demonstrated that most resistant plants did not have any of the known ACCase mutations (29). Similarly, across huge areas of Australia, non-target-site ACCase herbicide resistance is also common in resistant *L. rigidum* and *Avena* spp. (114, 115, and reviewed in section on Non-Target-Site Herbicide Resistance). A further complication is that individuals can exhibit both target-site and non-target-site ACCase herbicide resistance mechanisms.

### Glyphosate Resistance: EPSPS Pro-106 Mutations

Glyphosate is by far the world's most widely used and important herbicide. Glyphosate has a favorable environmental profile and controls a very broad spectrum of annual and perennial weeds in varied agricultural, industrial, and amenity situations. Glyphosate is a specific and potent inhibitor of the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSP). Glyphosate inhibition of EPSPS activity disrupts the shikimate pathway and inhibits aromatic amino acid production, ultimately causing plant death. Glyphosate has been globally and extensively used since 1974, and when reviewed in 1994, there were no reports of evolved glyphosate-resistant weeds (41). However, since first identified (132, 135), glyphosate resistance has evolved in at least 16 weed species in 14 different countries and is fast becoming a very significant problem in world agriculture (**Table 4; Figure 1**) (67; see Reference 129 for a review). A major factor accelerating the evolution of glyphosate-resistant weeds has been the advent of transgenic glyphosate-resistant crops such as soybean, maize, cotton,

**Table 4 Weed species that have evolved enhanced rates of cytochrome P450-mediated herbicide metabolism**

Species	Herbicide	Herbicide group	References <sup>a</sup>
<i>Amaranthus hybridus</i>	Chlorimuron	AHAS inhibitors	94
<i>Bromus tectorum</i>	Propoxycarbazone	AHAS inhibitors	119
<i>Alopecurus myosuroides</i>	Chlorotoluron Chlorsulfuron Flupyr-sulfuron Clodinafop Diclofop-methyl Propaquizafop Haloxypop Fenoxaprop- <i>p</i>	PSII inhibitors AHAS inhibitors  ACCase inhibitors	Also see 87
<i>Lolium rigidum</i>	Chlorotoluron Atrazine Diuron Metribuzin Simazine Chlorsulfuron Diclofop-methyl Tralkoxydim Pendimethalin	PSII inhibitors    AHAS inhibitors ACCase inhibitors  Dinitroaniline	163
<i>Lolium multiflorum</i>	Diclofop-methyl	ACCase inhibitors	19
<i>Avena sterilis</i>	Diclofop-methyl	ACCase inhibitors	
<i>Pbalaris minor</i>	Isoproturon	PSII inhibitors	
<i>Echinochloa phyllopogon</i>	Bispyribac-sodium Fenoxaprop- <i>p</i> -ethyl Thiobencarb	AHAS inhibitors ACCase inhibitors Thiocarbamates	10, 185, 198
<i>Stellaria media</i>	Mecoprop	Synthetic auxins	
<i>Digitaria sanguinalis</i>	Fluazifop- <i>P</i> -butyl Imazethapyr	ACCase inhibitors AHAS inhibitors	
<i>Sinapis arvensis</i>	Ethametsulfuron-methyl	AHAS inhibitors	

<sup>a</sup>Unless otherwise specified, see 54, 138, 146 for references.

and canola. These have been spectacularly adopted in North and South America. In these crops, glyphosate has replaced almost all other herbicides or other means of achieving weed control. From an evolutionary viewpoint, this singular reliance on glyphosate is an intense selection for any glyphosate resistance genes (128, 129). Unsurprisingly, widespread evolution of glyphosate resistance in weeds has quickly followed (Figure 4). The reader is referred to the 2008 special issue of *Pest Management Science*, Volume 64, which fully reviews glyphosate-resistant crops and weeds. Mechanisms endowing evolved glyphosate resistance in weeds have recently been reviewed,

showing that both target-site EPSPS gene mutation/amplification and non-target-site resistance (discussed below) have evolved (133, 143, 158).

Target-site glyphosate resistance, first identified in an *Eleusine indica* biotype (84), is due to a serine substitution at Pro-106 (Pro-106-Ser) in a highly conserved region of the EPSPS gene (9). Subsequently, threonine and alanine substitutions at Pro-106 have been first reported in glyphosate-resistant *E. indica* (112) and *Lolium* (189) populations, respectively. Now these three Pro-106 amino acid substitutions have been identified in *E. indica* and *Lolium* populations from various parts of



**Figure 4**

Evolved glyphosate-resistant *Sorghum halepense* infesting an Argentinean transgenic glyphosate-resistant soybean crop (with permission of M. Vila-Aiub; see 175).

the world (76; see Reference 143 for a review). These Pro-106 substitutions confer only a modest degree of glyphosate resistance.

#### **Will there be other EPSPS gene mutations?**

In considering whether resistance mutations other than Pro-106 will evolve, note that the EPSPS active site is highly conserved (116). The crystal structure of *Escherichia coli* EPSPS and molecular modeling show that glyphosate inhibits EPSPS by occupying the PEP binding site (45, 64, 155). Incisive work on *E. coli* EPSPS Pro-106 substitutions and the crystal structure of EPSPS-S3P-glyphosate reveals that Pro-106 substitutions cause a slight narrowing of the glyphosate/PEP binding site cavity, which endows glyphosate resistance but preserves EPSPS functionality (64). In contrast, substitutions at Gly-101 or Thr-102 confer high-level glyphosate resistance but reduce the volume of the glyphosate/PEP binding site,

and this significantly reduces affinity for PEP (45, 50). Thus, mutations enabling both glyphosate and PEP binding while retaining EPSPS functionality may be very rare. For example, only the Pro-106 substitutions were identified in a directed evolution strategy involving randomly mutated *Oryza sativa* EPSPS (*E. coli* expressed) in which only EPSPS mutants that conferred glyphosate resistance and retained EPSPS functionality were advanced (207). So far, there are no published studies on the effect of target-site EPSPS glyphosate resistance mutations on the fitness of resistant individuals. Such studies are needed for the various glyphosate resistance Pro-106 substitutions that have evolved in glyphosate-resistant weeds.

#### **EPSPS overexpression or amplification.**

It has been recently documented that highly glyphosate-resistant *Amaranthus palmeri* biotypes have up to 100-fold EPSPS gene

amplification resulting in up to 40-fold EPSPS overexpression (51). This EPSPS gene amplification is heritable and correlates with the expression level and glyphosate resistance segregating in F2 plants (51). This clear evidence of field-evolved glyphosate resistance endowed by EPSPS gene amplification is supported by laboratory selected glyphosate-resistant cell lines of several plant species that have EPSPS gene amplification (see Reference 41 for a review). A threefold increase in basal EPSPS mRNA and enzyme activity (not due to gene amplification) was observed in glyphosate-resistant *L. rigidum* (8), and a complementary role of constitutively higher EPSPS mRNA levels was suggested for several glyphosate-resistant *Conyza* biotypes, where reduced glyphosate translocation was found to be a major resistance mechanism (37). Thus, given the difficulty in mutating the EPSPS gene to obtain both resistance and enzyme functionality, we expect more examples of evolved glyphosate resistance due to EPSPS gene amplification. The high levels of EPSPS produced by massive gene amplification evident in glyphosate-resistant *A. palmeri* (51) should have a fitness cost, and this needs investigation. In conclusion, it is clear that EPSPS target-site glyphosate resistance is occurring due to Pro-106 resistance-endowing mutations, and glyphosate resistance endowed by EPSPS gene amplification has been reported. Given the persistent and widespread glyphosate reliance in many parts of the world, other glyphosate resistance mechanisms are likely. Indeed, non-target-site-based glyphosate resistance has evolved in several species (discussed in section Resistance Endowed by Restricted Rates of Herbicide Translocation, below).

### Resistance to Tubulin Assembly-Inhibiting Herbicides: Recessive Tubulin Gene Mutations

There are a number of structurally dissimilar, soil-active, pre-emergent herbicides (dinitroanilines, benzoic acids, phosphoramidates, pyridines, and carbamates) that mostly target germinating seeds, inhibiting

early cell division. The mode of action of this group of herbicides is to bind to plant tubulin dimers, disrupting microtubule growth (3, 12). Microtubules are polymers of  $\alpha$ - and  $\beta$ -tubulin dimers and are involved in many essential cellular processes including mitosis, cytokinesis, and vesicular transport. Dinitroaniline and other tubulin-inhibiting herbicides have been used for several decades but not on a grand scale, and evolved resistance has been reported in only 10 weed species (**Figure 1**) (67). Both target-site resistance and non-target-site resistance to tubulin herbicides exist, and the literature up to 1994 has been thoroughly reviewed (160). Here we focus on the biochemical and molecular basis of target-site dinitroaniline herbicide resistance, which has been studied in detail only in *Eleusine indica* and *Setaria viridis*. When reviewed in 1994, tubulin polymerization occurred normally in resistant plants in the presence of dinitroaniline herbicide (160). In 1998, two groups independently identified a  $\alpha$ -tubulin gene mutation resulting in a Thr-239-Ile substitution that conferred high-level resistance in *E. indica* (4, 184). This Thr-239-Ile substitution endows resistance to many dinitroaniline herbicides, and cross-resistance to phosphoramidate and pyridine herbicides, but increased sensitivity (negative cross-resistance) to some carbamate herbicides (see Reference 3 for a review). The same mutation was also reported in dinitroaniline-resistant *S. viridis*, where it conferred cross-resistance to a benzoic acid herbicide but negative cross-resistance to carbamate herbicides (30). A second mutation, Met-268-Thr, was found to provide lower-level dinitroaniline herbicide resistance in *E. indica* (184). Finally, in dinitroaniline-resistant *S. viridis*, a Leu-136-Phe mutation was also identified (30). Fitness cost studies with plants with each of these resistance mutations are required.

Importantly, target-site dinitroaniline herbicide resistance is inherited as a recessive single nuclear gene (72, 166, 177, 200, 201), and so only homozygous individuals survive the normal herbicide dose. In contrast, as most mutations conferring target-site resistance are

inherited as dominant/semidominant genes (discussed above), heterozygous individuals survive at normal herbicide dose. Therefore, this mechanism of resistance to tubulin-inhibiting herbicides is more difficult to evolve because the initially rare heterozygous resistant individuals are killed at normal herbicide dose. This helps explain the limited evolution of this mechanism of resistance, especially in cross-pollinated species.

### Resistance to Protoporphyrinogen IX Oxidase-Inhibiting Herbicides: A Novel Deletion Mutation

In plants, protoporphyrinogen IX oxidase (PPO) is a key enzyme in the biosynthesis of chlorophyll and heme. PPO catalyzes the oxidation of protoporphyrinogen (protogen) to protoporphyrin IX (Proto IX). In plants there are two nuclear-encoded PPO isoforms, PPO1 (targeted to the chloroplast and encoded by the gene PPX1) and the mitochondrial PPO2 (encoded by the gene PPX2). Several herbicides including the diphenylethers and oxidiazoles inhibit PPO. Some PPO herbicides have been used for many years but have not had the global use evident for major herbicides (discussed above). There has been little evolution of PPO herbicide resistance, with resistance reported in biotypes of only three weed species (**Figure 1**) (67). Investigations with a resistant *Amaranthus tuberculatus* biotype have revealed a novel and unexpected mutation in which resistance is conferred by an amino acid deletion. In resistant *A. tuberculatus* for the PPX2L gene that likely encodes both chloroplastic and mitochondrial PPO, there is the loss of a 3-bp codon, causing a deletion of glycine at position 210 (122). This is the only report of codon/amino acid deletion conferring herbicide resistance. The Gly-210 deletion in the PPO gene endows very high-level resistance to PPO herbicides with little effect on the affinity of PPO for its substrate protogen, but the deletion incurs tenfold-lower PPO activity than does the wild type (F.D. Dayan, P.R. Daga, S.O. Duke et al., unpublished data). Molecular dynamics

simulations, using the crystal structure of *Nicotiana tabacum* PPO2 in complex with herbicide (77), revealed that deletion of Gly-210 enlarged the volume of the PPO active site, causing a structural rearrangement of the substrate protogen binding domain (F.D. Dayan, P.R. Daga, S.O. Duke et al., unpublished data). The modeling supported the measurement that resistant PPO has no impact on substrate binding but suffers reduced catalytic efficiency. An obvious question is whether Gly-210 substitution rather than deletion would endow resistance. Modeling indicated that substitutions at Gly-210 provide either little or no resistance, or greatly reduce PPO functionality (F.D. Dayan, P.R. Daga, S.O. Duke et al., unpublished data). Therefore, although amino acid deletion is considered to be a much rarer evolutionary event ( $\sim 10^{-18}$ ) than substitution ( $\sim 10^{-9}$ ) (55), this was the mutation that evolved in this biotype of *A. tuberculatus*. The requirement for simultaneous loss of three nucleotides in the coding sequence of the target gene, plus the dual targeting of the gene product to chloroplasts and mitochondria, should limit the evolution of this deletion resistance mechanism, although it has been documented in a further four resistant *A. tuberculatus* populations (85). This unlikely yet novel resistance mechanism again demonstrates how the power of herbicide selection pressure can reveal rare and unexpected resistance-endowing genes. It is important to reveal to what extent the reduced PPO activity conferred by the Gly-210 deletion affects fitness of resistant plants, and whether there are other adverse pleiotropic effects of this deletion.

We recognize that we have not reviewed all cases of field-evolved target-site herbicide resistance. We have not reviewed resistance to synthetic auxin type herbicides (2,4-D and other auxin-type herbicide chemistries). These herbicides have been in use for more than 50 years, and notwithstanding the evolution of resistant weed populations (**Figure 1**), they remain remarkably effective. Unraveling the mechanistic basis of evolved resistance to synthetic auxin herbicides has been particularly difficult (22). Although there are current research advances

in plants on perception, signaling, and resistance to synthetic auxins (58, 63, 179), the exact molecular resistance mechanism has not been determined in any weed species. The mechanism of resistance to MCPA (4-chloro-2-ethoxyphenoxycetic acid) in a biotype of *Galeopsis tetrahit* is reduced MCPA translocation and enhanced MCPA metabolism, and at least two genes are involved in the resistance (183). It is hoped that in the next few years the precise biochemical and molecular basis of evolved resistance to synthetic auxin herbicides will be elucidated.

### NON-TARGET-SITE HERBICIDE RESISTANCE

Here we consider major non-target-site herbicide resistance mechanisms that have been selected in weed species. Evolved non-target-site herbicide resistance can be due to any one or a combination of mechanisms that limit to a nonlethal dose the amount of herbicide reaching a target site. Mechanisms include decreased herbicide penetration into the plant, decreased rates of herbicide translocation, and increased rates of herbicide sequestration/metabolism. Such mechanisms act to minimize the amount of herbicide reaching the target site (rendering the target site somewhat irrelevant).

### Cytochrome P450 Monooxygenases (P450s) and Evolved Herbicide Resistance

In pest insect species a major evolved resistance mechanism is increased P450 capacity to metabolize (detoxify) insecticides. There is fairly comprehensive understanding of the important role of P450s in insecticide resistance (88). In contrast, the role of P450s in endowing herbicide resistance in weeds is poorly understood. P450s are one of the largest superfamilies of enzymes and are found in almost all organisms, with plants having the highest number of P450 genes (e.g., 356 genes in rice versus 57 in humans). The many vital roles of plant P450s have been reviewed (157). Plant P450s are bound

to the endoplasmic reticulum (in a few cases to plastid membranes) and are involved in the synthesis of hormones, sterols and fatty acid derivatives and in many aspects of plant secondary metabolism. While P450s catalyze a wide diversity of reactions in plant metabolism, their role in herbicide conversion is usually hydroxylation or dealkylation. In most cases these reactions can be summarized as activation and insertion of an atom from molecular oxygen to form a more reactive product using electrons from NADPH, through the action of NADPH-P450 reductase. Thus some P450s will metabolize certain herbicides to products with reduced or modified phytotoxicity that are further inactivated, often by conjugation to glucose and subsequent transport into the vacuole (78). This is well known in crops such as wheat and maize, which tolerate herbicides across several modes of action due to substantial P450 mediated herbicide metabolism capacity (see References 159, 181 for reviews).

As crops can P450 metabolize many different herbicides, their use on large weed populations is a strong selection pressure for weed individuals possessing the same ability. Indeed, in weeds (as for insect pests), P450-based herbicide resistance is a very threatening resistance mechanism because P450 enzymes can simultaneously metabolize herbicides of different modes of action, potentially including never-used herbicides. Such resistance evolution was identified in the 1980s, with landmark reports that resistant *A. myosuroides* and *L. rigidum* biotypes displayed non-target-site cross-resistance across several herbicide modes of action, including herbicide groups never used (66, 107). Subsequently, *in vivo* studies on herbicide metabolism and P450 inhibitors in resistant biotypes showed that P450s catalyzed enhanced rates of metabolism of several herbicides (Table 4) (see References 60, 138, 146 for reviews). Note that evolved P450-based resistant *L. rigidum* populations can exhibit resistance across several (but not all) of the herbicides discussed in the section on Target-Site Herbicide Resistance, including PSII, AHAS, ACCase, and tubulin-inhibiting

herbicides (140). In addition to *L. rigidum* and *A. myosuroides* (61, 62), the evolution of resistance due to P450-catalyzed enhanced rates of herbicide metabolism has been demonstrated in resistant biotypes of a further nine weed species (**Table 4**). In these studies, resistance correlates with increased rates of in vivo herbicide metabolism and/or with full or partial reversal of resistance by P450 inhibitors (e.g., 1-aminobenzotriazole, piperonylbutoxide, tetracyclis, malathion). Further evidence of the importance of P450s in herbicide resistance evolution comes from studies conducted with herbicide-susceptible *L. rigidum* biotypes recurrently selected over three generations with a low dose of a P450-metabolizable herbicide (diclofop-methyl). At low dose susceptible plants can P450 metabolize diclofop-methyl and thus the plants were treated at a dose causing around 50% mortality. The survivors were grown for seed for the next generation, and the selection was repeated. In just three generations there was evolution of high-level, non-target-site resistance (110, 111; S. Manalil, R. Busi, M. Renton, S. Powles, manuscript in preparation). Importantly, there was concomitant evolution of cross-resistance to other P450-metabolizable herbicides of different modes of action. Although all of these studies (**Table 4**) clearly imply P450 involvement, definitive evidence requires that the P450 genes specifically responsible for resistance be identified in P450-based resistant weed biotypes. In resistant *L. rigidum* and *A. myosuroides*, all that is currently known is that more than one P450 gene is involved (16, 86, 145; R. Busi, M. Vila-Aiub, S. Powles, unpublished data). Interestingly, evolved P450-based herbicide resistance in *L. rigidum* can be associated with a fitness cost (171, 172).

To date, biochemical studies to characterize P450-based herbicide resistance in evolved resistant weed species have yielded little information. Herbicide-degrading P450 microsomes have not been successfully isolated from resistant *L. rigidum* or *A. myosuroides* (S. Powles, D. Werck-Reichhart, unpublished data) but have from resistant *E. phyllopogon*

(198). Sixteen P450 genes were isolated from a resistant *L. rigidum* biotype, but no attribution to herbicide metabolism was established (48). Three full-length P450s were obtained from resistant *L. rigidum* biotypes, one of which (CYP71R4), when expressed in yeast, metabolized a PSII herbicide (169); the other two still require functional characterization (N. Dillon, C. Preston, S. Powles, unpublished data). A major research frontier and rich research opportunity is to identify the P450s conferring resistance in weeds. Over the coming decade we expect that much will be elucidated, notwithstanding formidable technical challenges. P450 proteins can share as little as 16% amino acid identity, and there are more than 2000 plant P450 sequences in the P450 database (<http://drnelson.utmen.edu/cytochromep450.html>). One puzzling aspect is that, to date, P450-based evolved herbicide resistance has been reported mostly in grass weed species, with few reports in dicot species (**Table 4**). Apart from the fact that grass species have more P450 genes than dicots have, this may in part reflect the tendency of some research to examine only for target-site-based resistance. We emphasize that P450-based resistance is particularly alarming and threatening because resistance can occur across several herbicide modes of action and can extend to new herbicide discoveries, if these herbicides can be metabolized by P450s.

### Glutathione S-Transferases and Evolved Herbicide Resistance

Glutathione S-transferases (GSTs) (EC 2.5.1.18) are families of multifunctional enzymes that catalyze the conjugation of glutathione to a variety of electrophilic, hydrophobic substrates. GSTs have a particular role as a protective mechanism against oxidative stress by interacting with active oxygen species (38). GSTs are involved in stress response, and in some crop and weed species some herbicides can be detoxified by glutathione conjugation (see References 20, 44, 149 for reviews). Glutathione-conjugated herbicides can be

sequestered in the vacuole (95) or exuded via root tips (156). Herbicide-metabolizing GSTs have been purified and characterized from several crops (see References 20, 197 for reviews). The resolution of the 3-D structure of plant GST (including herbicide-induced GST), molecular modeling, and mutagenesis studies provide an understanding of the molecular basis of GST-catalyzed herbicide binding and how single amino acid substitution(s) can improve GST catalytic efficiency and affect substrate specificity for herbicides and xenobiotics (7, 11, 39).

Maize is very tolerant of triazine herbicides because of high activity of GSTs able to catalyze the conjugation of triazines to glutathione. It follows that widespread use of triazine herbicides could select for weeds with GSTs able to detoxify triazine herbicides. Indeed, evolved GST-mediated triazine herbicide resistance has been reported in *Abutilon theophrasti* (1, 56). Further studies revealed that increased GST (triazine) activity is due to higher catalytic capacity, rather than enzyme overexpression or the presence of a novel GST (127). This indicates a possible mutation in the GST gene that can improve herbicide binding and therefore GST catalytic efficiency. Atrazine resistance in this biotype is inherited as a single nuclear gene with partial dominance (2). In a resistant *Echinochloa phyllopogon* biotype it was demonstrated that fenoxaprop-*p*-methyl resistance can be due to glutathione-herbicide conjugation (10), although GST activity was not determined in this study. Studies with multiple resistant *A. myosuroides* biotypes with enhanced P450-catalyzed herbicide metabolism also reveal that they have higher GST activity, although there is limited evidence of higher capacity for GST-catalyzed herbicide conjugation (23, 24, 149). In these biotypes, it is possible that elevated GST activity has a secondary role in mitigating against oxidative stress. Thus, in conclusion, GST enzymes can play both a direct role (herbicide conjugation) and an indirect role (stress response) in evolved herbicide resistance. Given that herbicides select for all possible resistance mechanisms, further research is

required to establish the precise involvement of GSTs in evolved herbicide resistance.

### Resistance Endowed by Restricted Rates of Herbicide Translocation

Resistance evolution to the AHAS herbicides is a dramatic example of independent evolution of many resistance endowing mutations (currently 22 target-site mutations plus enhanced P450 metabolism). However, for some herbicides there are few options for resistance evolution. The chemistry and the modes of action of glyphosate and paraquat are such that neither herbicide can be sufficiently metabolized by plants (21, 46, 91, 162), and mutations of the target site are rare and limited for glyphosate (discussed above) and nonexistent for paraquat (see Reference 144 for a review). Therefore, evolution has found another way for plants to survive these herbicides, involving a restricted rate of herbicide movement (translocation) throughout the plant.

**Glyphosate resistance by restricted glyphosate translocation.** Upon entering plant leaves, glyphosate has considerable mobility via xylem and phloem (in general, glyphosate translocation follows photoassimilate translocation from source to sink). This is important as glyphosate translocation throughout the plant is necessary for its toxicity. Since first reported in glyphosate-resistant *Lolium* (91), restricted glyphosate translocation throughout the plant and to the roots has been confirmed in many resistant populations of *Lolium* and *Conyza* (e.g., 47, 176, 187; see References 133, 136, 143, 158 for reviews). Several populations of glyphosate-resistant *Sorghum halepense* also display restricted glyphosate translocation (M. Vila-Aiub, Q. Yu, S. Powles, unpublished data). Indeed, evolved glyphosate resistance due to restricted translocation throughout the plant is more common than EPSPS Pro-106 mutations (discussed above). Importantly, some *Lolium* populations have both an EPSPS Pro-106 target-site mutation and the restricted glyphosate translocation mechanism (143, 189),

and this will surely occur in other species. The underlying biochemical mechanism conferring restricted glyphosate translocation throughout the plant remains to be identified. We speculate that it could be a membrane transporter pumping glyphosate into vacuoles. All that is currently known is that the mechanism inherits as a single, nuclear, semidominant gene (see Reference 133 for a review). Significantly, in resistant *Lolium* biotypes the restricted glyphosate translocation mechanism has a fitness cost (124, 143; see Reference 173 for a review). There may be some other minor genes that contribute to non-target-site glyphosate resistance. There is evidence of reduced glyphosate leaf penetration in some resistant *Lolium* biotypes (105, 109) and one resistant *S. halepense* biotype (M. Vila-Aiub, Q. Yu, S. Powles, unpublished data).

**Paraquat resistance by restricted paraquat translocation.** The bipyridyl herbicide paraquat has been in global use for over 40 years. Paraquat rapidly enters leaves and then chloroplasts, where it disrupts photosystem I electron transport, reducing oxygen to damaging active oxygen states. Evolved paraquat resistance is evident in small areas in biotypes of 24 weed species (**Figure 1**) (67). The mechanisms for evolved paraquat resistance have been thoroughly reviewed (144). Enhanced activity of enzymes that detoxify reactive oxygen species has been proposed as a paraquat resistance mechanism (see Reference 54 for a review). Many studies establish that restricted paraquat translocation is a resistance mechanism in biotypes of several weed species (see References 138, 144 for reviews; see also References 141, 161, 170, 188, 189). Importantly, the restricted paraquat translocation mechanism works well at modest temperatures but fails at high temperatures (148).

Paraquat resistance due to restricted translocation inherits as a single nuclear, semidominant gene in several species (see Reference 144; see also Reference 193). While it is clear that evolved paraquat resistance in many species is due to restricted paraquat translocation rates, neither the mechanism nor the site

of paraquat sequestration is known. Potential sites for paraquat sequestration in plant leaves are the cell wall and the vacuole. No differential paraquat cell wall binding has been detected (82, 137). Similarly, no difference was found in paraquat uptake across the leaf plasmalemma (130). However, there is some supportive evidence for the hypothesis of paraquat sequestration into leaf vacuoles (74, 81, 106, 186; Q. Yu, S.B. Huang, and S. Powles, unpublished data). In bacteria, several multidrug transporter genes have been isolated that confer paraquat resistance by paraquat extrusion or sequestration (e.g., 147, 186). Recently, two differentially expressed EST sequences were identified in paraquat-resistant *C. canadensis*. One EST is homologous to polyamine and amino acid transporters (putrescine transporter PotE from *E. coli*, cationic acid transporter CAT4 from *A. thaliana*) and the other is homologous to multidrug resistance protein EmrE from *E. coli* and vacuolar H<sup>+</sup>-ATPase subunit C of DET3 from *A. thaliana* (74). Thus the current hypothesis is that a tonoplast membrane transporter is able to pump paraquat into vacuoles. It is hoped that this will soon be resolved at the biochemical/genetic level.

It must be emphasized that restricted glyphosate or paraquat translocation are independent mechanisms. Glyphosate and paraquat differ greatly in molecular structure, electron charge, and mobility within plants, and glyphosate-resistant plants are not resistant to paraquat, or vice versa (91, 176, 188). However, both mechanisms can exist in the same plant (189).

## CONCLUSIONS AND FUTURE PROSPECTS

Herbicide resistance in plants is a stark example of rapid evolution. The first global wave of resistance evolution was widespread target-site triazine (PSII) herbicide resistance. Triazine herbicides are competitive inhibitors with the normal substrate and bind within the catalytic site (**Figure 2**). Globally, target-site resistance was found to be the result of a

single mutation (*psbA* Ser-264-Gly), which prevents triazine binding but also reduces substrate binding, thus conferring high-level resistance but at a substantial fitness cost. Also, non-target-site PSII herbicide resistance due to P450 and/or GST-mediated metabolism has evolved. Greater diversity in resistance mechanisms became evident in the next global wave of resistance, AHAS herbicide resistance. The AHAS gene can be easily mutated such that a remarkable 22 AHAS resistance-endowing gene mutations have occurred to date (**Table 2**). AHAS herbicides are not competitive inhibitors with the AHAS substrate and do not bind within the catalytic site, and several resistance mutations do not change AHAS functionality. Clearly, AHAS target-site resistance is very different from triazine resistance. Also, non-target-site resistance due to P450-mediated AHAS herbicide metabolism is a potent resistance mechanism. The next global wave of resistance was grass weed resistance to ACCase herbicides. In a situation intermediate between triazine and AHAS herbicide resistance, eight different ACCase resistance-endowing amino acid substitutions have been identified (**Table 3**), with fitness cost that ranged from negligible to substantial. ACCase herbicides bind on an herbicide binding domain that is close to and overlaps the catalytic site. Thus, a number of resistance mutations are possible for target-site ACCase resistance, but not as many as with AHAS target-site resistance. Similarly, as many ACCase herbicides are subject to P450 degradation, much metabolism-based resistance has evolved.

The current global wave of resistance is the evolution of resistance to glyphosate, the world's most widely used and important herbicide. Glyphosate resistance evolution will be a major issue in the coming decade because of massive glyphosate selection in the large areas devoted to transgenic glyphosate-resistant crops. To date, a non-target-site mechanism of restricted glyphosate translocation is most common. Target-site EPSPS gene resistance seems more difficult to evolve as glyphosate binds within the EPSPS catalytic site, and

there may be very few mutations that confer glyphosate resistance while retaining EPSPS functionality. However, target-site glyphosate resistance evolution due to substitutions at EPSPS Pro-106 is occurring, as well as EPSPS gene amplification to endow glyphosate resistance. Thus, despite it being difficult for plants to evolve glyphosate resistance, the huge glyphosate selection pressure being exerted globally has resulted to date in three different Pro-106 EPSPS gene mutations, together with EPSPS gene amplification, and a reduced translocation resistance mechanism. Clearly, even with an herbicide for which resistance evolution is difficult, if the selection pressure is widespread, persistent, and intense, then resistance mechanisms will evolve in large weed populations.

The greatest challenge posed by herbicide-resistant weeds is the accumulation in individuals of many resistance mechanisms, both target-site and non-target-site. This is evident now for *L. rigidum* across large areas of Australia and for *A. myosuroides* in western Europe, and it is becoming prevalent in other prominent weed species in various parts of the world. *L. rigidum* or other weeds possessing multiple herbicide resistance, including non-target-site-enhanced P450 metabolism of many herbicides, are difficult to control chemically. Although target-site gene mutations that endow herbicide resistance can be precisely identified, our current understanding of non-target-site-based herbicide resistance is very limited. At the molecular level, little is known as to the P450 and/or GST genes/enzymes endowing enhanced metabolism-based herbicide resistance, or as to the molecular basis for reduced translocation of glyphosate or paraquat. Thus, there are significant research challenges and opportunities in unraveling non-target-site resistance mechanisms. Especially, as P450-mediated herbicide resistance and other non-target-site resistance mechanisms are becoming increasingly prominent and threatening, this is a current herbicide resistance research frontier.

There has been insufficient attention and appreciation of the role of herbicide dose

in resistance evolution, and yet where herbicides are used at sublethal dose (some plants are affected but survive), there can be rapid resistance evolution (110, 111). High herbicide dose results in high mortality but selects for rare resistance genes capable of endowing high-level resistance. However, selection at lower herbicide dose (most plants killed but some survivors) selects for all possible resistance-endowing genes, both weak and strong. Especially in cross-pollinated species this can allow the rapid accumulation of resistance genes. Considerable research attention to the role of herbicide dose (selection intensity) on resistance evolution is justified. Aside from an applied perspective there are likely to be fundamental discoveries made. Herbicides are powerful selective and environmental stress agents, and an intriguing possibility is whether herbicide stress could unleash in survivors epigenetic gene expression (heritably changed state of gene expression without change in DNA sequence). Indeed, early research demonstrated that a small number of triazine herbicide-susceptible *Chenopodium album* individuals possessed but did not express the *psbA* gene Ser-264-Gly mutation (26). However, after treatment with triazine herbicide at a dose enabling survivors, the next generation was strongly triazine-resistant and expressed the *psbA* gene Ser-264-Gly mutation (26). This puzzling result could be an epigenetic

gene expression. Whether the stress induced by sublethal herbicide dose may unleash epigenetic gene expression is an area worthy of research investigation.

Finally, we believe that unraveling the precise details of the biochemical, genetic, and molecular means by which plants evolve herbicide resistance will contribute to wiser use of precious herbicide resources, new innovations, and more sustainable strategies for pest weed management. Through this knowledge we believe that there will be future chemical innovations such as P450 synergists to overcome metabolism-based resistance, judicious herbicide combinations, and conceptualization of new resistance-breaking herbicide structures to overcome target-site resistance. Similarly, this fundamental knowledge is essential in creating realistic population genetics/management simulation models and practical control strategies to achieve sustainability through integrated and diverse weed control strategies that maximize herbicide longevity. As there are no foreseeable new technologies that can rival herbicides for weed management in world cropping, herbicide sustainability is an imperative that must be achieved to help guarantee world food supply. Thus, the major challenge to herbicide sustainability posed by the global evolution of herbicide-resistant weeds demands considerable ongoing public and private sector multidisciplinary research focus.

### SUMMARY POINTS

1. The rapid evolution of herbicide-resistant weeds threatens the sustainability of excellent herbicide technology essential in world food production.
2. Herbicide resistance evolves due to mutated target-site genes, and/or non-target-site genes, and individuals can accumulate many resistance genes, especially in cross-pollinated species.
3. While the molecular basis of target-site resistance can be precisely determined, non-target-site resistance is becoming increasingly prevalent and threatening, but the molecular genetic basis remains largely unknown. Thus, there are significant research challenges and opportunities in unraveling non-target-site resistance mechanisms, especially the role of cytochrome P450 enzymes and membrane transporters.

4. Much remains to be discovered on the genetics of herbicide resistance evolution, and there is the possibility that herbicide stress unleashes epigenetic resistance gene expression in plants.
5. Fundamental understanding of the molecular mechanisms endowing evolved herbicide resistance will enable innovations that, together with integrated control strategies, will help minimize and manage resistance evolution.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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