Review TRENDS in Plant Science Vol.12 No.1

Non-target-site herbicide resistance: a family business

Joshua S. Yuan[1], Patrick J. Tranel[2] and C. Neal Stewart Jr[1]

[1] Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996, USA
[2] Department of Crop Sciences, University of Illinois, Urbana, IL 61801, USA

We have witnessed a dramatic increase in the frequency and diversity of herbicide-resistant weed biotypes over the past two decades, which poses a threat to the sustainability of agriculture at both local and global levels. In addition, non-target-site mechanisms of herbicide resistance seem to be increasingly implicated. Non-target-site herbicide resistance normally involves the biochemical modification of the herbicide and/or the compartmentation of the herbicide (and its metabolites). In contrast to herbicide target site mutations, fewer non-target mechanisms have been elucidated at the molecular level because of the inherently complicated biochemical processes and the limited genomic information available for weedy species. To further understand the mechanisms of non-target-site resistance, we propose an integrated genomics approach to dissect systematically the functional genomics of four gene families in economically important weed species.

Introduction – herbicide resistance as a global threat

Weeds are a ubiquitous threat to agriculture, costing US farmers up to US$20 billion annually [1]. To agriculturalists, weeds are defined as highly competitive plants that persistently adapt to cropping systems and cause crop loss and damage. Herbicide application has been a prominent weed control measure in recent decades but, increasingly, herbicide-resistant weeds are challenging this practice. Herbicide resistance refers to the ability of a plant biotype to survive and reproduce under a normally lethal dose of herbicide. We have witnessed a dramatic increase in the number of weeds showing herbicide resistance during the past two decades. More than 300 biotypes of weeds have evolved resistance to one or more of all the major groups of herbicides (see http://www.weedscience.org), among which, resistance to glyphosate is currently of greatest concern. The widespread adoption of herbicide-resistant crops, such as Roundup-Ready™ soybean, corn, cotton and oilseed rape (http://monsanto.com) – genetically modified (GM) crops impervious to glyphosate – has greatly improved the effectiveness of weed management. However, greater glyphosate usage has played a role in the evolution of glyphosate resistance in weedy species [2]. It was originally expected that resistance to glyphosate would evolve slowly or not at all [3], but this belief has been dispelled in recent years [2]. Glyphosate-resistant horseweed (*Conyza canadensis*) was identified after three years of planting glyphosate-resistant soybean in Delaware (USA) [4]. Three of the ten most notorious weed species have already evolved glyphosate resistance after less than a decade of cultivating genetically modified crops [1]. Once resistance is significantly frequent within a population, it might spread rapidly to other populations by pollen or seed, and potentially can be transmitted to other species via hybridization [5,6]. An increase in the application frequency of a particular herbicide will probably be accompanied by commensurate resistance to that herbicide [6]. Herbicide-resistant weed biotypes have been found in >270 000 fields among 56 different countries all around the world (http://www.weedscience.org). Therefore, herbicide-resistant weeds have become an increasing global threat to agriculture [6,7], making it urgent to understand the basis of resistance, particularly those associated with non-target metabolic and transport mechanisms. In this Review article, we aim to review the most prevalent genes and pathways involved in non-target herbicide resistance and propose an integrated functional genomics approach for dissecting the non-target-site resistance.

Mechanisms of herbicide resistance

There are two primary mechanisms of herbicide resistance in weeds: (i) resistance caused by mutations in target sites of the herbicide (target-site resistance) and (ii) resistance caused by mutations in non-target sites (non-target-site resistance). Increased gene expression could also be the bases for both target-site and non-target-site resistance [8]. Most herbicides are designed to target specific enzymes or proteins and, thus, target-site resistance is mostly monogenic and involves a point-mutated target enzyme. It is therefore relatively easy to study the molecular mechanisms of target-site resistance. With regards to resistance management, the evolution of target-site resistance depends on repeated usage of a single herbicide (or group of related herbicides) and/or the exclusion of other weed control tactics [9]; it could be argued that the same is true for non-target-site resistance. Previous research has also indicated a dose effect in herbicide resistance development, where high dose application tends to promote target-site resistance development, and low dose application tends to promote non-target-site metabolic resistance [10,11]. Because of historical factors, wheat was adopted early on as a model plant to investigate the effect of P450 proteins on selective herbicides [11,12]. With regards to molecular evolution, the occurrence of herbicide resistance

Corresponding author: Stewart, C.N. Jr (nealstewart@utk.edu).
Available online 11 December 2006.

 1360-1385/$ – see front matter doi:10.1016/j.tplants.2006.11.001

depends on the abundance of resistance alleles in nature and the molecular structure of the target enzyme [13]. For example, resistance to herbicides that inhibit acetolactate synthase (ALS) represents the largest group of resistant weeds with 95 documented cases, which might be partially caused by the high frequency of resistant ALS alleles in nature. The 'Clearfield' imidazolinone- (a type of ALS inhibitor) resistant crops were generated from breeding selection using natural or mutagenized alleles. The existence of natural ALS-inhibitor resistance alleles in many weedy species such as rigid ryegrass has led to rapid and widespread evolution of resistant biotypes [9]. Moreover, multiple mutation sites can account for increased ALS inhibitor resistance, and a recent 3D structure study of the protein has helped elucidate mechanisms behind four frequently mutated amino acids [13]. Unlike acetolactate synthase-, photosystem II (PSII)- and acetyl-CoA carboxylase (ACCase)-inhibitors, some herbicides, such as glyphosate, have low to no known natural variation for target-site resistance alleles, which has led to a limited number of cases of target-site resistance. In this scenario, non-target-site herbicide resistance might represent the main mechanism of resistance for a herbicide such as glyphosate. Wider use of glyphosate might result in more non-target herbicide resistant weeds.

Compared with target-site resistance, non-target-site herbicide resistance might pose a greater threat to agriculture because of the often unexpected multi-herbicide resistance and multi-gene involvement in the mechanisms [7,14]. Indeed, the mechanisms of non-target-site resistance are myriad because of the underlying apparent genomic plasticity among weeds.

Non-target resistance: all in the family

Herbicides are chemicals that are normally not the natural substrate for enzymes or transporters involved in resistance. However, to respond to constantly changing environments, plants have evolved sophisticated detoxification systems against toxic chemicals [15]. Non-target herbicide resistance can be caused by a plant detoxification process that follows a four-phase schema [16–18]. Phase I is detoxification, in which herbicide molecules are activated so that certain functional groups can be exposed for phase II enzymes. Oxidation is a typical phase I detoxification reaction, which can be carried out by P450 monooxygenases or mixed function oxidases [19–21]. Phase II detoxification generally involves conjugation of a bulky hydrophilic molecule to the activated xenobiotic using thiols or sugars, which enables the end product of phase II detoxification to be recognized by the phase III transporters [22–24]. Phase III detoxification involves transporting the conjugated molecule into the vacuole or extracellular space by active transport. ABC transporters are the most common group of transporters involved in phase III detoxification [18,25]. Phase IV detoxification involves further degradation of the conjugated molecule in the vacuole or extracellular spaces. Many plant detoxifying proteins might be involved in non-target-site herbicide resistance. However, to date, participation in non-target herbicide resistance has been well established for only four gene families: P450s, GSTs, glycosyltransferases and ABC transporters.

Cytochrome P450 monooxygenases

The P450 gene family encodes the most versatile class of enzymes involved in the phase I metabolic detoxification of herbicides. P450s encode the heme-protein-dependent enzyme systems that normally catalyze oxygen- and NADPH-dependent mono-oxygenation reactions (Figure 1). Electrons from NADPH are singly transferred to P450s via cytochrome P450 reductase. The variety of reactions mediated by P450s, including hydroxylations, epoxidations, dealkylations, isomerizations, decarboxylations and deaminations [20,26], results in oxygenated products that are normally more reactive or more soluble, thus setting the stage for subsequent detoxification reactions [27].

Plant P450 genes surpass those of animals both in number and diversity, which is assumed to be of adaptive significance for a sessile lifestyle. P450 gene diversity facilitates the processing of a wide range of chemicals, an important consideration with regard to the involvement of this gene family in herbicide resistance. *Arabidopsis* has 246 P450 genes and 26 pseudogenes comprising 44 subfamilies. Comparative genomic analysis of P450 genes reveals that the gene family is also diverse in other species [28]. Gene duplications and divergence, particularly the multiple local tandem duplications, indicate a dynamic evolution of P450 genes, which might allow for rapid evolution of substrate specificity as well as regulation of expression. In addition to genome diversity, the evidence for diverse substrate specificity of single P450 enzymes is

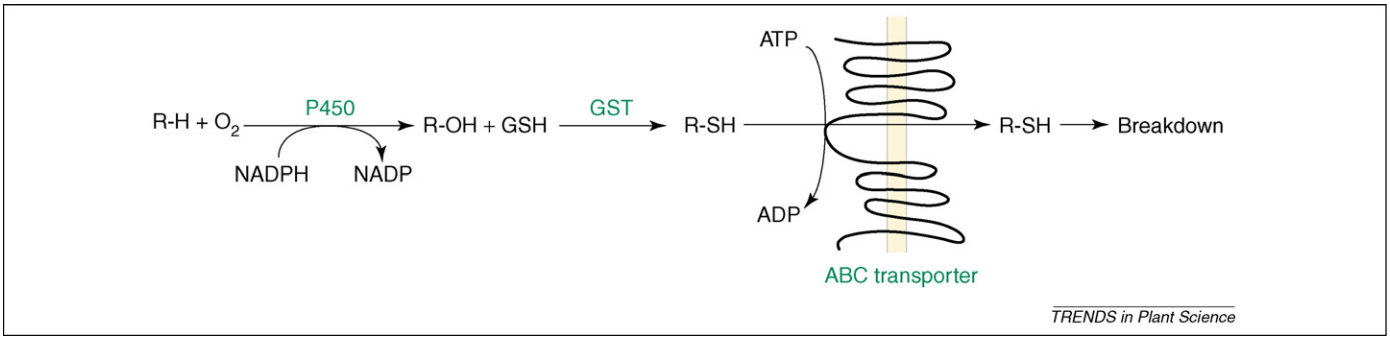

Figure 1. Schema of P450, GST, glycosyltransferase and ABC transporter gene-encoded resistance activities. The schema falls into a four-step detoxification process, the monooxygenase activity for P450 genes, the GST conjugation reaction for GST genes, the ATP-dependent transport of small molecules via ABC transporters, followed by subsequent detoxification in vacuoles.

Table 1. Non-target-site herbicide resistances mediated by P450s, GSTs or glycosyltransferases[a]

Gene family	Common name	Binomial	Herbicide	Refs
P450	Blackgrass	*Alopecurus myosuroides*	Chlorotoluron	[26]
P450	Chickweed	*Stellaria media*	Mecaprop	[74]
P450	Rigid ryegrass	*Lolium rigidum*	Diclofop-methyl	[75]
P450	Rigid ryegrass	*Lolium rigidum*	Diuron, atrazine, simazine	[76]
P450	Rigid ryegrass	*Lolium rigidum*	Chlorsulfuron	[77]
P450	Blackgrass	*Alopecurus myosuroides*	Chlorotoluron	[78]
P450	Sterile oat	*Avena sterilis*	Diclofop-methyl	[79]
P450	Littleseed canarygrass	*Phalaris minor*	Isoproturon	[80]
P450	Wild mustard	*Sinapis arvensis* (syn. *Brassica kaber*)	Ethametsulfuron-methyl	[81]
P450	Large crabgrass	*Digitaria sanguinalis*	Fluazifop-P-butyl	[82]
P450	Blackgrass	*Alopecurus myosuroides*	Flenoxaprop-p-ethyl	[31]
P450	Ryegrass	*Lolium* spp.	Diclofop-methyl	[83]
P450	Italian ryegrass	*Lolium multiflorum*	Diclofop-methyl, fluazifop-P-butyl, tralkoxydim, isoproturon, cycloxydim	[30]
P450	Blackgrass	*Alopecurus myosuroides*	Aryloxyphenoxypropionates (fops), flupyrsulfuron, fenoxaprop-P, ureas, haloxyfop, clodinafop	[31]
P450	Late watergrass	*Echinochloa phyllopogon*	Bispyribac-sodium, fenoxaprop-ethyl, thiobencarb	[21]
GST	Velvetleaf	*Abutilon theophrasti*	Atrazine	[22,84,85]
GST	Blackgrass	*Alopecurus myosuroides*	Fenoxaprop-ethyl and moderately resistant to chlorotoluron, diclofop-methyl, fluazifop-P-butyl and tralkoxydim	[38,39]
GST	Italian ryegrass	*Lolium multiflorum*	Diclofop-methyl, fluazifop-P-butyl, tralkoxydim, isoproturon, cycloxydim	[30]
GST	Blackgrass	*Alopecurus myosuroides*	Aryloxyphenoxypropionates (fops), flupyrsulfuron, fenoxaprop-P, ureas, haloxyfop, clodinafop	[31]
Glycosyl-transferase	Blackgrass	*Alopecurus myosuroides*	Multiple herbicides including 2,4,5-TCP and others	[55]

Abbreviation: TCP, trichlorophenol.
[a]Only the cases with confirmed enzyme activity correlating to the herbicide resistance are included in this table.

another important consideration in assessing the role of P450s in the evolution of herbicide resistance.

The function of P450s in non-target herbicide resistance has been well established through the correlation of P450 enzyme activity with herbicide resistance in weeds. P450 involvement in blackgrass (*Alopecurus myosuroides*) resistance to chlorotoluron was identified through exogenous application of a P450 enzyme inhibitor and by analyzing the herbicide metabolites that accumulated following herbicide treatment [29]. Similar approaches have been used to identify other cases in which P450s mediated non-target-site herbicide resistance (Table 1). There is also data to suggest that P450s are involved in metabolic cross resistance, which significantly contributes to the difficulty in managing weed populations with non-target resistance mechanisms [21,30,31]. Moreover, research studies have also indicated the coordination of P450 enzymes with Phase II detoxifying enzymes such as GSTs and glycosyltransferases [30–32]. Further evidence for the involvement of P450s in non-target herbicide resistance comes from safener application data. Safeners are chemicals added to herbicides to protect some crops from herbicide damage by mostly unknown, but non-target mechanisms. Safener application can induce expression in some P450 genes along with other detoxifying genes including GSTs and ABC transporters in maize.

In addition to the correlative data in weeds, more direct evidence for P450 enzyme involvement in non-target herbicide resistance comes from cloning and characterizing P450 genes and characterizing the protein–herbicide interactions in non-weedy species. One of the first P450 genes identified for herbicide resistance was cloned from Jerusalem artichoke *Helianthus tuberosus* [33]. The cytochrome P450 CYP76B1 can be strongly induced by herbicides and other xenobiotics and it catalyzes double N-dealkylation reactions metabolizing a variety of xenobiotics and several phenylurea herbicides [33]. Subsequently, several plant P450 gene products were shown to detoxify a range of herbicides in crop and model plant species [26,27].

Even though no P450 genes have yet been identified from herbicide-resistant weeds, P450 genes from other species have been used to engineer herbicide-resistant crops. For example, the overexpression of a yeast CYP51A1 in tobacco caused a bypass of the endogenous sterol biosynthesis pathway conferring resistance to triazole herbicides [34]. Since these early experiments, several transgenic plants with P450-based herbicide resistance have been produced (Table 2). Mammalian P450 genes have become recent favorites for overexpression to produce herbicide resistance in transgenic crops [26]. The transgenic work also helped to elucidate the mechanism behind P450 enzyme-induced herbicide resistance in weeds because P450s in both transgenic plants and naturally occurring in weeds have resulted in resistance to multiple herbicides (Tables 1 and 2). It was previously shown that several P450 genes might be involved in multiple herbicide resistance, and it has been demonstrated that a single P450 gene in transgenic plants can confer resistance to up to 13 different herbicides [20,35].

Glutathione *S*-transferases

A second well-established non-target herbicide resistance gene family is the glutathione *S*-transferase (GST) gene family. Plant GSTs are multifunctional enzymes that catalyze the conjugation of glutathione (γ-glutamyl-cysteinyl-glycine) or homoglutathione (in legumes) to various substrates (R-X) to form a polar *S*-glutothionylated product

Table 2. Herbicide-resistant transgenic plants with increased P450, GST or ABC transporter gene expression

Gene family	Donor species	Recipient	Gene	Herbicides	Refs
P450	Yeast	Tobacco	CYP51A1	γ-Ketotriazole	[34]
P450	Soybean	Tobacco	CYP71A10	Fluometuron, linuron, chlorotoluron, diuron	[86]
P450	Human	Tobacco	CYP1A1	Chlorotoluron	[87]
P450	Jerusalem artichoke	Tobacco and *Arabidopsis thaliana*	CYP76B1	Chlorotoluron	[88]
P450	Rat	Potato	CYP1A1	Atrazine, chlorotoluron	[89,90]
P450	Human	Rice	CYP2B6	Thirteen herbicides	[35]
GST	Maize	Wheat	GST-27	Atrazine, oxyfluorfen	[52]
GST	Soybean	Tobacco	GmGSTU21 and GmhGS	Fomesafen	[53]
GST	Maize	Tobacco	GST I	Alachlor	[51]
ABC transporter	*Ochrobactrum anthropi*	Tobacco	PqrA	Paraquat	[91]
ABC transporter	Pea and *A. thaliana*	*A. thaliana*	AtPgp1 and psNTP9	Dicamba, pendimethalin, oryzalin, MSMA	[70]

Abbreviation: MSMA, monosodium methane arsonate.

(R-SG) (Figure 1). Because the R-X substrates are often hydrophobic and electrophilic toxic chemicals, GSTs are considered to be important detoxification components involved in Phase II detoxification, even though GSTs might directly conjugate herbicides too. The conjugation reaction leads to R-SG products that are often transported into the vacuole by Phase III proteins such as ABC transporters [24,36]. As with P450s, the diversity and dynamic evolution of the GST gene family allow them to detoxify a wide range of chemicals and be involved in the synthesis of diverse secondary metabolites.

GSTs were first implicated in herbicide resistance in weeds in the 1970s, when a relationship was elucidated between a GSH conjugate and atrazine resistance in several grasses [37]. Further evidence that GSTs are involved in non-target-site herbicide resistance comes from GST activity assays in herbicide-resistant weeds. GST activity is normally studied by using a model substrate, such as 1-chloro-2,4-dinitrobenzene (CDNB), whereby the conjugation of GST with artificial substrates is detectable by light absorbance. Correlations between herbicide resistance in a weed and increased GST activity were established in velvetleaf (*Abutilon theophrasti*), in which increased glutathione conjugation of atrazine was observed in the resistant biotype [22]. GST-mediated herbicide resistance can also target multiple herbicides, sometimes even more diverse than P450-mediated resistance [30,31,38–40]. In some cases, an increase in GST activity is accompanied by increased GST gene expression [38,41], whereas in other cases, herbicide resistance has resulted from an increase in GST enzyme activity. As with P450s, further evidence for GST-mediated non-target herbicide resistance comes from safener application data that show that induced GST gene expression has been found [42–45]. Besides the studies in weeds, functional characterizations of GST genes in crops also indicate that GSTs have a role in herbicide metabolism. In 1979, a GST enzyme was purified and characterized for its activity toward herbicide detoxification [46]. Subsequently, many GST enzymes were purified and characterized for their activity on a variety of herbicides in soybean, wheat, maize and other crops [47–49]. Recently, comparative analysis of rice genomic sequences with maize and wheat GSTs led to the molecular cloning of a rice GST gene whose product had activity toward chloroacetanilide herbicides [50]. A transgenic approach has been useful to study the overexpression of GST genes to confer herbicide resistance [51–53] (Table 2). In one case, increased resistance resulted from coordinated overexpression of both GST and thiol synthase (e.g. homoglutathione synthase) genes because GST activity requires available thiol [53].

Glycosyltransferases

In addition to GSTs, glycosyltransferases are another family of enzymes involved in Phase II herbicide detoxification. Like GSTs, glycosyltransferases can conjugate herbicides directly. Glycosyltransferases comprise a large gene family in which proteins conjugate a sugar molecule to a wide range of lipophilic small molecule acceptors including plant hormones, secondary metabolites, and xenobiotics such as herbicides [23]. Glycosylation can occur at -OH, -COOH, -NH_2, and -SH, and glycosyltransferases can, thus, be classified as a *O*-glycosyltransferase or a *N*-glycosyltransferase according to the acceptor of glycosylation [54]. Both *O*-glycosyltransferases and *N*-glycosyltransferases have been suggested for their roles in herbicide detoxification [54,55]. The conjugation reactions enable glycosyltransferases 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. Glycosyltransferases exist as a gene superfamily with diverse members. They are found in all kingdoms and can be classified into 78 subfamilies. As for GSTs and P450s, diversity is an important consideration for glycosyltransferase-mediated non-target herbicide resistance because it enables the enzymes to use a wide range of sugar acceptors including herbicides [23,54].

Glycosyltransferases have been shown to detoxify a variety of toxic chemicals, including pollutants and herbicides [55–59]. The first evidence that glycosyltransferases have a role in non-target-site herbicide resistance in weeds was induced glycosyltransferase activity in multiple herbicide-resistant *Alopecurus myosuroides* [55]. Further evidence of glycosyltransferase conferring herbicide resistance was obtained by the characterization of glycosyltransferases with activity toward herbicides in crop and model species. In 1992, two soybean glycosyltransferases were shown to glycosylate the primary major bentazone metabolite, 6-hydroxybentazone [60]. Additional glycosyltransferases have since been cloned and characterized for their activity toward herbicides such

as 2,4,5-trichlorophenol [57,61]. As with P450s, these are also among the genes induced by safener application, which also indicates a role in herbicide detoxification [42,62].

ABC transporters

In contrast to the P450, GST and glycosyltransferase gene families, which are involved in herbicide biochemical modification through metabolism, ABC transporters confer herbicide resistance through compartmenting herbicides and their metabolites, which can be considered as Phase III detoxification (Figure 1). ABC transporters are targeted to membranes and have one or two ATP binding cassettes for active transport using ATP hydrolysis. In higher plants, ABC transporters have been characterized for a wide range of functions including excretion of toxic compounds, sequestration of secondary metabolites, translocation of fatty acids and phospholipids, as well as transporting of chlorophyll catabolites, auxins and heavy metals to maintain cell homeostasis [63]. ABC transporters are also one of the most diverse gene families and far surpass those found in non-plant species, both in number and in diversity. Substrate diversity is an important consideration of the function for ABC transporters in herbicide resistance.

Even though herbicide metabolites have long been identified in plant vacuoles, limited research has linked ABC transporters with non-target herbicide resistance in weeds. Nonetheless, ABC transporter activity toward herbicide metabolites has been well established in model species and crop plants. Plant ABC transporters were shown to be able to transport glutathionate-conjugated chemicals in 1993 [16]. A similar experiment showed that the glucoside conjugate of the herbicide derivative (5-hydroxyphenyl) primisulfuron could be sequestered in barley mesophyll vacuoles via ABC transporters [64]. AtMRP1 was cloned and characterized as the first ABC transporter gene shown to be able to transport the GS-conjugated herbicide metolachlor [65]. In addition, several ABC transporters from *Arabidopsis thaliana* and other species have been shown to transport different herbicides and herbicide metabolites [25,63,66]. Interestingly, reduced translocation has been indicated as one mechanism for glyphosate resistance [67,68], and this might involve ABC transporters (C.N. Stewart Jr *et al.* unpublished). ABC transporters can also be upregulated upon safener application, thereby resulting in coordinated overexpression of glutathione-conjugates, ABC transporters and GST enzymes [44]. A GS-conjugate-transporting ABC transporter AtOPT6 was also up-regulated in response to the herbicide primisulfuron [69]. Recent experiments have also indicated that glyphosate upregulates several *Conyza canadensis* ABC transporters in resistant biotypes only (C.N. Stewart Jr *et al.*, unpublished). More direct evidence of ABC transporter-mediated herbicide resistance comes from genetic engineering experiments. Overexpression of AtPgp1, a multi-drug-resistant family member, and its garden pea homolog psNTP9 have been shown to confer multi-herbicide resistance in *Arabidopsis* [70]. Besides herbicide resistance, kanamycin resistance has also been found in transgenic plants overexpressing an ABC transporter gene [71].

Coordination among the detoxifying gene family proteins

Transgenic expression of a single gene in the P450, GST, glycosyltransferase and ABC transporter gene families can induce herbicide resistance, which indicates that non-target-site herbicide resistance can be monogenic [26,70]. However, there is evidence that coordinated regulation of detoxifying genes confers herbicide resistance in weeds. Safener application can lead to a co-upregulation of P450, GST, glycotransferase and ABC transporter genes, indicating a coordinated detoxification process [42,62,64,72]. For example, in *Alopecurus myosuroides*, the conjugation of diclofop-methyl with glucose in a resistant biotype was found to be greatly reduced upon treatment with the P450 monooxygenase inhibitor 1-aminobenzotriazole, indicating that P450-based oxidation might be a pre-requirement for glycosylation [32]. Recently, enzyme activity assays revealed that a resistant *A. myosuroides* biotype lost resistance upon treatment with a P450 inhibitor, whereas two other biotypes lost resistance only upon treatment with both a P450 inhibitor and a GST inhibitor [31]. For Phase II and III coordination, GSTs and glycosyltransferases normally first conjugate the toxin or herbicide and then the conjugated product can be transported into vacuoles by an ABC transporter with specificity to the GSH or sugar conjugate. It has been shown that glycosylation is required for transport of chlorsulfuron-derived 5-hydroxychlorsulfuron into vacuoles via ABC transporters, and glutathionation is required for transport of chlorimuron-ethyl metabolites into vacuoles via ABC transporters [18]. Overall, it seems that a coordinated increase in the activity of detoxifying enzymes is one of the main mechanisms of non-target herbicide resistance. Moreover, the non-target herbicide resistance mechanism might also work with the target mechanisms to promote the survival of the weeds under herbicide treatment [8].

Integrated genomics approach to dissect non-target herbicide resistance

Most of the non-target-site herbicide resistance cases have been established using enzyme assays and metabolite analysis, but few resistance genes have been actually cloned and characterized from weeds (discounting *Arabidopsis*, which we do not consider to be a weed [1]). Many important questions regarding the mechanisms of non-target herbicide resistance remain unanswered. Does resistance result from gene transcriptional regulation, an increase in enzyme activity, altered substrate specificity, or some combination of these three? Does increased enzyme activity involve a site mutation? If so, what is the mutation, and how does it confer stronger activity? It is important to answer these questions to develop more effective herbicides, to understand the evolution of resistance for management, and to gain insights into similar physiological processes useful in phytoremediation and xenobiotic detoxification. Gene discovery is the key to answering these questions. The main obstacle for cloning and characterizing non-target herbicide resistance genes in weeds is the limited genomic and genetic resources from weedy species. Chhandak Basu *et al.* first proposed a comprehensive package for weed genomics [1]. A functional

genomics approach has recently been successfully applied in herbicide resistance studies and led to the identification of several resistance genes [45,62,73]. We hereby propose an integrated functional genomics approach to identify genes involved in non-target herbicide resistance in weedy species (Box 1) to address this global threat to weed management sustainability. P450, GST, glycosyltransferase and ABC transporter gene families have been implicated in non-target herbicide resistance. There is only limited genomic information about weedy species and, as a result, few gene candidates from weedy species have been identified and characterized for their role in non-target herbicide resistance. Given that non-target herbicide resistance is an ever-growing problem in

Box 1. An integrated functional genomics strategy for non-target gene discovery in herbicide resistant weeds

The genome of a weedy species has not been sequenced yet; therefore, we believe that the most useful information might come from sequencing ESTs from cDNA libraries of model weeds [1]. Basically, cDNA libraries could be constructed from herbicide-resistant (R biotype) and non-resistant (S biotype) biotypes of weeds such as *Conyza canadensis* or *Amaranthus* species (Figure I). In addition, comparisons could be made between cDNA libraries taken from herbicide-treated versus non-treated resistant biotypes. A sequence-tag-based selection could help to select for GST, P450, glycosyltransferase and ABC transporter gene families. The cDNA library and the sequences might be useful in several ways. First, the sequences could be used for comparative genome analysis based on sequences from known species for ortholog identification. Moreover, protein structure modeling might be accomplished if full-length cDNA sequence is available. Second, the cDNA libraries could be used to produce cDNA-based microarrays. If enough sequence information is available, the sequence information could be assembled into unigene sets for designing long-oligo arrays. Microarray analysis, the most powerful functional genomics approach for global gene profiling, has been widely applied in pathological, physiological and developmental studies. Microarrays could be used to study the gene profiling of relevant gene families in response to different herbicide doses in comparisons of resistant and non-resistant plants, thus helping to unravel non-target herbicide resistance mechanisms. Third, EST sequences from cDNA libraries might be compared directly for different frequencies in herbicide-resistant and non-resistant weeds under herbicide treatment. The comparative approach is an expensive version of gene profiling but might also identify candidate genes for further study. This approach would be supported by new high-throughput sequencing technologies. Once a candidate gene has been identified by one of the above methods, the full-length cDNA of the gene could be cloned by 5′ RACE and 3′ RT-PCR if the full-length sequence is not available in the cDNA library. The cloned gene could be further characterized through biochemical-, vesicle transport-, and transgenic overexpression analysis. Besides a cDNA-based genomics study, vacuole metabolite profiling could also provide important complementary information. Vacuole metabolite analysis has helped to identify herbicide conjugates, and the comparative metabolite profiling of herbicide-resistant and non-resistant weed biotypes should help to identify herbicide metabolites and, thus, predict the possible pathway(s) involved in the detoxification process. The metabolite profiling data could be integrated with the microarray gene profiling data to correlate the metabolic products with the pathways involved [92].

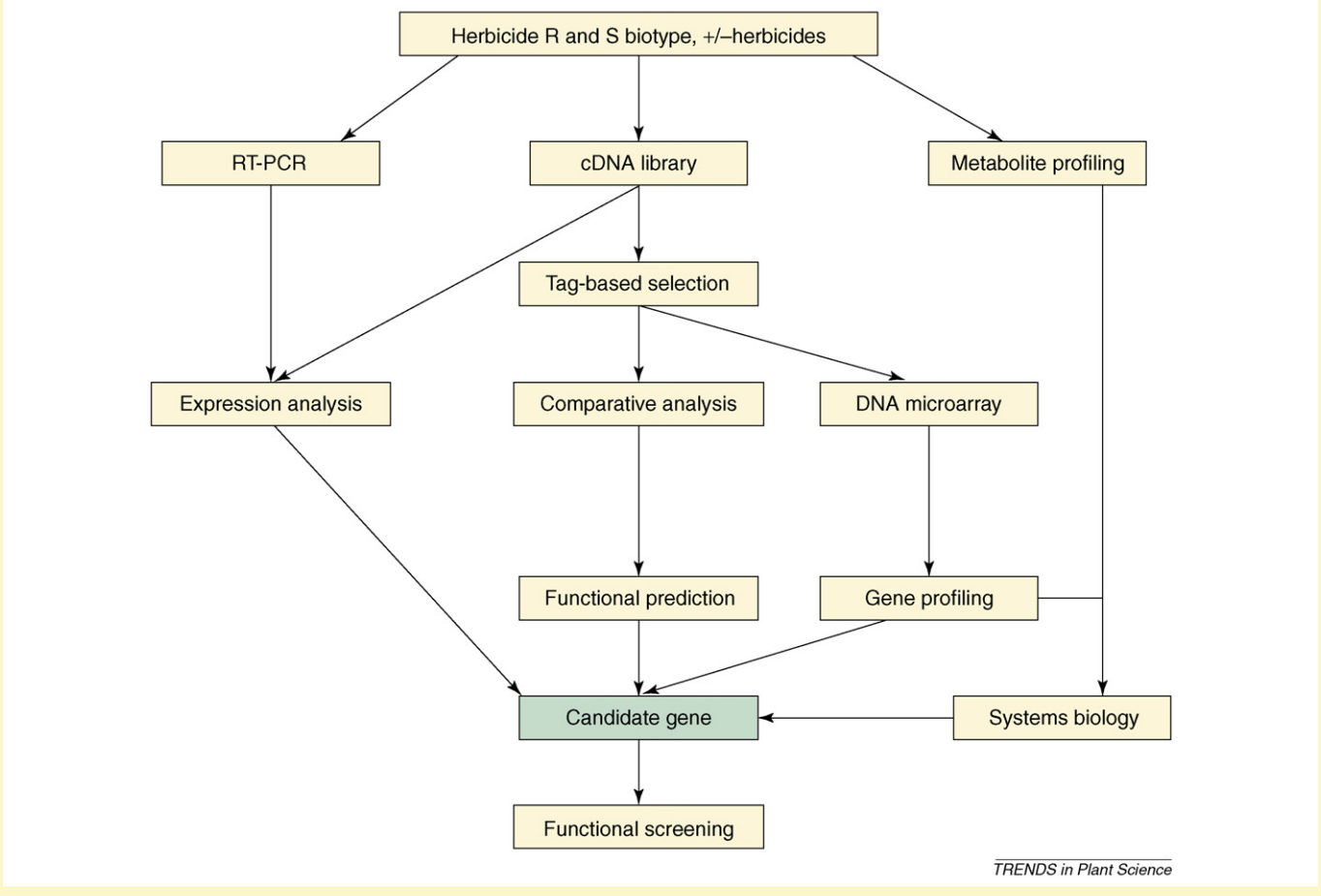

Figure I.

agriculture, it is important to begin integrated functional genomics research to develop tools as part of the solution. Considering the importance of non-target site herbicide resistance in agriculture, we expect a strong return on investment to the farmer and consumer from the development of genomic resources and approaches to study this growing problem.

Acknowledgements

We thank Tennessee Agriculture Experimental Station for support. We deeply appreciate the insightful suggestions of Jonathan Gressel and two anonymous reviewers.

References

1 Basu, C. *et al.* (2004) Weed genomics: new tools to understand weed biology. *Trends Plant Sci.* 9, 391–398
2 Dill, G.M. (2005) Glyphosate-resistant crops: history, status and future. *Pest Manag. Sci.* 61, 219–224
3 Watkinson, A.R. *et al.* (2000) Predictions of biodiversity response to genetically modified herbicide-tolerant crops. *Science* 289, 1554–1557
4 Van Gessel, M.J. (2001) Glyphosate-resistant horseweed from Delaware. *Weed Sci.* 49, 703–705
5 Rieger, M.A. *et al.* (2002) Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science* 296, 2386–2388
6 Owen, M.D. and Zelaya, I.A. (2005) Herbicide-resistant crops and weed resistance to herbicides. *Pest Manag. Sci.* 61, 301–311
7 Preston, C. (2004) Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. *Weed Sci.* 52, 448–453
8 Dinelli, G. *et al.* (2006) Physiological and molecular insight on the mechanisms of resistance to glyphosate in *Conyza canadensis* (L.) Cronq. biotypes. *Pest. Biochem. Physiol.* 86, 30–42
9 Tranel, P.J. and Wright, T.R. (2002) Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci.* 50, 700–712
10 Gardner, S.N. *et al.* (1998) A revolving dose strategy to delay the evolution of both quantitative vs. major monogene resistances to pesticides and drugs. *Int. J. Pest Manage.* 44, 161–180
11 Gressel, J. (2002) *Molecular Biology of Weed Control,* Taylor & Francis
12 Gressel, J. (1997) Genetic engineering can either exacerbate or alleviate herbicide resistance, In *Proceedings of the 50th NZ Plant Protection Conference 1997*, pp. 298–306, The New Zealand Plant Protection Society (http://www.hortnet.co.nz/publications/nzpps/journal/50/nzpp50_298.pdf)
13 McCourt, J.A. *et al.* (2006) Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. *Proc. Natl. Acad. Sci. U. S. A.* 103, 569–573
14 Preston, C. *et al.* (1996) Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. *Pestic. Biochem. Physiol.* 54, 123–134
15 Shimabukuro, R.H. *et al.* (1971) Glutathione conjugation: an enzymatic basis for atrazine resistance in corn. *Plant Physiol.* 47, 10–14
16 Martinoia, E. *et al.* (1993) ATP-dependent glutathione S-conjugate export pump in the vacuolar membrane of plants. *Nature* 364, 247–249
17 Sandermann, H. (2004) Molecular ecotoxicology of plants. *Trends Plant Sci.* 9, 406–413
18 Bartholomew, D.M. *et al.* (2002) Alternate energy-dependent pathways for the vacuolar uptake of glucose and glutathione conjugates. *Plant Physiol.* 130, 1562–1572
19 Werck-Reichhart, D. *et al.* (2000) Cytochromes for engineering herbicide tolerance. *Trends Plant Sci.* 5, 116–123
20 Schuler, M.A. and Werck-Reichhart, D. (2003) Functional genomics of P450s. *Annu. Rev. Plant Biol.* 54, 629–667
21 Yun, M.S. *et al.* (2005) Cytochrome P-450 monooxygenase activity in herbicide-resistant and -susceptible late watergrass (*Echinochloa phyllopogon*). *Pestic. Biochem. Physiol.* 83, 107–114
22 Anderson, M.P. and Gronwald, J.W. (1991) Atrazine resistance in a velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione S-transferase activity. *Plant Physiol.* 96, 104–109
23 Bowles, D. *et al.* (2005) Glycosyltransferases: managers of small molecules. *Curr. Opin. Plant Biol.* 8, 254–263
24 Reade, J.P.H. *et al.* (2004) A role for glutathione S-transferases in resistance to herbicides in grasses. *Weed Sci.* 52, 468–474
25 Klein, M. *et al.* (2006) The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. *FEBS Lett.* 580, 1112–1122
26 Inui, H. and Ohkawa, H. (2005) Herbicide resistance in transgenic plants with mammalian monooxygenase genes. *Pest Manag. Sci.* 61, 286–291
27 Morant, M. *et al.* (2003) Plant cytochromes: tools for pharmacology, plant protection and phytoremediation. *Curr. Opin. Biotechnol.* 14, 151–162
28 Nelson, D.R. *et al.* (2004) Comparative genomics of rice and *Arabidopsis*. Analysis of 727 cytochrome genes and pseudogenes from a monocot and a dicot. *Plant Physiol.* 135, 756–772
29 Kemp, M.S. *et al.* (1990) Herbicide resistance in *Alopecurus myosuroides*. In *Managing Resistance to Agrochemicals: From Fundamental Research to Practical Strategies* (Green, M.B. *et al.*, eds), pp. 376–393, Washington, D.C., American Chemical Society
30 Cocker, K.M. *et al.* (2001) Resistance to ACCase-inhibiting herbicides and isoproturon in UK populations of *Lolium multiflorum*: mechanisms of resistance and implications for control. *Pest Manag. Sci.* 57, 587–597
31 Letouze, A. and Gasquez, J. (2003) Enhanced activity of several herbicide-degrading enzymes: a suggested mechanism responsible for multiple resistance in blackgrass (*Alopecurus myosuroides Huds.*). *Agronomie* 23, 601–608
32 Menendez, J. and DePrado, R. (1996) Diclofop-methyl cross-resistance in a chlorotoluron-resistant biotype of *Alopecurus myosuroides*. *Pestic. Biochem. Physiol.* 56, 123–133
33 Robineau, T. *et al.* (1998) The chemically inducible plant cytochrome CYP76B1 actively metabolizes phenylureas and other xenobiotics. *Plant Physiol.* 118, 1049–1056
34 Grausem, B. *et al.* (1995) Functional expression of *Saccharomyces cerevisiae* CYP51A1 encoding lanosterol-14-demethylase in tobacco results in bypass of endogenous sterol biosynthetic pathway and resistance to an obtusifoliol-14-demethylase herbicide inhibitor. *Plant J.* 7, 761–770
35 Hirose, S. *et al.* (2005) Transgenic rice containing human CYP2B6 detoxifies various classes of herbicides. *J. Agric. Food Chem.* 53, 3461–3467
36 Dixon, D.P. *et al.* (2002) Plant glutathione transferases. *Genome Biol.* 3, REVIEWS3004
37 Jensen, K.I.N. *et al.* (1977) Detoxification of atrazine in three Gramineae subfamilies. *Weed Sci.* 25, 212–220
38 Cummins, I. *et al.* (1999) A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. *Plant J.* 18, 285–292
39 Hall, L.M. *et al.* (1997) Mechanisms of resistance to aryloxyphenoxypropionate herbicides in two resistant biotypes of *Alopecurus myosuroides* (blackgrass): herbicide metabolism as a cross-resistance mechanism. *Pestic. Biochem. Physiol.* 57, 87–98
40 Hatton, P.J. *et al.* (1999) Glutathione transferases involved in herbicide detoxification in the leaves of *Setaria faberi* (*giant foxtail*). *Physiol. Plant.* 105, 9–16
41 Cummins, I. *et al.* (1997) Glutathione transferases in herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). *Pestic. Sci.* 51, 244–250
42 Hatzios, K.K. and Burgos, N. (2004) Metabolism-based herbicide resistance: regulation by safeners. *Weed Sci.* 52, 454–467
43 Smith, A.P. *et al.* (2004) Proteomic analysis of *Arabidopsis* glutathione S-transferases from benoxacor- and copper-treated seedlings. *J. Biol. Chem.* 279, 26098–26104
44 DeRidder, B.P. and Goldsbrough, P.B. (2006) Organ-specific expression of glutathione S-transferases and the efficacy of herbicide safeners in *Arabidopsis*. *Plant Physiol.* 140, 167–175
45 Zhang, O. and Riechers, D.E. (2004) Proteomic characterization of herbicide safener-induced proteins in the coleoptile of *Triticum tauschii* seedlings. *Proteomics* 4, 2058–2071
46 Guddewar, M.B. and Dauterman, W.C. (1979) Purification and properties of a glutathione-S-transferase from corn which conjugates S-triazine herbicides. *Phytochemistry* 18, 735–740

47 Cummins, I. *et al.* (1997) Purification of multiple glutathione transferases involved in herbicide detoxification from wheat (*Triticum aestivum* L.) treated with the safener fenchlorazole-ethyl. *Pestic. Biochem. Physiol.* 59, 35–49

48 Dixon, D. *et al.* (1997) Characterisation of multiple glutathione transferases containing the GST I subunit with activities toward herbicide substrates in maize (*Zea mays*). *Pestic. Sci.* 50, 72–82

49 Andrews, C.J. *et al.* (2005) Purification and characterisation of a family of glutathione transferases with roles in herbicide detoxification in soybean (*Glycine max L.*); selective enhancement by herbicides and herbicide safeners. *Pestic. Biochem. Physiol.* 82, 205–219

50 Cho, H.Y. and Kong, K.H. (2005) Molecular cloning, expression, and characterization of a phi-type glutathione S-transferase from *Oryza sativa*. *Pestic. Biochem. Physiol.* 83, 29–36

51 Karavangeli, M. *et al.* (2005) Development of transgenic tobacco plants overexpressing maize glutathione S-transferase I for chloroacetanilide herbicides phytoremediation. *Biomol. Eng.* 22, 121–128

52 Milligan, A.S. *et al.* (2001) The expression of a maize glutathione S-transferase gene in transgenic wheat confers herbicide tolerance, both *in planta* and *in vitro*. *Mol. Breed.* 7, 301–315

53 Skipsey, M. *et al.* (2005) Manipulation of plant tolerance to herbicides through co-ordinated metabolic engineering of a detoxifying glutathione transferase and thiol cosubstrate. *Plant Biotech. J.* 3, 409–420

54 Bowles, D. *et al.* (2006) Glycosyltransferases of lipophilic small molecules. *Annu. Rev. Plant Biol.* 57, 567–597

55 Brazier, M. *et al.* (2002) O-glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible blackgrass (*Alopecurus myosuroides*). *Phytochemistry* 59, 149–156

56 Brazier, M. *et al.* (2003) Partial purification and characterisation of a 2,4,5-trichlorophenol detoxifying O-glucosyltransferase from wheat. *Phytochemistry* 64, 419–424

57 Loutre, C. *et al.* (2003) Isolation of a glucosyltransferase from *Arabidopsis thaliana* active in the metabolism of the persistent pollutant 3,4-dichloroaniline. *Plant J.* 34, 485–493

58 Messner, B. *et al.* (2003) *Arabidopsis* glucosyltransferases with activities toward both endogenous and xenobiotic substrates. *Planta* 217, 138–146

59 Poppenberger, B. *et al.* (2003) Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *J. Biol. Chem.* 278, 47905–47914

60 Leah, J.M. *et al.* (1992) Isolation and characterization of 2 glucosyltransferases from *Glycine max* associated with bentazone metabolism. *Pest. Sci.* 34, 81–87

61 Brazier-Hicks, M. and Edwards, R. (2005) Functional importance of the family 1 glucosyltransferase UGT72B1 in the metabolism of xenobiotics in *Arabidopsis thaliana*. *Plant J.* 42, 556–566

62 Edwards, R. *et al.* (2005) Differential induction of glutathione transferases and glucosyltransferases in wheat, maize and *Arabidopsis thaliana* by herbicide safeners. *Zeitschrift Fur Naturforschung C* 60, 307–316

63 Schulz, B. and Kolukisaglu, H.U. (2006) Genomics of plant ABC transporters: the alphabet of photosynthetic life forms or just holes in membranes? *FEBS Lett.* 580, 1010–1016

64 Gaillard, C. *et al.* (1994) A herbicide antidote (safener) induces the activity of both the herbicide detoxifying enzyme and of a vacuolar transporter for the detoxified herbicide. *FEBS Lett.* 352, 219–221

65 Lu, Y.P. *et al.* (1997) AtMRP1 gene of *Arabidopsis* encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. *Proc. Natl. Acad. Sci. U. S. A.* 94, 8243–8248

66 Liu, G. *et al.* (2001) Enhanced multispecificity of *Arabidopsis* vacuolar multidrug resistance-associated protein-type ATP-binding cassette transporter, AtMRP2. *J. Biol. Chem.* 276, 8648–8656

67 Feng, P.C.C. *et al.* (2004) Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. *Weed Sci.* 52, 498–505

68 Koger, C.H. and Reddy, K.N. (2005) Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). *Weed Sci.* 53, 84–89

69 Cagnac, O. *et al.* (2004) AtOPT6 transports glutathione derivatives and is induced by primisulfuron. *Plant Physiol.* 135, 1378–1387

70 Windsor, B. *et al.* (2003) Multiherbicide tolerance conferred by AtPgp1 and apyrase overexpression in *Arabidopsis thaliana*. *Nat. Biotechnol.* 21, 428–433

71 Mentewab, A. and Stewart, C.N., Jr (2005) Overexpression of an *Arabidopsis thaliana* ABC transporter confers kanamycin resistance to transgenic plants. *Nat. Biotechnol.* 23, 1177–1180

72 Persans, M.W. *et al.* (2001) Characterization of maize cytochrome monooxygenases induced in response to safeners and bacterial pathogens. *Plant Physiol.* 125, 1126–1138

73 Gachon, C.M.M. *et al.* (2005) Transcriptional co-regulation of secondary metabolism enzymes in *Arabidopsis*: functional and evolutionary implications. *Plant Mol. Biol.* 255, 229–245

74 Coupland, D. *et al.* (1990) Uptake, translocation, and metabolism of mecoprop in a sensitive and a resistant biotype of *Stellaria media*. *Pestic. Biochem. Physiol.* 36, 61–67

75 Christopher, J.T. *et al.* (1991) Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*): II. Chlorsulfuron resistance involves a wheat-like detoxification system. *Plant Physiol.* 95, 1036–1043

76 Burnet, M.W.M. *et al.* (1993) Increased detoxification is a mechanism of simazine resistance in *Lolium rigidum*. *Pestic. Biochem. Physiol.* 46, 207–218

77 Christopher, J.T. *et al.* (1994) Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. *Pestic. Biochem. Physiol.* 49, 172–182

78 Hall, L.M. *et al.* (1995) Mechanism of resistance to chlorotoluron in two biotypes of the grass weed *Alopecurus myosuroides*. *Pestic. Biochem. Physiol.* 53, 180–192

79 Maneechote, C. *et al.* (1997) A diclofop-methyl-resistant *Avena sterilis* biotype with a herbicide-resistant acetyl-coenzyme A carboxylase and enhanced metabolism of diclofop-methyl. *Pestic. Sci.* 49, 105–114

80 Singh, S. *et al.* (1998) Effect of ABT on the activity and rate of degradation of isoproturon in susceptible and resistant biotypes of *Phalaris minor* and in wheat. *Pestic. Sci.* 53, 123–132

81 Veldhuis, L.J. *et al.* (2000) Metabolism-based resistance of a wild mustard (*Sinapis arvensis* L.) biotype to *ethametsulfuron-methyl*. *J. Agric. Food Chem.* 48, 2986–2990

82 Hidayat, I. and Preston, C. (2001) Cross-resistance to imazethapyr in a fluazifop-P-butyl-resistant population of *Digitaria sanguinalis*. *Pestic. Biochem. Physiol.* 71, 190–195

83 Bravin, F. *et al.* (2001) Resistance to diclofop-methyl in two *Lolium* spp. populations from Italy: studies on the mechanism of resistance. *Weed Res.* 41, 461–473

84 Gray, J.A. *et al.* (1996) Increased glutathione conjugation of atrazine confers resistance in a Wisconsin velvetleaf (*Abutilon theophrasti*) biotype. *Pestic. Biochem. Physiol.* 55, 157–171

85 Plaisance, K.L. and Gronwald, J.W. (1999) Enhanced catalytic constant for glutathione S-transferase (atrazine) activity in an atrazine-resistant *Abutilon theophrasti* biotype. *Pestic. Biochem. Physiol.* 63, 34–49

86 Siminszky, B. *et al.* (1999) Expression of a soybean cytochrome monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1750–1755

87 Shiota, N. *et al.* (2000) Expression of human cytochromes 1A1 and 1A2 as fused enzymes with yeast NADPH-cytochrome oxidoreductase in transgenic tobacco plants. *Biosci. Biotechnol. Biochem.* 64, 2025–2033

88 Didierjean, L. *et al.* (2002) Engineering herbicide metabolism in tobacco and *Arabidopsis* with CYP76B1, a cytochrome enzyme from Jerusalem artichoke. *Plant Physiol.* 130, 179–189

89 Yamada, T. *et al.* (2002) Enhancement of metabolizing herbicides in young tubers of transgenic potato plants with the rat CYP1A1 gene. *Theor. Appl. Genet.* 105, 515–520

90 Yamada, T. *et al.* (2002) Inducible cross-tolerance to herbicides in transgenic potato plants with the rat CYP1A1 gene. *Theor. Appl. Genet.* 104, 308–314

91 Jo, J. *et al.* (2004) Paraquat resistance of transgenic tobacco plants over-expressing the *Ochrobactrum anthropi* pqrA gene. *Biotechnol. Lett.* 26, 1391–1396

92 Rischer, H. *et al.* (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. *Proc. Natl. Acad. Sci. U. S. A.* 103, 5614–5619