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Evolved Glyphosate Resistance in Plants: Biochemical and Genetic Basis of Resistance[1]

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Abstract: Resistance to the herbicide glyphosate is currently known in at least eight weed species from many countries. Some populations of goosegrass from Malaysia, rigid ryegrass from Australia, and Italian ryegrass from Chile exhibit target site–based resistance to glyphosate through changes at amino acid 106 of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. Mutations change amino acid 106 from proline to either serine or threonine, conferring an EPSPS weakly resistant to glyphosate. The moderate level of resistance is sufficient for commercial failure of the herbicide to control these plants in the field. Conversely, a nontarget site resistance mechanism has been documented in glyphosate-resistant populations of horseweed and rigid ryegrass from the United States and Australia, respectively. In these resistant plants, there is reduced translocation of glyphosate to meristematic tissues. Both of these mechanisms are inherited as a single, nuclear gene trait. Although at present only two glyphosate-resistance mechanisms are known, it is likely that other mechanisms will become evident. The already very large and still increasing reliance on glyphosate in many parts of the world will inevitably result in more glyphosate-resistant weeds, placing the sustainability of this precious herbicide resource at risk.

Nomenclature: Glyphosate; goosegrass, *Eleusine indica* (L.) Gaertn. #[3] ELEIN; horseweed, *Conyza canadensis* (L.) Cronq. # ERICA; rigid ryegrass, *Lolium rigidum* Gaud. # LOLRI; Italian ryegrass, *Lolium multiflorum* Lam. # LOLMU.

Additional index words: EPSPS, herbicide resistance, herbicide translocation.

Abbreviations: ACCase, acetyl-coenzyme A carboxylase; ALS, acetolactate synthase; ESPS, 5-enolpyruvylshikimate-3-phosphate synthase.

INTRODUCTION

Glyphosate is the world's most important herbicide because it is extremely versatile, controls a wide spectrum of annual and perennial weeds, has low mammalian toxicity, and has no soil activity. Since its 1974 introduction, glyphosate has grown to dominate world herbicide usage. Major agricultural uses of glyphosate include field crop weed control, intercrop row weed control, and weed control around perennial trees and vines. Major nonagricultural uses include weed control along roadsides, irrigation channels, in recreational areas, and for woody weed control. The pioneering work of scientists at the Monsanto Corporation, resulting in the commercialization of crops genetically modified to be glyphosate resistant, has been a development of major economic impact. Glyphosate-resistant crops (Roundup Ready[4]) express a bacterial EPSPS that is insensitive to glyphosate and sometimes express a gene endowing glyphosate metabolism (reviewed by Padgette et al. 1996).

Since 1996, the already high levels of glyphosate usage have dramatically increased with the adoption of genetically modified glyphosate-resistant crops. Glyphosate-resistant crops have been massively adopted in the Americas, with more than 80% of soybean [*Glycine max* (L.) Meer.] and cotton (*Gossypium hirsutum* L.) planted in the United States, 98% of soybean planted in Argentina, 99% of the soybean planted in Uruguay, and more than 60% of the soybean grown in Paraguay (James 2004) being glyphosate resistant. Of course, the widespread adoption of glyphosate-resistant crops has increased the use of glyphosate in these cropping systems as well as increasing the adoption of zero tillage, partic-

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[3] Letters following this symbol are a WSSA-approved computer code from *Composite List of Weeds,* Revised, 1989. Available only on computer disk from WSSA, 810 East 10th Street, Lawrence, KS 66044-8897.

[4] Roundup Ready, glyphosate-resistant crops, Monsanto Corporation, 800 North Lindbergh Boulevard, Saint Louis, MO 63167.

Table 1. Glyphosate-resistant weed populations and mechanisms of resistance identified in resistant populations.

Weed species	Scientific name	Country	Mechanisms of resistance identified[a]
Common ragweed	*Ambrosia artemisiifolia*	United States	—
Flaxleaf fleabane	*Conyza bonariensis*	South Africa	—
		Spain	—
Canada horseweed	*Conyza canadensis*	United States	Reduced translocation
Goosegrass	*Eleusine indica*	Malaysia	Target site
Italian ryegrass	*Lolium multiflorum*	Brazil	—
		Chile	Target site
		United States	—
Rigid ryegrass	*Lolium rigidum*	Australia	Reduced translocation
			Target site
		United States	Target site
		South Africa	—
Buckhorn plantain	*Plantago lanceolata*	South Africa	—

[a] Cells with a "—" have not yet had a mechanism of resistance identified.

ularly in canola (*Brassica napus* L.) (Devine and Buth 2001) and soybean crops (Gianessi et al. 2002). The combined effects of increasing glyphosate applications, glyphosate use later in the growing season, and adoption of zero tillage seeding creates a significantly increased risk of glyphosate resistance evolution (Neve et al. 2003b).

A relatively recent development has been the evolution of glyphosate-resistant weeds. When reviewed a decade ago, there were no cases of evolved glyphosate resistance, despite 20 yr of use (Dyer 1994). Because glyphosate had been used for 2 decades before the evolution of resistance was first published, there was, perhaps understandably, a degree of complacency about the evolution of resistance. Some considered the evolution of glyphosate resistance in weeds to be very unlikely (Bradshaw et al. 1997; Jasieniuk 1995; Waters 1991). The reasons for this, coherently argued by Bradshaw et al. (1997), were based on the observations that glyphosate resistance had not been documented after 20 yr of use, that plants do not readily metabolize glyphosate, and that site-directed plant EPSPS mutants had reduced enzyme activity and provided little resistance. Lastly, considerable effort, eventually requiring the sourcing of genes from bacteria, was required to create glyphosate-resistant crops that had commercial utility. However, as presented below, since the first reports of evolved glyphosate resistance, (Powles et al. 1998; Pratley et al. 1999) glyphosate resistance is now known in at least eight different weed species in many countries (Table 1) (see Heap 2005).

In light of these arguments, it is instructive to examine the glyphosate-resistance mechanisms that are known in weed populations. Genetically modified glyphosate-resistant crops are well covered by several reviews (see Padgette et al. 2000; Saroha et al. 1998) and will not be further considered here because this review focuses on the mechanisms and genetics of glyphosate resistance evident in weedy plant species. Equally, not covered in this review are studies on weedy plant species that are naturally tolerant of glyphosate (never or only ever partially controlled by glyphosate). For example, Weller and colleagues have identified a number of factors, which in combination, may contribute to glyphosate tolerance in field morningglory (*Convolvulus arvensis* L.) (Westwood and Weller 1997; Westwood et al. 1997; see also Yuan et al. 2002 for Chinese foldwing [*Dicliptera chinensis*]). Two resistance mechanisms that have now been clearly demonstrated in weed species that have evolved resistance to glyphosate in fields are a weak target site mutation and a reduced glyphosate translocation mechanism.

Target Site–Based Resistance to Glyphosate in Weedy Plant Species. With few exceptions, herbicides are toxic to susceptible plant species because they inhibit enzyme functions essential to plant survival. The specific plant enzyme inhibited by a particular herbicide is defined as the herbicide target site. Therefore, herbicides can be classified by the specific target site enzyme they inhibit. At the broad level, evolved herbicide resistance in plants can be separated into either target site or non-target site–based resistance (for definitions see Hall et al. 1994). Target site–based resistance is where resistance is provided by gene mutation conferring a change to a target site enzyme such that the herbicide no longer effectively inhibits the normal enzyme function. Resistance arises following the enrichment of the mutation in populations. The mutation is normally a specific nucleotide substitution within a crucial coding region encoding a different amino acid that results in a structural, charge, or hydrophobicity change in the herbicide target site en-

zyme rendering it less sensitive to inhibition by the herbicide. Of course, for any such mutation to be enriched in populations the altered target site enzyme must retain substantial enzyme functionality, and any adverse effects of the mutation on plant fitness must not be excessive.

Target site–based resistance has been documented in many weed species. For example, resistance to the triazine herbicides is frequently target site–based, mostly as a result of a single nucleotide change (mutation) of the *psbA* gene encoding a glycine-to-serine change at amino acid 264. This mutation provides high-level target site resistance to triazine herbicides and has evolved in many plant species in various parts of the world (reviewed by Gronwald 1994; Trebst 1996). Similarly, resistance to acetolactate synthase (ALS)–inhibiting herbicides is often target site–based, with several different functional mutations of the ALS gene characterized as endowing resistance (reviewed by Saari et al. 1994; Tranel and Wright 2002). Target site–based resistance to the acetyl coenzyme A carboxylase (ACCase)–inhibiting herbicides is also widespread (reviewed by Delye 2005; Devine 1997) and recently, mutations of the nuclear-encoded, plastid-expressed ACCase gene endowing target site resistance to ACCase herbicides have been identified (Brown et al. 2003; Christoffers et al. 2002; Delye et al. 2003, 2005; Zhang and Devine 2000).

The chloroplastic enzyme EPSPS has long been known to be the enzyme target site for glyphosate (Amhrein et al. 1983; Jaworkski 1982; Steinrucken and Amhrein 1980). EPSPS is the penultimate enzyme of the shikimate pathway and catalyzes the reversible reaction of shikimate-3-phosphate with phosphoenolpyruvate to produce EPSP and inorganic phosphate. Glyphosate is a specific and potent inhibitor of EPSPS (Steinrucken and Amhrein 1980). Considerable research has elucidated the precise nature by which glyphosate interacts with EPSPS (see Schönbrunn et al. 2001). Inhibition of EPSPS results in the accumulation of shikimic acid; the plant is starved of EPSP and ensuing metabolic products, such as the aromatic amino acids phenylalanine, tyrosine, and tryptophan (see review by Herrman and Weaver 1999).

The first published examples of evolved target site–based glyphosate resistance were Malaysian populations of goosegrass. Many glyphosate-resistant goosegrass populations have evolved in Malaysia with the likely contributory factor being that in 1994 the glyphosate price in Malaysia dropped by two-thirds, resulting in greatly increased adoption, higher use rates and, most important, more frequent application in Malaysian plantation and horticulture agriculture (Lee and Ngim 2000). Within the bountiful, all-year-round, tropical Malaysian growing season, goosegrass produces four generations per year and over the period 1994 to 1998 glyphosate was applied to goosegrass populations six to eight times per year in plantations. Evolved glyphosate resistance followed this persistent glyphosate usage. First reported by Lee and Ngim (2000), persistent and strong glyphosate-selection pressure in Malaysia resulted in many glyphosate-resistant goosegrass populations evolving (Lee 1999; Thai and Chiong 1999; Tran et al. 1999). Now, glyphosate no longer provides control of goosegrass in many parts of Malaysia (J. Ngim, personal communication). These resistant goosegrass populations have only moderate levels of resistance, with the glyphosate LD_{50} for resistant plants being from twofold to eightfold greater than for susceptible plants.

Excellent research rapidly identified that a target site change was the mechanism endowing glyphosate resistance in some Malaysian goosegrass populations. Baerson et al. (2002) showed that the glyphosate concentration required to inhibit EPSPS (I_{50}) from resistant biotypes was five times higher than for EPSPS from susceptible goosegrass. The specific activity of EPSPS was found to be the same in resistant and susceptible biotypes, and because glyphosate treatment did not induce EPSPS, the glyphosate resistance mechanism was established as the reduced sensitivity of EPSPS to glyphosate (Baerson et al. 2002). The molecular basis of the reduced ability of glyphosate to inhibit EPSPS in the resistant biotypes was revealed as the result of a mutation of the EPSPS gene, leading to a proline-to-serine substitution at amino acid 106 (Pro106–Ser). Interestingly, this same Pro106–Ser change had been identified as endowing moderate levels of glyphosate resistance in laboratory mutagenic studies with *Salmonella* and *Petunia* (Comai et al. 1983; Padgette et al. 1991). Parallel studies with other Malaysian glyphosate-resistant goosegrass biotypes revealed the same Pro106–Ser substitution in the EPSPS gene, and this research also identified that proline-to-threonine (Pro106–Thr) substitution also endows glyphosate resistance (Ng et al. 2003, 2004, 2005). Thus, at least two different mutations of the EPSPS gene, leading to amino acid changes at Pro106, are now known to endow glyphosate resistance in goosegrass populations. The inheritance of the EPSPS Pro106 target site glyphosate-resistance mutations in goosegrass have been established, as expected, as a single gene that is nuclear-encoded and incompletely dominant (Ng et al. 2004).

It should be expected that the mutation of Pro106 in EPSPS found in glyphosate-resistant goosegrass would

occur in other weed species. Indeed, this mutation has now been found in Australian (Wakelin and Preston 2005) and Chilean (Perez-Jones et al. 2005) glyphosate-resistant ryegrass (*Lolium* spp.) populations. A resistant EPSPS is also involved in glyphosate resistance in a Californian population of rigid ryegrass (Simarmata and Penner 2004); however, the mutation in this population is not yet identified. Although the Pro106 mutations endow only moderate levels of glyphosate resistance, it is important to realize that target site mutations endowing plant survival at the prevailing commercial glyphosate use rate will be enriched in populations under selection. All mechanisms (both "weak" and "strong") that contribute to plant survival can be enriched in plant populations under herbicide selection. It is important to emphasize that it is not valid to extrapolate that glyphosate resistance will always be target site–based nor that all cases of glyphosate target site resistance will involve mutations only at amino acid 106. Evolution teaches us that any mechanism that endows survival can be selected for. Time will establish whether, and to what extent, other glyphosate-resistance–endowing mechanisms can evolve and be enriched in plant populations under glyphosate selection.

An important agronomic and ecological question is whether there is any adverse effect on plants conferred by the EPSPS gene mutation endowing glyphosate resistance. The *psbA* mutation endowing target site–based triazine resistance results in reduced agroecological fitness (reviewed by Holt and Thill 1994). Conversely, some mutations endowing target site–based resistance to ALS or ACCase herbicides have little or no fitness costs. Some studies comparing the Pro106-Ser glyphosate-resistant goosegrass with susceptible biotypes reveal some differences, but it is not yet evident whether or not there are any fitness costs associated with this target site EPSPS–based resistance mechanism (Ismail et al. 2002; Lee 1999). Such studies are required.

Nontarget Site Reduced Glyphosate-Translocation Resistance Mechanism. One of the important features of glyphosate is its systemic action. Glyphosate has considerable mobility within plants, and in general, glyphosate translocation patterns mirror photoassimilate translocation by accumulating in sink tissues (Arnaud et al. 1994; Bromilow et al. 1993). There is evidence that this rapid and widespread translocation of glyphosate is important in achieving herbicide efficacy (Claus and Behrens 1976). Therefore, it is possible that changes in the pattern of translocation of glyphosate could endow resistance in plants.

Glyphosate resistance first appeared in Australian populations of rigid ryegrass (Powles et al. 1998; Pratley et al. 1999). Extensive studies with one population, NLR70, showed resistance was not due to a resistant EPSPS or to glyphosate degradation (Lorraine-Colwill et al. 2002). There were no differences between resistant and susceptible populations in glyphosate absorption into leaf tissue; however, patterns of glyphosate translocation were different. Glyphosate applied to susceptible plants tended to accumulate in the lower part of the plant and, to a lesser extent, in the roots, whereas in resistant plants, glyphosate accumulated in the tip of the treated leaf, with little translocation to the roots (Lorraine-Colwill et al. 2002). Further studies with four different Australian glyphosate-resistant rigid ryegrass populations found the same patterns of reduced glyphosate translocation (Wakelin et al. 2004).

Glyphosate inhibition of EPSPS results in an accumulation of precursors of the chorismic acid pathway, most notably shikimate (Amrhein et al. 1980; Lydon and Duke 1988). Therefore, accumulation of shikimate is an indicator of whether glyphosate is reaching the target enzyme. Experiments on rigid ryegrass showed shikimate accumulation in both resistant and susceptible plants, but a more rapid decline of shikimate in resistant plants (Lorraine-Colwill et al. 1999). This is an indication that glyphosate transiently inhibits EPSPS in resistant rigid ryegrass and supports the concept that glyphosate is being moved away from the target site in resistant plants more rapidly than in susceptible plants.

There are now 44 known glyphosate-resistant *Lolium* populations in Australia (see Preston 2005), as well as populations in California (Simarmarta et al. 2003), Chile (Perez and Kogan 2003), and South Africa (A. Cairns, unpublished data). Experiments have now been conducted on several of these populations to determine resistance mechanisms. Studies with several resistant Australian populations have shown consistent differences in glyphosate translocation between resistant and susceptible populations. The resistant populations have increased glyphosate accumulation in the treated leaf and decreased accumulation in the stem, stem meristem, and roots compared with susceptible plants (Lorraine-Colwill et al. 2002, Wakelin et al. 2004).

Studies investigating the mechanism of glyphosate resistance in other *Lolium* populations have not identified such large differences in glyphosate translocation. Feng et al. (1999) studied a population from Victoria, Australia, and Perez et al. (2004), working with Chilean *Lolium* populations, both found no differences in glyphosate ab-

sorption or translocation between resistant and susceptible plants. Simarmata et al. (2003), investigating a glyphosate-resistant Californian *Lolium* population observed significantly more glyphosate in treated leaves of resistant plants 2 and 3 d after treatment but observed no other significant differences in absorption or translocation of glyphosate between resistant and susceptible plants. It is very likely that these varying results indicate that different mechanisms are responsible for glyphosate resistance in different *Lolium* populations. Diversity in resistance mechanisms is exactly what we would expect, especially in a highly genetically diverse species, such as *Lolium*.

Studies of inheritance of glyphosate resistance have been conducted on several resistant *Lolium* populations. Working with the original NLR70 population with the reduced glyphosate-translocation resistance mechanism, Lorraine-Colwill et al. (2001) showed resistance was nuclear-encoded and displayed incomplete dominance, and second generation (F_2) and backcross populations indicated that a single gene encoded resistance. Further experiments have been conducted with seven additional glyphosate-resistant *Lolium* populations all crossed to the same susceptible population. First-generation (F_1) plants all show resistance to glyphosate but to varying degrees (Lorraine-Colwill et al. 2002; Wakelin and Preston, unpublished data). In each case, resistance is nuclear-encoded; however, dominance varies from high to moderate.

Populations of horseweed resistant to glyphosate first appeared in glyphosate-resistant soybean fields in Delaware in 2000 (Van Gessel 2001). Since then, populations have occurred in many glyphosate-resistant crop fields across many U.S. states (Main et al. 2004; Mueller et al. 2003). Given the ease with which the wind-borne seed of horseweed can be dispersed, the widespread areas of glyphosate-resistant crops across parts of the United States, and the propensity of this species to proliferate in minimal-tillage systems, it is inevitable that glyphosate-resistant horseweed will become a problem across large areas. Experiments on the mechanism of glyphosate resistance in horseweed populations reveal glyphosate treatment results in increased leaf shikimate concentrations in both resistant and susceptible populations (Feng et al. 2004; Koger et al. 2005; Mueller et al. 2003), indicating EPSPS remained susceptible to glyphosate. Feng et al. (2004) and Koger and Reddy (2005) also measured absorption and translocation of glyphosate. No differences were found in absorption of glyphosate, but translocation of glyphosate to the roots was greatly reduced in the resistant populations. Export of glyphosate out of the treated leaf was much reduced in resistant plants, leading to accumulation of glyphosate in the treated leaf. Therefore, a very similar glyphosate-resistance mechanism, involving reduced glyphosate translocation, is apparent in certain resistant *Conyza* and *Lolium* biotypes.

Inheritance studies on the original population of glyphosate-resistant horseweed from Delaware have shown resistance is nuclear-encoded, and the trait is semidominant. F_2 and backcross segregation patterns are consistent with single gene inheritance (Zelaya et al. 2005).

Inefficient translocation of herbicides as a mechanism of herbicide resistance has rarely been observed in weeds (Preston 2002). It is of considerable interest, therefore, that this mechanism appears to account for glyphosate resistance in several *Conyza* and *Lolium* populations. It is probable that reduced translocation is a common mechanism endowing glyphosate resistance in weeds. The precise biochemical and molecular basis of this reduced glyphosate-translocation mechanism remains to be elucidated.

An important question is whether the reduced glyphosate-translocation resistance mechanism evident in *Conyza* and *Lolium* populations has any effects on the fitness of resistant plants. No studies have yet been published for glyphosate-resistant *Conyza*. A number of fitness studies are underway with glyphosate-resistant *Lolium* populations. The reduced glyphosate-translocation resistance mechanism in the *Lolium* populations studied thus far show that this resistance mechanism may incur a fitness cost (Pedersen et al. 2006; Preston et al., unpublished data; Wakelin et al., unpublished data). Further studies to examine the fitness costs of glyphosate resistance in *Conyza*, *Lolium*, and other resistant species as they develop are justified.

CONCLUSIONS

In the (limited) studies thus far conducted on plants with evolved glyphosate resistance, it is evident that at least two very different mechanisms can endow glyphosate resistance. Target site–based, evolved resistance to glyphosate has been shown to occur through mutations in the EPSPS gene, with mutations changing Pro106 to Ser or Thr. Conversely, nontarget site–based, evolved resistance to glyphosate has been shown to be the result of reduction in translocation of glyphosate to the meristematic regions of resistant plants. Both the target site and the nontarget site glyphosate-resistance mechanisms are inherited as single gene, nuclear traits.

Of course, especially in cross-pollinated species, both mechanisms could co-occur in individual resistant plants or populations. It is likely that other glyphosate-resistance mechanisms will be documented in the future.

In conclusion, we now know that resistance to glyphosate will evolve where glyphosate-selection pressure is high. Large areas of the United States, Argentina, and Brazil, where glyphosate-resistant crops are intensively grown, are under particularly strong glyphosate-selection pressure. Glyphosate-resistant weed populations are now appearing at an alarming rate in the United States. Glyphosate-resistant horseweed (*Conyza*) populations are widespread, and the first glyphosate-resistant ragweed (*Ambrosia artemisiifolia*) populations (R. Smeda, personal communication) and common lambsquarters (*Chenopodium album*) (J. Stachler and M. Loux, personal communication) have appeared in the United States. There is also evidence glyphosate resistance may occur in the important pigweed (*Chenopodium album* L.) weed complex (Zelaya and Owen 2005), with resistant populations of the economically damaging Palmer amaranth (*Amaranthus palmeri*) now apparent (J. Wilcut, personal communication). Clearly, the previous complacency about the evolution of glyphosate resistance cannot now be justified, and it must be recognized that intense glyphosate-selection pressure, such as in continuous glyphosate-resistant crops, increases the risks of glyphosate-resistant weed populations evolving (Powles 2003). Other high-risk situations for glyphosate-resistance evolution is intense glyphosate use for burn-down weed control before crop seeding in minimum or zero tillage systems (Neve et al. 2003b) and intense glyphosate reliance in horticultural systems, especially trees, nuts, and vines.

Because glyphosate has a pivotal role in world agriculture, its continued efficacy is essential, and the looming threat that resistance poses to glyphosate sustainability requires action. Solutions will not be easy to identify or implement. Although solutions will be regional- and agroecosystem-based, common features will include greater diversity in agroecosystems and restraint in glyphosate usage. The aim must be to preserve glyphosate efficacy so that this unique herbicide will continue to contribute to world food and fiber production.

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