

Evolution of Glyphosate-Resistant Crop Technology

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New and improved glyphosate-resistant (GR) crops continue to be rapidly developed. These crops confer greater crop safety to multiple glyphosate applications, higher rates, and wider application timings. Many of these crops will also have glyphosate resistance stacked with traits that confer resistance to herbicides with other modes of actions to expand the utility of existing herbicides and to increase the number of mixture options that can delay the evolution of GR weeds. Some breeding stacks of herbicide resistance traits are currently available, but the trend in the future will be to combine resistance genes in molecular stacks. The first example of such a molecular stack has a new metabolically based mechanism to inactivate glyphosate combined with an active site-based resistance for herbicides that inhibit acetolactate synthase (ALS). This stack confers resistance to glyphosate and all five classes of ALS-inhibiting herbicides. Other molecular stacks will include glyphosate resistance with resistance to auxin herbicides and herbicides that inhibit acetyl coenzyme A carboxylase (ACCase) and 4-hydroxyphenyl pyruvate dioxygenase (HPPD). Scientists are also studying a number of other herbicide resistance transgenes. Some of these new transgenes will be used to make new multiple herbicide-resistant crops that offer growers more herbicide options to meet their changing weed management needs and to help sustain the efficacy of glyphosate.

Nomenclature: Glyphosate.

Key words: Genetically modified crops, GMO, herbicide resistant crops, glyphosate, ALS, dicamba, imidazolinone, glufosinate, sulfonyleurea.

In 1970, glyphosate, a broad-spectrum and highly translocated foliar herbicide, was discovered (Franz et al. 1996). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; 2.5.1.26), the sixth enzyme in the shikimate biosynthetic pathway that produces the essential aromatic amino acids (tryptophan, tyrosine, and phenylalanine) and subsequently phenolics, lignins, tannins, and other phenylpropanoids (EXTOXNET 2007; WSSA 2007). Glyphosate sales started in 1974 and by 1995 had reached 4.5 million kg in the United States. Since then, glyphosate use has increased more than 10-fold because of price reductions from the expiration of key patents and the development of glyphosate-resistant (GR) crops. To date, companies have sought regulatory approval for nine GR crops: soybean [*Glycine max* (L.) Merr], cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.), Argentine canola (*Brassica napus* L.), Polish canola (*Brassica rapa* L.), alfalfa (*Medicago sativa* L.), sugarbeet (*Beta vulgaris* L.), creeping bentgrass (*Agrostis stolonifera* L.), and wheat (*Triticum aestivum* L.) (AGBIOS 2008). All except creeping bentgrass and wheat have been grown commercially.

Biotechnology brought big business and huge investments into agriculture and revolutionized the way biologists do research (Charles 2001). The ability to manipulate the plant genome directly gave scientists new ways to create crops resistant to glyphosate and other herbicides (Table 1). In 1983, scientists at Monsanto and Washington University isolated the common soil bacteria, *Agrobacterium tumefaciens* strain CP4, which is highly tolerant to glyphosate because its EPSPS is less sensitive to inhibition by glyphosate than EPSPS found in plants (Watrud et al. 2004). By 1986, they had successfully inserted the *cp4 epsps* gene into the plant genome and obtained GR plants. Within 10 yr, GR soybean was commercialized. The initial GR crops were the most quickly

adopted technology in the history of agriculture (James 2007). This rate of adoption continues at more than 10% yr⁻¹ in both developing and developed countries. In 2007, 12 million growers in 23 countries planted 114.3 million ha of biotech crops (AGBIOS 2008; James 2007).

The introduction of GR crops transformed the way many growers manage weeds. Growers chose GR crops because glyphosate made weed control easier and more effective, increased profit, required less tillage, and did not restrict crop rotations. Before the introduction of GR crops, growers routinely used many different herbicides with many different modes of action, e.g., 10 different herbicide modes-of-action in soybean. Now, many growers rely only on glyphosate (Foresman 2008; Gustafson 2008). Applying glyphosate alone over wide areas on highly variable and prolific weeds made the evolution of resistant weeds inevitable (Owen 2001; Thill and Lemerle 2001). The first reports of GR weeds increased concerns about long-term sustainability of glyphosate and started a large effort in private and public laboratories to develop alternative options. The battle was on to preserve glyphosate (Service 2007). Part of that effort was to develop a new generation of transgenic GR traits with improved properties and to stack those traits with resistance traits to other herbicide modes of action so growers have more options to manage the evolution of resistant weeds.

Glyphosate-Resistant Crops

The first GR crop systems provided growers a new way to use glyphosate. Glyphosate was simple to use, effective, and inexpensive. Key glyphosate patents were expiring and generic manufacturers were starting to sell glyphosate at very low prices. In the United States, a use patent extended protection of glyphosate on GR crops (Shah et al. 1990), but the price of glyphosate still declined. This low price severely reduced demand for the more expensive selective herbicides. Compet-

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Table 1. Summary of transgenic herbicide-resistant crops that have trade names.

Crop	Resistance trait	Trait genes	Promoters	Trait designation	Company	First sales ^a
Alfalfa	Glyphosate	<i>cp4 epsps</i>	FMV 35S	J101, J163	Monsanto	2005
Canola	Glyphosate	<i>cp4 epsps and goxu247</i>	FMV 35S and FMV 35S	GT73	Monsanto	1996
	Glufosinate	<i>pat</i>	CaMV 35S	HCN92	Bayer	1995
Cotton	Bromoxynil	<i>bxn</i> nitrilase		BXN	Calgene	1995
	Glyphosate	<i>cp4 epsps</i>	FMV 35S	MON1445	Monsanto	1996
		Two <i>cp4 epsps</i>	FMV/TSF1 and 35S/ACT8	MON88913	Monsanto	2006
		Modified-maize <i>epsps</i>	Ph4a748At	GHB614	Bayer	TBD
Corn	Glufosinate	<i>bar</i>	CaMV 35S	LLCotton25	Bayer	2005
	Glyphosate	Three modified maize <i>epsps</i>	Os.Act1	GA21	DeKalb	1998
		Two <i>cp4 epsps</i>	Os.Act1a and CAMV e35S	NK603	Monsanto	2001
		<i>gat</i> and <i>zm-bra</i>	<i>ubiZM1</i> and <i>zmALS</i>	98140	Pioneer	TBD
		Glufosinate	<i>pat</i>	CaMV 35S	T25	Bayer
Soybean	Glyphosate	<i>cp4 epsps</i>	CaMV e35S	GTS 40-3-2	Monsanto	1996
		<i>cp4 epsps</i>	FMV e35S/TSF1	MON89788	Monsanto	TBD
	Glyphosate and ALS	<i>gat</i> and <i>gm-bra</i>	SCP1 and SAMS	356043	Pioneer	TBD
		Glufosinate	<i>pat</i>	CaMV 35S	A2704-12	Bayer
Sugarbeet	Glyphosate	<i>cp4 epsps</i>	FMV e35S	H7-1	Monsanto	2007
	Glufosinate	<i>pat</i>	CaMV 35S	T120-7	Bayer	TBD

^a Abbreviation: TBD, to be determined.

itors reduced herbicide prices but could not regain sales. Some selective herbicides survived and have recently regained significant sales because of the evolution of GR weeds. In addition, the need to control GR feral crops is forcing some growers back to more diversified weed management systems.

Another key issue for the success of GR crops was capturing the value of technology in the marketplace, particular on self-pollinating crops like soybean where growers could readily replant seed from the previous harvest. In the United States, Monsanto charged growers a per-bag technology fee and enforced their patents by requiring soybean growers to sign a contract that waived their right to replant seed. The contract was a risky innovation that initially contained provisions to monitor growers' land for 3 yr. Although growers objected, they signed, and the contract did not significantly restrict sales. The contract did succeed in dramatically reducing grower-saved seed and thus helped maintain the value of the technology.

Growers adopted GR crop technology even faster in Argentina than in the United States. Laws in Argentina did not provide protection for the GR soybean technology, so growers could save seed from the previous crop and not pay a royalty fee. The amount of saved seed that growers planted increased to more than 80%. In Brazil, the government denied registration for many years, while their growers obtained GR seed from Argentina. Brazil passed provisions that allowed growers to plant saved seed without paying royalties. Monsanto threatened lawsuits in countries that were receiving the exported seed and eventually Brazil agreed to allow collection of royalty payments at elevators when growers sold their grain. This arrangement may become the model in other countries that have weak laws for protecting germplasm and trait technologies.

Glyphosate-Resistant Soybean. *GTS 40-3-2.* The first GR soybean field test was in 1989 with the 44-3-2 event (APHIS 2008). Soybean with the commercialized glyphosate-tolerant soybean GTS 40-3-2 event¹ was first field tested in 1991 and first sold in 1996. The marketing strategy was to distribute the trait as broadly as possible. Adoption of the technology was very rapid, and glyphosate resistance is now in more than 1,000 commercial soybean varieties. Success was due to a collaboration, with Monsanto providing the gene and the money, Agracetus

providing the transformation technique, and Asgrow and Jacob Hartz contributing the germplasm and breeding expertise.

The GTS 40-3-2 soybean line was produced by inserting the *cp4 epsps* gene into the group V maturity cultivar 'A5403' using particle acceleration (biolistic) transformation (Padgett et al. 1995). The selectable marker was a *gus* gene that codes for the productions of β -glucuronidase, but the marker was not integrated into the genome. The event has the enhanced cauliflower mosaic virus (CaMV) e35S promoter with the duplicated enhancer regions, which constitutively express the gene throughout the life cycle, and a chloroplast transit peptide from *Petunia hybrida*, which directs CP4 EPSPS to the chloroplast, where the entire shikimate pathway is located. The GTS 40-3-2 event did not significantly alter soybean morphology, agronomic characteristics (such as time to flowering and pod set), or tendency to become a weed.

Demand for GR soybean was high, encouraging rapid completion of the minimum number of backcrosses to incorporate the trait into commercial varieties. Many growers found that the initial varieties yielded less than the nontransgenic, conventional varieties. University research indicated that GR soybean yielded 5 to 7% less than conventional varieties, but it was never determined whether this "yield drag" was due to the trait, to the genes associated with the insertion point, or to the first commercial varieties retaining too much of the lower-yielding A5403 genetics, where the trait originated (Elmore et al. 2001). Today, GR soybean is the conventional soybean, and isolines with and without the trait are not widely available for independent evaluation.

MON89788. The first new transgenic trait in soybean in more than a decade, MON89788,² was approved by regulatory agencies in 2007. The initial promotions for MON89788 claimed higher yield than GTS 40-3-2, the first GR trait. In GTS 40-3-2, the male reproductive tissues, such as the tapetum and developing pollen, were a strong sink for any residual glyphosate in the plant and were susceptible to being injured (Cajacob et al. 2007). MON89788 contains the same form of EPSPS but has a different insertion site and stronger constitutive viral promoters and regulatory elements to enhance expression in both the male reproductive and vegetative tissues (Watrud et al. 2004).

MON89788 was produced with *Agrobacterium*-mediated transformation of meristematic tissue in an elite cultivar 'A3244', maturity group III variety. The plasmid PV-GMGOX20 used for transformation contained the *aroA* gene from *A. tumefaciens* encoding for CP4 EPSPS (Watrud et al. 2004). Expression of the *cp4 epsps* results in a single 455 amino acid protein. A chimeric promoter combines the enhancer sequences from the 35S promoter of the figwort mosaic virus (FMV) and a promoter from the *Tsfl* gene from *Arabidopsis thaliana*, which codes for the elongation factor, EF1 α control expression. The plasmid has a chloroplast transit peptide (CTP2) coding sequence from the *epsps* gene of *A. thaliana* to translocate EPSPS to the chloroplasts. MON89788 did not significantly alter morphology, agronomic characteristics (such as time to flowering and pod set, or vigor) or tendency to become a weed. The only significant difference compared with A3244 was slightly reduced height, but this reduction was within the range of normal commercial variability. Commercialization is expected in 2009.

356043. Pioneer is developing a GR soybean, designated 356043,³ which has been genetically modified to express GAT4601 (glyphosate acetyltransferase) and GM-HRA, modified version of a soybean acetolactate synthase (ALS) to confer resistance to glyphosate and ALS-inhibiting herbicides. Glyphosate acetyl transferase (*gat*) catalyzes the acetylation of glyphosate to the inactive N-acetyl glyphosate (NAG). The *gat4601* gene is based on the sequences of three weakly active N-acetyltransferase isozymes from the soil bacterium *Bacillus licheniformis* (Weigmann) Chester (Castle et al. 2004). The *gat* enzymes are members of the GCN-5 family of N-acetyltransferases, also known as the GNAT family. The GNAT family is one of the largest enzyme families with more than 10,000 representatives from plants, animals, and microbes. To increase the glyphosate acetylation activity, a collection of recombinant *gat* genes were expressed in *Escherichia coli* and screened for glyphosate acetylation. Recombinants with the highest glyphosate acetylation then went through iterative rounds of gene shuffling, a technique for molecular recombination between genes and directional screening to improve the properties of enzymes (Ness et al. 2002). At two points during the shuffling process, site-directed mutagenesis introduced additional amino acid substitutions, identified through analysis of related sequences, to augment the naturally occurring 12 amino acid differences among the natural isozymes. Shuffling of the *gat* genes provided a route to highly active *gat* alleles that provide a high level of resistance to glyphosate (Castle et al. 2004; Siehl et al. 2007).

The 356043 event was produced from the 'Jack' soybean cultivar using particle acceleration (biolistic) transformation. Expression of the *gat4601* gene in soybean is driven by a constitutive synthetic-core promoter SCP1, comprising a portion of the CaMV 35S promoter (Odell et al. 1985), and the Rsyn7-Syn II core synthetic consensus promoter (Bowen et al. 2003). The ALS-resistance *gm-hra* gene was the selectable marker for the event in tissue culture. The 356043 has been in field tests since 2003 (APHIS 2008), with a commercial release anticipated in 2011 pending regulatory approvals.

Glyphosate-Resistant Corn. *GA21*. Efforts to develop GR corn began in the late 1980s. The first commercial event was

GA21,⁴ which relies on maize *epsps* genes (*zm-epsps*) modified to be insensitive to glyphosate (Spencer et al. 2000). It contains a single insertion of three complete copies in tandem of the modified corn *epsps* gene cassette and three incomplete copies. Although these partial expression cassettes could theoretically result in truncated coding regions, no unexpected mRNA or protein was detected. The *GA21* corn line was obtained by inserting the gene from a plasmid designated pDPG434 into a cell culture of the inbred 'AT', using particle acceleration (biolistic) transformation. Transformed cells were selected with glyphosate, so there are no additional marker genes. *GA21* retained the agronomic characteristics of its parental inbred line.

A key difficulty in developing commercially acceptable glyphosate resistance was the unavailability of an efficient promoter and a chloroplast transit peptide. The rice (*Oryza sativa* L.) actin 1 (Os.Act1) promoter controls expression. The amino acid sequence of the modified EPSPS protein is 99.3% identical to the wild-type corn enzyme, without any sequences homologous with known toxins or allergens. *GA21* uses the optimized transit peptide (OTP) sequence, based on the chloroplast transit sequences of *Helianthus annuus* and *Zea mays* ribulose 1,5-bisphosphate carboxylase oxygenase (Ru-BisCo) gene to direct the EPSPS to the chloroplast. Strong sink tissues, such as developing pollen and tapetum, were vulnerable to injury because of accumulated glyphosate (CaJacob et al. 2007).

Monsanto acquired DeKalb in 1996, after DeKalb had developed commercially acceptable GR corn lines. United States regulatory agencies approved the original GR corn event in November 1997. First sales were in 1998 but were slowed because the European Union delayed import approvals. That coupled with legal uncertainties over the ownership of *GA21*, which prevented DeKalb branded sales in 2000, forced Monsanto to replace *GA21* with a new, second-generation event. Sale of *GA21* stacked with insect resistance (and glufosinate resistance) started in the 2003 growing season.

NK603. A new event, *NK603*,⁵ was developed with stronger constitutive viral promoters and regulatory elements to enhance expression in vulnerable tissues. *NK603* was produced from a proprietary inbred line designated as AW \times CW using particle acceleration (biolistic) transformation. It contains two copies of the *epsps cp4* gene, one in each of two expression cassettes, one driven by the Os.Act1 promoter, and the other by the enhanced CaMV e35S promoter with the enhancer region duplicated for high expression (CaJacob et al. 2007). The CP4 EPSPS sequence was modified slightly to improve activity but still has greater than 99.4% homology in nucleotide sequence and greater than 99.7% homology in amino acid sequence to the native *A. tumefaciens epsps cp4* gene. Both cassettes contain the CTP2 transit peptide from *A. thaliana* EPSPS. The rapid substitution of *NK603* for *GA21* in the marketplace, facilitated by grower incentives and intellectual property settlements, was a major accomplishment for Monsanto.

98140. Pioneer is developing GR corn 98140,⁶ which was produced by *Agrobacterium*-mediated transformation of the inbred 'introEF09B'. The 98140 line has been genetically modified to express the *gat4621* gene that codes for

glyphosate acetyltransferase. In 98140, the corn ubiquitin (ubiZM1) promoter (Christensen and Quail 1996) drives the expression of the *gat4621* gene. Transformed cells in tissue culture were selected with glyphosate so there are no extra marker genes, but the *gat4621* gene is molecularly stacked with a modified version of a maize ALS gene (*zm-hra*), which confers resistance to ALS-inhibiting herbicides. The increased resistance to glyphosate and some ALS-inhibiting herbicides is more than 1,000-fold compared with an untransformed isolate (Green et al. 2008). Commercial release is anticipated in 2010, pending regulatory approvals.

Glyphosate-Resistant Cotton. *MON1445*. Cotton is very susceptible to weed competition (Coble and Byrd 1992), and growers did not have many effective POST selective herbicides before GR cotton was developed by Delta and Pineland in 1997. However, crop safety with the first trait was marginally acceptable and only allowed application over the canopy up to the four-leaf growth stage. Applications after the four-leaf stage were associated with fruit abortion and subsequent yield loss. Still, growers appreciated the yield benefit of controlling weeds early and rapidly accepted the technology.

The first commercial GR trait, *MON1445*,⁷ was genetically engineered into 'Coker 312' with *Agrobacterium*-mediated transformation (AGBIOS 2008). The plasmid for *MON1445* contained two glyphosate resistance genes, *epsps cp4* and *gox*, a gene that encodes for the glyphosate oxidoreductase (GOX) enzyme, which catalyzes the breakdown of glyphosate into aminomethylphosphonic acid (AMPA) and glyoxylate. However, the *gox* gene was not integrated into the genome, so the commercial product contains only the *epsps cp4* gene. The *epsps cp4* gene sequence from *A. tumefaciens* strain CP4 was modified slightly to improve protein expression but did not change the EPSPS CP4 amino acid sequence. The CoMVb promoter from a modified FMV and the transit peptide CTP2 from *A. thaliana* drive the expression. *MON1445* lacked sufficient expression of the *epsps cp4* gene in the developing pollen and tapetum (Dill et al. 2008). An antibiotic-resistance gene that encodes for neomycin phosphotransferase II was the marker to select transformed plants in tissue culture. Another selectable marker gene that codes for 3''(9)-O-aminoglycoside adenylyltransferase was used to select transformed bacterial colonies but is not expressed in cotton. *MON1445* does significantly alter morphology, agronomic characteristics (such as time to flowering and pod set or vigor), or the tendency to become a weed.

MON88913. *MON88913*⁸ was developed to express resistance during reproductive growth phases, so growers could apply glyphosate after the four-leaf growth stage. *MON88913* contains two *cp4 epsps* genes with chimeric promoters to provide stronger expression in the 4- to 12-leaf growth vegetative stages and in the sensitive reproductive tissue (CaJacob et al. 2007). The genes from the binary plasmid PV-GHGT35 were inserted into the variety Coker 312 using *Agrobacterium*-mediated transformation of hypocotyl tissue. The plasmid contains two tandem *cp4 epsps* gene cassettes (AGBIOS 2008). In the first cassette, FMV/TSF1, a chimeric promoter containing the *A. thaliana* *tsf1* promoter, the encoding elongation factor EF1 α gene, and enhancer sequences from the FMV 35S promoter, regulate gene expression. CaMV 35S/ACT8, a chimeric promoter from

the *act8* gene of *A. thaliana* and the CaMV 35S promoter, regulate the expression of the second *cp4 epsps* gene. Growers rapidly accepted *MON88913*, because of its greater crop safety and flexibility for applying glyphosate, and planted 0.9 million ha in 2006, its first year of sales (Dill et al. 2008).

GBH614. A new GR cotton trait event,⁹ *GHB614*, uses a modified-maize *epsps* gene (*2mepsps*) (CFIA 2008; Ellis et al. 2008). The double-mutant 2mEPSPS enzyme differs from the naturally occurring maize EPSPS by two amino acids. The genes were inserted into the variety Coker 312 using *Agrobacterium*-mediated transformation. The event has a single copy of the *2mepsps* gene. The constitutive Ph4a748At promoter (Chaboute et al. 1987) and the TPotpC transit peptide (Lebrun et al. 1996) control expression. Other than glyphosate resistance, *GBH614* is agronomically similar to its parent line.

Glyphosate-Resistant Canola. *GT73*. Canola, both Argentine and Polish types, is the second largest oilseed crop in the world. Early in its development, canola is not very competitive, and weeds can substantially reduce yields (Kirkland 1995). Additionally, contamination by wild mustard (*Sinapis arvensis* L.) seeds reduced grain and oil quality by increasing erucic acid and glucosinolate levels. Weed control in canola can be expensive, requiring up to four herbicide applications. The first commercial GR canola called *GT73*,¹⁰ also known as *RGT73*, was launched in 1996. In both Canada and the United States, growers rapidly accepted GR canola. A second-generation GR canola is in the early stages of development (Dill et al. 2008).

GT73 contains two different GR genes, the *epsps cp4* gene from *A. tumefaciens* and a *gox* gene, *goxv247*, from strain LBAA of the bacterium *Ochrobactrum anthropi* (AGBIOS 2008). The *goxv247* gene produces a modified-GOX enzyme that improves the degradation of glyphosate to AMPA and glyoxylate. A 35S promoter from a modified virus (FMV) drives the constitutive expression of both *epsps cp4* and *goxv247*. The chloroplast transit peptides for CTP2 are from the small subunit of the ribulose-1,5-bisphosphate carboxylase gene of *A. thaliana* and the *epsps* gene of *A. thaliana*. *GT73* uses *Agrobacterium*-mediated transformation to insert the genes into the cultivar 'Westar' (Watrud et al. 2004).

Combining resistance mechanisms conferred a high level of resistance to glyphosate. Other than resistance to glyphosate, *GT73* has similar agronomic characteristics to its parental inbred line. There are presently no field-isolation requirements, but transgenes can move through pollen to both conventional canola and related weeds species. The most important related weed is wild mustard, but hybridization can occur with India mustard [*Brassica juncea* (L.) Czern.], Ethiopian mustard (*Brassica carinata* A. Braun), black mustard [*Brassica nigra* (L.) W.D.J. Kock], annual wallrocket [*Diplotaxis muralis* (L.) DC.], wild radish (*Raphanus raphanistrum* L.), and common dogmustard [*Erucastrum gallicum* (Willd.) O.E. Schulz]. The frequency of outcrossing is very low, and hybrids are usually less fit, but a transgene has become established in a stable wild population (Beckie et al. 2006; Warick et al. 2008).

Glyphosate-Resistant Alfalfa. *J101 and J163*. Monsanto and Forage Genetics International collaborated to develop GR

alfalfa that was deregulated in 2006 (AGBIOS 2008). Two events,¹¹ J101 and J163, were obtained through *Agrobacterium*-mediated transformation into proprietary alfalfa clone 'R2336'. The events have a single copy of the *epsps cp4* gene. An enhanced FMV 35S promoter and the petunia heat shock protein 70 5' untranslated leader sequence, with the CTP2 transit peptide, regulate expression. The United States returned J101 and J163 to regulated status following a preliminary court injunction related to the environmental impact assessment on March 12, 2007, and commercial sales have stopped (APHIS 2007).

Glyphosate-Resistant Sugarbeet. *H7-1*. GR sugarbeet was released for sale in 2007. The event H7-1¹² was obtained through *Agrobacterium*-mediated transformation into the sugarbeet line '3S0057'. The event has a single copy of the *epsps cp4* gene, driven by an enhanced FMV 35S promoter with the CTP2 transit peptide from *A. thaliana*, which controls expression of the trait. Glyphosate was used to select the resistant lines, so there are no marker genes.

Other Glyphosate-Resistant Crops. Regulatory approvals have been sought for at least two other GR crops. Monsanto and The Scotts Company developed GR creeping bentgrass, primarily for use in turf on golf course fairways and greens. The creeping bentgrass event, ASR368, was produced as the parent line B99061R using particle acceleration (biolistic) transformation (AGBIOS 2008). The event uses two *epsps cp4* genes and two enhanced promoters for both vegetative and reproductive tissues. The promoters are the enhanced 35S promoter from the CaMV and the actin1 promoter from rice. Each CP4 EPSPS enzyme is associated with the CTP2 transit peptide.

Deregulation of the perennial GR creeping bentgrass was requested in 2005 but has not been approved (APHIS 2005). One of the main issues is concern about feral escapes and pollen flow to native plants (Mallory-Smith and Zapiola 2008; Watrud et al. 2004). The grass-seed industry was also divided on the threat of transgenic seed contaminating nontransgenic seed production in Oregon, which would stop exports to countries that do not accept any transgenic seed. Concerns increased in 2004, when researchers confirmed transgene flow to a related species, redtop bentgrass (*Agrostis gigantea* Roth), and feral transgenic creeping bentgrass escapes outside a test area that had been terminated. Efforts continue to eliminate these escapes (Mallory-Smith and Zapiola 2008).

Monsanto also produced GR spring wheat in the variety 'Bobwhite'. The event coded MON71800 was obtained through *Agrobacterium*-mediated transformation (AGBIOS 2008). It uses two *epsps cp4* genes and two distinct promoters for both vegetative and reproductive tissues. The two promoters are the enhanced 35S promoter from the CaMV and actin1 promoter from rice. Each CP4 EPSPS enzyme is associated with the CTP2 transit peptide. Crop tolerance to glyphosate was high, but the wheat industry feared customer rejection of a transgenic product and was reluctant to accept the technology (Grain Industry Working Group 2003). In 2004, Monsanto deferred all further efforts to introduce GR wheat as a single trait. In the future, the trait could still be introduced in a stack with an output trait that creates value to the end user that the grain industry and their customers are more likely to accept.

New Glyphosate-Resistance Mechanisms. New GR traits can have value if the mechanism of resistance is improved or if seed companies are allowed greater freedom to operate or avoid royalty payments. Several companies continue to work on discovering such new GR mechanisms. For example, a recent patent application describes new bacterial enzymes that metabolically inactivate glyphosate. The enzymes referred as GDC-1 and GDC-2 have homology to known decarboxylases and use a pyrophosphate cofactor (Hammer et al. 2007).

Another example is a recently discovered class of EPSPS enzymes that has high resistance to glyphosate (high K_i) and maintains affinity (K_m) to the natural substrate phosphoenolpyruvate (PEP) (Vande Berg et al. 2008). The *epsps* gene, *aroA1398*, was cloned from an uncharacterized bacterial strain designated ATX1398. The primary sequence for the *aroA1398* is quite distinct from other *epsps* genes, with only 22% homology with the *cp4 epsps* genes while exhibiting 800-fold more resistance at the enzyme level to glyphosate than corn EPSPS. Resistant corn plants were obtained through *Agrobacterium*-mediated transformation. The expression cassette contains a novel constitutive promoter from eastern gamagrass (*Tripsacum dactyloides* L.) and a chloroplast transit peptide. Glyphosate sprayed at rates eight times the recommended field rate did not injure T1 corn plants at the V4 and V8 growth stages.

Zhou et al. (2006) recently obtained a target-site resistance gene by mutating the rice *epsps* gene with an error-prone polymerase chain. The EPSPS mutation was Pro to Leu at position 106 (P106L), a mutation known to occur naturally and to bestow glyphosate resistance in weeds (Perez-Jones et al. 2007). When P106L was expressed in an EPSPS-deficient *E. coli* strain, the mutation decreased affinity to glyphosate about 70-fold and affinity to PEP, 4.6-fold. P106L gave resistance at the whole-plant level, when expressed in tobacco (*Nicotiana tabacum* L.).

Nontransgenic crop resistance to glyphosate is also possible. As more weeds evolve resistance to glyphosate naturally, more opportunities will occur to transfer resistance from weeds to crops with traditional breeding techniques. For example, Simarmata et al. (2005) identified two nuclear genes in a natural population of rigid ryegrass (*Lolium rigidum* Gaud.) that increase resistance to glyphosate more than 100-fold. Rigid ryegrass can form hybrids with several *Lolium* and possibly some *Festuca* species and thus allow transfer of this nontransgenic glyphosate resistance to turfgrass (Penner and Simarmata 2007).

Glyphosate Resistance Stacked with Other Resistance Mechanisms

The rapid evolution of resistant weeds is requiring that growers diversify their weed management practices and use combinations of herbicides, tillage practices, and herbicide resistance traits. Where diversity is maintained, the utility of glyphosate can be maintained (Powles 2008). The use of multiple herbicide-resistant crops will be an important part of that diversity (Green et al. 2008). Some breeding stacks of herbicide resistance mechanisms exist in the marketplace, but so far, herbicide-resistant crops have been primarily for single types of herbicides (Table 1). However, the era of the single herbicide-resistant trait in major crops is probably over. The next wave of technology will molecularly stack herbicide-

Table 2. Examples of non-glyphosate herbicide-resistance transgenes that are not currently being sold.

Herbicide/herbicide class ^a	Characteristics	References
2,4-D	Microbial degradation enzyme	Bisht et al. 2004
ALS-inhibitors	Resistant ALS from many sources	Bedbrook et al. 1995
Aryloxyphenoxypropionate ACCase inhibitor and phenoxy acid (auxin)	Microbial, aryloxyalkanoate dioxygenase	Wright et al. 2005
Asulam	Microbial dihydropteroate synthase	Surov et al. 1998
Bromoxynil/oxynil	BXN nitrilase, <i>Klebsiella pneumonias</i>	Stalker et al. 1996
Dalapon	Microbial degradation enzyme	Buchanan-Wollaston et al. 1992
Dicamba	<i>Pseudomonas maltophilia</i> , O-demethylase	Herman et al. 2005
HPPD inhibitors	Over-expression, alternate pathway, and increasing flux of pathway	Matringe et al. 2005
Phenylurea	P450, <i>Glycine max</i> and <i>Helianthus tuberosus</i>	Siminszky et al. 1999
Paraquat	Chloroplast superoxide dismutase	Didierjean et al. 2002
Phenmedipham	Microbial degradation enzyme	Sen Gupta et al. 1993
PDS inhibitors	Resistant microbial and <i>Hydrilla verticillata</i> PDS	Streber et al. 1994
PPO inhibitors	Resistant microbial and <i>Arabidopsis thaliana</i> PPO	Arias et al. 2005
Multiple herbicides	Glutathione S-transferase, <i>Escherichia coli</i>	Li and Nicholl 2005
	P450, <i>Zea mays</i>	Skipsey et al. 2005
		Dam et al. 2007

^a Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid ; ALS, acetolactate synthase; ACCase, acetyl coenzyme A carboxylase; HPPD, hydroxyphenylpyruvate dioxygenase; PDS, phytoene desaturase; PPO, protoporphyrinogen oxidase.

resistance transgenes so seed companies can efficiently provide growers with more weed management options.

The first example of a molecular stack of multiple herbicide resistance is glyphosate and ALS resistance (Green et al. 2008). Development has also started for molecular stacks of glyphosate resistance with other resistance mechanisms, and more are in the research stage, including glutathione S-transferase (GST) conjugation (Skipsey et al. 2005) and cytochrome P450-based herbicide metabolism (Hirose et al. 2005) (Table 2). Interestingly, the latter metabolic transgenes often give resistance to only some members of a herbicide family and thus can allow the use of the same mode of action to control weeds in one season and then allow other herbicides with that mode of action to control any feral crop in the next.

Glufosinate Resistance. Glufosinate is a broad spectrum, contact herbicide that inhibits glutamine synthetase (GS; EC 6.3.1.2), an enzyme that catalyzes the conversion of glutamate plus ammonium to glutamine as part of nitrogen metabolism in plants (EXTOXNET 2007; WSSA 2007). One theory is that ammonia levels increase rapidly after glufosinate application in sensitive plants causing membrane disruption, inhibition of photosynthesis, chloroplast disruption, and eventual plant death (Vasil 1996). Resistance to glufosinate is broadly available in a number of crops.¹³ The *bar* or the *pat* genes encode for two homologous enzymes, phosphinothricin acetyltransferases (PAT) and bialaphos acetyltransferase (BAR), which acetylate and thus inactivate glufosinate (Herouet et al. 2005). These enzymes can give resistance to whole plants or be used as selectable markers for transformed cells. The *pat* gene was originally isolated from the common soil microorganism, *Streptomyces viridochromogenes*, and the *bar* gene isolated from the soil microorganism *Streptomyces hygroscopicus*. N-acetyl-glufosinate is not deacylated in plants (Kriete et al. 1996).

Glufosinate-resistant crops are likely to become more important because glufosinate has a broad weed spectrum and no known resistant weeds. U.S. regulatory agencies approved several glufosinate-resistant soybean events in 1999, but European regulatory agencies delayed approving the trait because of concerns about the antibiotic marker. Efforts continue to get glufosinate-resistant soybean, A2704-12, fully

approved, and commercialization is possible in the near future.

First sales of glufosinate-resistant corn were in 1997. The trait is commonly used as a marker to select insect resistance traits, and thus, many lines with a protein from the bacterium *Bacillus thuringiensis* (Bt) are resistant to glufosinate. Glufosinate-resistant cotton was launched in 2004 with a cotton-specific glufosinate formulation. The *bar* gene was integrated into the variety Coker 312 through *Agrobacterium*-mediated transformation (AGBIOS 2008). The CaMV 35S promoter controls expression of the trait. Glufosinate-resistant canola was launched in 1996 in open-pollinated varieties, with a weed management program that was inferior to glyphosate, but the trait was successful because it preceded glyphosate resistance. Glufosinate resistance is now stacked with two other transgenes that control pollination for hybrid seed production.

ALS-Inhibiting Herbicide Resistance. The enzyme ALS (EC 4.2.1.6) has two substrates, 2-ketobutyrate and pyruvate, used to produce the essential branched-chain amino acids, i.e., Val, Leu, and Ile (EXTOXNET 2007; WSSA 2007). ALS is a nuclear-encoded enzyme that moves to its active site in the chloroplast with the help of a chloroplast-transit peptide. The five chemical classes of commercially used ALS-inhibiting herbicides (sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinylthiobenzoates, and sulfonylamino-carbonyl-triazolinones) all bind to ALS, but not all at the same attachment points. Binding blocks substrate access to a deeply buried active site (McCourt et al. 2006). Resistance to ALS-inhibiting herbicides most commonly occurs by point mutations in the ALS genes. Depending on the mutation, the ALS enzyme becomes insensitive to some, or all, of the ALS herbicides.

ALS-inhibiting herbicides can complement glyphosate or even compete directly with low-cost, broad-spectrum, soil-residual mixtures where weed resistance has not become a problem. Resistance to ALS-inhibiting herbicides is now commercial in at least six crops (Shaner et al. 2007; Tan et al. 2005). ALS-resistant corn, regenerated from tissue culture selection, was commercialized as imidazolinone-resistant corn in 1992 (Anderson and Georgeson 1989). Subsequently,

pollen mutagenesis was used to develop other commercial ALS-resistant corn lines; microspore selection was used in canola; and seed mutagenesis was used in soybean, lentil (*Lens culinaris* Medik.), wheat, and rice. Incorporation of a gene from a weedy relative was used to create resistant sunflower (*Helianthus annuus* L.). The amino acid changes (in reference to the *A. thaliana* sequence) are Ala205Val in sunflower, Trp574Val in corn and canola, Ser653Asn in corn, canola, lentil, wheat, and rice, Pro197Ala in soybean, and Gly654Glu in rice.

In soybean, a soybean *hrra* gene (*gm-hrra*) was molecularly stacked with glyphosate resistance and used in tissue culture to select for the 356043 event. This HRA protein is composed of 656 amino acids with the two-point mutations, Pro to Ala at position 197 and Trp to Leu at position 574 (Lee et al. 1988). The *S*-adenosyl-L-methionine synthetase (SAMS) soybean promoter drives constitutive expression of the *gm-hrra* gene. Particle acceleration (biolistic) bombardment introduced a single copy of these genes and other DNA regulatory sequences into soybean somatic embryos. Genetic segregation data over five generations demonstrated that *gm-hrra* and its natural *als* genes were stably inherited. Resistance is high to most ALS-inhibiting herbicides (Green et al. 2008).

The 91840 corn line has a modified version of a maize ALS encoded by a corn *hrra* gene, *zm-hrra*, to confer resistance to ALS-inhibiting herbicides. It also has the two HRA amino acids substitutions present in the soybean HRA. Expression of the *zm-hrra* gene is functionally linked to the native maize ALS promoter. HRA gives high resistance to all ALS herbicides in corn (Green et al. 2008).

Another wave of transgenic ALS-resistance traits from BASF and Dow has been in field tests for a number of years (APHIS 2008). BASF and Embrapa, Brazil's crop science institute, have announced that they are codeveloping transgenic ALS traits in soybean and sugarcane (*Saccharum officinarum* L.) with commercialization in soybean planned around 2011. The soybean trait is a modified gene from *A. thaliana*.

HPPD-Inhibiting Herbicide Resistance. Herbicides that inhibit HPPD (EC 1.13.11.27) control a number of important weed species and have soil residual activity that complements glyphosate performance (Matringe et al. 2005). HPPD converts 4-hydroxyphenyl pyruvate to homogentisate, a key step in plastoquinone biosynthesis, and its inhibition causes bleaching symptoms on new growth. These symptoms result from an indirect inhibition of carotenoid synthesis because of the involvement of plastoquinone as a cofactor of phytoene desaturase (PDS; EC 1.14.99.-). Tissue damage is slower to appear on older tissue as it depends on carotenoid turnover (EXTOXNET 2007; WSSA 2007). There are no reports yet of any weeds resistant to HPPD-inhibiting herbicides.

Resistance to HPPD-inhibiting herbicides has been field tested since 1999 (APHIS 2008). In 2001, the trait was approved for field tests in a stack with a glyphosate-resistance trait. In November 2007, Bayer announced collaboration with Mertec and M.S. Technologies to develop soybean with glyphosate-, glufosinate-, and HPPD-inhibitor resistance.

ACCase and Auxin Resistance. ACCase (EC 6.4.1.2) catalyzes the adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA from malonyl-CoA, in the first step of

fatty acid synthesis. Cells produce acetyl-CoA in the cytoplasm, chloroplasts, mitochondria, and peroxysomes. Acetyl-CoA does not cross membranes. ACCase-inhibiting herbicides inhibit ACCase in most monocot species (Via-Ajub et al. 2005). The first ACCase-resistant crop, sethoxydim-resistant corn, was not transgenic and had an altered ACCase enzyme that was generated from tissue culture selection (Somers 1996).

Auxin herbicides are the oldest class of synthetic herbicides, 60 yr old, and are still widely used. New herbicides in this class are still introduced each decade. Auxin herbicides mimic the natural plant growth hormone or auxin, indole-3-acetic acid. Auxin herbicides disrupt natural growth and development processes and can cause plant death, particularly in broadleaf species (EXTOXNET 2007; WSSA 2007). Because auxin herbicides act at multiple receptors, designing a target-site modified auxin-resistant crop is extremely difficult. To date, only resistance mechanisms based on metabolic inactivation have been technically successful.

A new family of traits provides resistance to certain auxin- and ACCase-inhibiting herbicides with chemical structure similarities that allow aryloxyalkanoate dioxygenase (AAD) to inactivate both (Wright et al. 2005, 2007). The AAD gene was isolated from a gram-negative soil bacteria, *Alcaligenes eutrophus*, and codes for a 2-ketoglutarate-dependent dioxygenase that degrades the alkanoate side chain of the synthetic auxin herbicide 2,4-D to a hydroxyl (Fukumori and Hausinger 2007). Various AAD gene sequences code 2-ketoglutarate-dependent dioxygenases that differentially inactivate the two herbicide types. Some are active on phenoxyalkanoate auxins, and some are active on aryloxyphenoxypropionate (AOPP) ACCase inhibitors, whereas others inactive both. One, called AAD-12, inactivates pyridyloxyacetate synthetic auxins, such as triclopyr and fluroxypyr, but not AOPP ACCase herbicides. These traits will be combined with current glyphosate and glufosinate resistance traits (Simpson et al. 2008).

Dicamba Resistance. Dicamba is a widely used, low-cost auxin herbicide that does not persist very long in the soil (EXTOXNET 2007; WSSA 2007). In 2003, researchers used a bacterial gene for an enzyme that deactivates dicamba to make resistant plants (Behrens et al. 2007). The transgene, dicamba monooxygenase (DMO), from the soil bacterium *Pseudomonas maltophilia* (strain DI-6), encodes for Rieske nonheme monooxygenase that converts dicamba to 3-6-dichlorosalicylic acid (DCSA). The bacterial complex, dicamba O-demethylase, has three components, which include the monooxygenase, a reductase, and a ferredoxin. It shuttles electrons from reduced nicotinamide adenine dinucleotide (NADH) through a reductase to a ferredoxin and finally to the terminal component, DMO. The ferredoxin component of dicamba O-demethylase resembles the ferredoxin found in chloroplasts. The researchers targeted the monooxygenase for expression in the chloroplasts, where the enzyme would have a steady stream of electrons from reduced ferredoxin, generated by photosynthesis. Transgenic soybean events tolerated 2.8 kg ha⁻¹ dicamba with no apparent loss in agronomic performance (Behrens et al. 2007). Commercialization in a three-way stack with glyphosate and glufosinate resistance for soybean and corn could be as early as 2012.

PPO-Inhibiting Herbicide Resistance. Protoporphyrinogen oxidase (PPO) is an essential enzyme that catalyzes the last

common step in the production of heme and chlorophyll, the oxidation of protoporphyrinogen IX to protoporphyrinogen IX. PPO-inhibiting herbicides cause the accumulation of protoporphyrinogen. Then, exposure to light causes the formation of singlet oxygen and other oxidative chemicals that cause rapid burning and desiccation of leaf tissue and growth inhibition (Lee et al. 1993). The soil residual and fast action of PPO-inhibitor herbicides complement the slow and systemic activity of glyphosate. Several strategies to make crops resistant to PPO-inhibiting herbicides have been successful, and the technology has even received a trade name (Li and Nicholl 2005).¹⁴

PPO enzyme mutations tend to reduce its enzymatic activity, which could help explain the relatively slow evolution of resistant weeds to this 40-yr-old herbicide class (Li et al. 2003). It also explains the need for a strong promoter to develop PPO-resistant crops that rely on PPO mutations. For example, the first PPO-resistant corn used a double mutant PPO, PPO-1, from *A. thaliana* with its native promoter (PTX). Similarly, PPO inhibitor-resistant rice used overexpression of the naturally resistant *Bacillus subtilis* PPO gene. Other approaches, such as increasing gene copy number and tissue culture to select for overexpression of wild-type PPO genes, have also been successful (Li and Nicholl 2005).

P450 Metabolic Resistance. Cytochrome P450 monooxygenase (P450) enzymes are located on the cytoplasmic side of the smooth endoplasmic reticulum and metabolically inactivate herbicides such as acetanilides, bentazon, ALS-inhibiting herbicides (imidazolinones, sulfonamides, sulfonyleureas, and triazolopyrimidines), isoxazoles, phenoxy, and urea herbicides (Barrett et al. 1997; Hatzios and Burgos 2004). Plants have a diverse array of more than 50 families of P450 enzymes, with more than 500 genes (Barrett et al. 1997). P450 enzymes catalyze ring hydroxylation, epoxidation, sulfoxidation, dealkylation, and alkyl oxidation reactions. Some P450 functions include the biosynthesis of lignins, ultraviolet protectants, pigments, defense compounds, fatty acids, hormones, and signaling molecules and degradation of internally and externally produced toxic compounds. P450 enzymes are often inducible by herbicide safeners (Davies and Caseley 1999). Because of their roles in a diversity of metabolic processes, P450 transgenes are potential tools as selectable markers for transformation (Ishida et al. 1996) or as traits for developing herbicide-resistant crops (Ohkawa et al. 1999; Siminzky et al. 1999).

Didierjean et al. (2002) showed that a P450 transgene could give resistance to diuron. More recently, Williams et al. (2006) identified four closely linked P450 genes on the short arm of chromosome 5 in corn. At least one of these genes functions to deactivate a number of herbicides. Soybeans that express the transgene show resistance to a range of herbicides (Dam et al. 2007).

Summary

GR crop technologies continue to evolve. The commercial successes of the first GR crops encouraged companies to make large investments to expand research capabilities and develop new GR transgenes. Researchers have overcome many of the technical difficulties that slowed commercialization of initial

transgenic traits and have discovered many new transgenes. Some of these transgenes have improved characteristics but transgenes with equivalent characteristics also have value if companies can avoid royalty payments and give their breeders greater freedom to operate. The number of GR crops and GR transgenes will almost certainly continue to increase.

Growers have very rapidly accepted GR crops. Glyphosate resistance is the dominant transgenic trait being used on the 114 million ha of transgenic crops in 23 countries. Such widespread use and reliance on glyphosate has stimulated the evolution of resistant weeds and increased concerns about the long-term sustainability of glyphosate. Experts agree that growers cannot rely on glyphosate only and must use more diverse weed management systems. Unfortunately, chemical companies are not commercializing any selective herbicides with new modes of action. Therefore, companies are rapidly developing crops that combine glyphosate resistance with resistance to herbicides with other modes of action to meet this need. These multiple herbicide-resistant crops will give growers more options to manage the evolution of resistant weeds with currently available herbicide technology. When used properly with other herbicides and resistance mechanisms, glyphosate and GR crops will continue to play an important role in weed management.

Sources of Materials

¹ GTS 40-3-2 glyphosate-resistant soybean event, trade name Roundup Ready[®], Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

² MON89788 glyphosate-resistant soybean event, trade name Roundup Ready2Yield[®], Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

³ 356043 glyphosate and ALS-inhibiting herbicide resistant soybean, trade name Optimum[®] GAT[®], Pioneer Hi-Bred, 7100 NW 62nd Avenue, Johnston, IA 50131.

⁴ GA21 glyphosate-resistant corn, trade names Roundup Ready[®], Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167; and Agrisure[™] GT, Syngenta Seeds, 7500 Olson Memorial Highway, Golden Valley, MN 55427.

⁵ NK603 glyphosate-resistant corn, trade name Roundup Ready[®] 2, 800 N. Lindbergh, Monsanto Company, St. Louis, MO 63167.

⁶ 98140 glyphosate and ALS-inhibiting herbicide resistant corn, trade name Optimum[®] GAT[®], Pioneer Hi-Bred, 7100 NW 62nd Avenue, Johnston, IA 50131.

⁷ MON1445 glyphosate-resistant cotton event, trade name Roundup Ready[®], Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

⁸ MON88913 glyphosate-resistant cotton, trade name Roundup Ready[®] Flex, Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

⁹ GHB614 glyphosate-resistant cotton, trade name Glyto1[™], Bayer CropScience, 2 TW Alexander Drive, Research Triangle Park, NC 27709.

¹⁰ GT73 and GT200 glyphosate-resistant canola, trade name Roundup Ready[®], Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

¹¹ J101 and J163 glyphosate-resistant alfalfa, trade name Roundup Ready[®], Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

¹² H7-1 glyphosate-resistant sugarbeet, trade name Roundup Ready®, Monsanto Company, 800 N. Lindbergh, St. Louis, MO 63167.

¹³ Glufosinate-resistant crops, trade name LibertyLink®, Bayer CropScience, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709.

¹⁴ PPO-inhibiting herbicide resistance, trade name Acuron™, Syngenta Seeds, Syngenta Seeds, 7500 Olson Memorial Highway, Golden Valley, MN 55427.

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