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Response of 98140 Corn with *gat4621* and *hra* Transgenes to Glyphosate and ALS-Inhibiting Herbicides

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The transgenic corn line 98140 has a high level of resistance to glyphosate and all five chemical classes of herbicides that inhibit acetolactate synthase (ALS). The dual herbicide resistance is due to a molecular stack of two constitutively expressed genes: *gat4621,* which produces a glyphosate acetyltransferase that rapidly inactivates glyphosate, and *hra,* which produces a highly resistant ALS. On a rate basis, the positive 98140 isoline with a single copy of the *gat4621* gene is over 1,000-fold more resistant to glyphosate than a negative isoline without the transgene. Similarly, the positive 98140 isoline with the *hra* gene is over 1,000-fold more resistant to ALS-inhibiting herbicides such as chlorimuron and sulfometuron at the whole-plant and enzyme level. The *gat4621* and *hra* genes do not change the natural tolerance of corn to selective herbicides, so new corn hybrids based on 98140 will give growers more options to manage weeds and delay the evolution of herbicide-resistant weeds.

Nomenclature: Chlorimuron; glyphosate; sulfometuron; corn, *Zea mays* L.

Key words: AHAS, EPSPS, maize, imidazolinone, pyrimidinylthiobenzoate, sulfonylamino–carbonyl–triazolinone, triazolopyrimidine, sulfonylurea.

Glyphosate-resistant (GR) crops have transformed the way many growers manage weeds by providing a new and more effective, flexible, and economical way to control weeds. However, after a decade of widespread use, weeds are adapting to GR crop systems and glyphosate applied alone is losing effectiveness. Now, growers must diversify their weed management programs to maintain the effectiveness of glyphosate. Unfortunately, no commercial herbicide with a new mode of action has been discovered since the early 1980s (Stuebler et al. 2008). Fortunately, the trend with crop technology has changed from single to multiple herbicide resistance traits, which expand weed management options with existing herbicides and help extend the utility of glyphosate (Green et al. 2008). For example, Pioneer Hi-Bred International, Inc. is developing a multiple herbicide–resistant corn with a molecular stack of a metabolic system that inactivates glyphosate and a highly resistant acetolactate synthase (ALS; EC 4.1.3.18).[1]

Soon after the discovery of glyphosate and ALS-inhibiting herbicides, scientists started work to develop resistant crops (Dill et al. 2008; Thompson 2007). Nontransgenic approaches were not successful for GR crops (Franz et al. 1996). In 1983, scientists found a highly resistant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.26) in a soil bacterium, *Agrobacterium tumefaciens* strain CP4, and used its *cp4 epsps* gene to develop GR soybean [*Glycine max* (L.) Merr] in 1996 (CaJacob et al. 2007). Today, other transgenic GR crops that have been commercialized include cotton (*Gossypium hirsutum* L.), corn, Argentine canola (*Brassica napus* L.), Polish canola (*Brassica rapa* L.), alfalfa (*Medicago sativa* L.), and sugarbeet (*Beta vulgaris* L.). Nontransgenic approaches to develop ALS-resistant crops were more successful with ALS-resistant corn commercialized in 1992. Today, other ALS-resistant crops include soybean, canola, wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and sunflower (*Helianthus annuus* L.) (Tan et al. 2005).

A new metabolic system to inactivate glyphosate is being developed on the basis of the *gat4621* gene that codes for an enhanced glyphosate acetyltransferase enzyme (Castle et al. 2004). The *gat4621* gene is derived from the sequences of three weakly active *N*-acetyltransferase isozymes from the soil bacterium *Bacillus licheniformis* (Weigmann) Chester. To increase the conversion of glyphosate to *N*-acetylglyphosate (NAG), a collection of recombined *gat* genes were expressed in *Escherichia coli* and screened for activity. Recombinants with the highest activity then went through 11 iterative rounds of gene shuffling, a technique for molecular recombination of genes and directional screening to improve enzymatic activity (Ness et al. 2002). At two points, site-directed mutagenesis introduced additional amino acid substitutions to augment the 12 amino acid differences among the natural isozymes (Castle et al. 2004; Siehl et al. 2007).

The first transgenic ALS gene was *hra*, a highly herbicide resistant allele with two mutations: tryptophan to leucine at position 574 and proline to alanine at position 197 (Bedbrook et al. 1995). The tryptophan to leucine substitution confers high resistance to a wide range of ALS-inhibiting herbicides, whereas the proline substitution confers additional resistance to specific sulfonylurea and triazolopyrimidine herbicides (Tranel and Wright 2002). The *hra* and the *gat4621* genes were combined in a molecular stack, and *Agrobacterium*-mediated transformation was used to insert the genes into the corn line 98140.[1] In 98140, a corn ALS promoter (zmALS) drives expression of a corn *hra,* and a corn ubiquitin promoter (ubiZM1) drives expression of *gat4621*. In addition, the PHP24279 vector has three copies of the CaMV 35S enhancer region from cauliflower mosaic virus that contribute to the constitutive expression of both the *hra* and *gat4621* genes.

Currently, most ALS-inhibiting herbicides depend on rapid metabolic inactivation for crop safety. The widespread introduction of a target-site *hra* gene in corn could eliminate crop safety concerns for many ALS herbicides. Used alone, *hra* with ALS-inhibiting herbicides will not provide an effective or sustainable weed management system (Tranel and Wright 2002). However, a stack of glyphosate and ALS herbicide resistance that maintains natural tolerance to commonly used

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selective herbicides would create more biological opportunities to combine herbicide modes of action, particularly combinations with soil residual activity, to manage weeds and delay the evolution of GR weeds (Green et al. 2008). In this study, we investigate the level of resistance that a 98140 hybrid with *gat4621* and *hra* has to glyphosate, a wide range of ALS herbicides, and currently registered, naturally selective corn herbicides.

Material and Methods

Whole-Plant Tests. The 98140 corn used in these studies was one of the first experimental hybrids created from the initial source of the *gat* and *hra* transgenic event that is being commercially developed, the inbred 'introEF09B'. This 98140 hybrid is hemizygous, having a single copy of the *gat* and *hra* transgenes. Seeds were planted 2 cm deep in 14-cm square plastic containers. For the preemergence studies, two seeds were planted in containers filled with Tama silt loam (fine-silty, mixed, superactive, mesic Aquic Argiudolls) soil with 3% organic matter. For the postemergence studies, four seeds were planted in each container and thinned after emergence to two uniform plants. These containers were filled with a synthetic growth medium.[2]

All containers were watered and fertilized for rapid growth. The fertilizer solution[3,4,5] was diluted into the watering system with an injector system.[6] Metal halide lights with 160 μmol m^{-2} s^{-1} of photosynthetically active radiation supplemented natural intensity during a 16-h photoperiod when light intensity was below 500 μmol m^{-2} s^{-1}. Day temperature was 28 ± 2 C and night temperature was 22 ± 2 C. Relative humidity ranged from 50 to 90%. For the preemergence studies, corn was sprayed within 24 h after planting. For the postemergence studies, corn was sprayed 10–11 days after planting at the V2 to V3 growth stage.

The commercial herbicide formulations used in the studies are listed in Table 1. Spray preparations of the potassium salt of glyphosate and the 28 ALS-inhibiting herbicides were made with deionized water and applied with a tallow amine surfactant with 15 mol of ethoxylation (TAE-15)[8] applied at 0.25% (w/w) of the spray volume. The soluble liquid formulation of glyphosate had no added surfactants. Glyphosate rates are expressed as the amount of acid. All treatments were applied in a spray chamber at a spray volume of 374 L ha^{-1} with an 8002E flat fan noozle[7] at a height of 51 cm and 138 kPa spray pressure. For ALS herbicides, 0.1% (w/w) tripotassium phosphate (K_3PO_4) was added to solubilize any herbicide particles (Green and Cahill 2003). For the postemergence study with other herbicide types, the adjuvant condition was 1% (w/w) of a high-quality crop oil concentrate (COC)[9] and 2.5% (w/w) ammonium sulfate (AMS).

The experimental design was completely randomized with four replications and the test was repeated. For the preemergence studies, visual ratings and shoot fresh weights were taken 24 d after herbicide application. For the postemergence studies, visual ratings and shoot fresh weights were taken 14 d after application. Plants were visually evaluated on a scale of 0 (no effect or no symptoms) to 100% injury (dead). Leaf malformation, chlorosis, and necrosis; reduction of leaf expansion; and overall plant size were key parameters for visual ratings. Percent fresh weight growth inhibition was determined by comparing treated plants to the untreated control. Fresh weight and visual evaluations for ALS-inhibiting herbicides were highly correlated (more than 97%), so only fresh weight measurements are reported. Both visual and fresh weight results are reported for tests that involved herbicides with other modes of action.

Enzyme Assays. ALS enzyme was obtained from corn grown in similar fashion to the whole-plant assay. After 2 wk, 30 g of leaf tissue was harvested, mixed with 300 mg of polyvinyl polypyrrolidone, then frozen rapidly with liquid nitrogen. The frozen mixture was ground to a fine powder and stored at −80 C. The enzyme was extracted from the powder by addition of two volumes of 100 mM K_2HPO_4, pH 7.5, containing 0.5 mM $MgCl_2$, 10% glycerol, 1 mM pyruvate, 0.5 mM thiamine pyrophosphate, and 10 μM FAD (extraction buffer). The homogenate was filtered through eight layers of cheesecloth and centrifuged at 25,000 × *g* for 20 min. AMS was used to precipitate the enzyme from the supernatant. Protein that precipitated between 25 and 75% saturation was collected by centrifugation. The pellet was resuspended in 70% AMS and then flash frozen in liquid nitrogen and stored at −80 C.

The enzyme was prepared for assay by thawing aliquots of the AMS suspension, recovering the pellet after centrifugation and dissolving the protein in the extraction buffer. The enzyme was then desalted on PD10 columns equilibrated with the same buffer. Assays were performed essentially as described before (Ray 1984; Singh et al. 1988), wherein acetolactate is converted to acetoin by acidification with 6 N sulfuric acid and the acetoin is colorized by reaction with 0.5% creatine and 5% naphthol solution at 60 C for 15 m. Acetoin absorbance at 525 nm was converted to percentage of control after correction for the enzyme. Protein was determined with the Bradford assay (Bradford 1976).

Growth Time Course. Corn was grown similarly to other postemergence studies. Glyphosate application was made midafternoon to minimize the effect of diurnal variations in leaf growth (Tang and Boyer 2008). The extensiometer system for measuring plant growth used linear variable differential transformers (LVDT)[11] interfaced with a computerized data acquisition system[12] to measure leaf elongation (Bogoslavsky and Neuman 1998; Degli Agosti et al. 1997). Multiple plants were monitored simultaneously. At 7 d, seedlings were selected for uniformity of stage and the emergence of a newly expanding leaf extending vertically from the whorl. The leaf tip was connected to a small alligator clip connected to a microfilament thread looped over a low-resistance pulley and joined to the core of a LVDT. A small counterweight helped overcome frictional resistance. The electrical output of the LVDT varied linearly with changes in the position of the tip of the growing leaves. Without resetting, the LVDT can measure a displacement of up to 38.1 mm with an accuracy of 0.25 mm. Leaf growth was measured every 15 m from 5 h before to 28 h after herbicide applications. The positive isoline with the *gat* gene and the negative isoline without *gat* were evaluated in separate experiments that were done under similar conditions. Results are the means of five replications.

Statistical Analysis. For the single–herbicide rate studies, data were analyzed with a general linear model procedure[10] to

Table 1. Commercial herbicides used in biological tests.

Mode of action[a]	Common name	Registered trade name	% Active	Physical state	Manufacturer
EPSPS inhibitor	Glyphosate	Touchdown Hi-Tech	52.3	Liquid	Syngenta Crop Protection
ALS inhibitor	Imazethapyr	Pursuit	70	Dry	BASF Agricultural Products
	Imazapic	Cadre	70	Dry	BASF Agricultural Products
	Imazapyr	Arsenal	28.7	Liquid	BASF Agricultural Products
	Imazaquin	Scepter	17.3	Liquid	BASF Agricultural Products
	Pyrithiobac	Staple	85	Dry	DuPont Crop Protection
	Flucarbazone	Everest	70	Dry	Arysta LifeScience
	Amidosulfuron	Gratil	75	Dry	Bayer CropScience
	Azimsulfuron	Gulliver	50	Dry	DuPont Crop Protection
	Bensulfuron	Londax	50	Dry	DuPont Crop Protection
	Chlorimuron	Classic	25	Dry	DuPont Crop Protection
	Chlorsulfuron	Glean	75	Dry	DuPont Crop Protection
	Ethametsulfuron	Muster	75	Dry	DuPont Crop Protection
	Flupyrsulfuron	Lexus	50	Dry	DuPont Crop Protection
	Iodosulfuron	Husar	5	Dry	Bayer CropScience
	Metsulfuron	Ally	60	Dry	DuPont Crop Protection
	Nicosulfuron	Accent	75	Dry	DuPont Crop Protection
	Primisulfuron	Tell	75	Dry	Syngenta Crop Protection
	Prosulfuron	Peak	57	Dry	Syngenta Crop Protection
	Rimsulfuron	Titus	75	Dry	DuPont Crop Protection
	Sulfometuron	Oust	75	Dry	DuPont Crop Protection
	Sulfosulfuron	Maverick	75	Dry	Monsanto Company
	Thifensulfuron	Harmony	75	Dry	DuPont Crop Protection
	Triasulfuron	Amber	75	Dry	Syngenta Crop Protection
	Tribenuron	Express	50	Dry	DuPont Crop Protection
	Trifloxysulfuron	Envoke	75	Dry	Syngenta Crop Protection
	Triflusulfuron	Upbeet	50	Dry	DuPont Crop Protection
	Chloransulam	FirstRate	84	Dry	Dow AgroSciences
	Flumetsulam	Python	80	Dry	Dow AgroSciences
HPPD inhibitor	Isoxaflutole	Balance Pro	40.5	Liquid	Bayer CropScience
	Mesotrione	Callisto	40	Liquid	Syngenta Crop Protection
PSII inhibitor	Atrazine	AAtrex	90	Dry	Syngenta Crop Protection
	Bentazon	Basagran	44	Liquid	Agriliance
	Bromoxynil	Buctril	33.4	Liquid	Bayer CropScience
Mitosis inhibitor	Acetochlor	Harness	74.8	Liquid	Monsanto Company
	Alachlor	Lasso	45.1	Liquid	Monsanto Company
	Dimethenamid	Frontier	60	Liquid	BASF Agricultural Products
	Pendimethalin	Prowl	37.4	Liquid	BASF Agricultural Products
PPO inhibitor	Carfentrazone	Aim	40	Dry	FMC Corporation
	Flumiclorac	Resource	10.1	Liquid	Valent U.S.A. Corporation
Synthetic auxin	2,4-D	Unison	19.6	Liquid	Helena Corporation
	Clopyralid	Stinger	40.9	Liquid	Dow AgroSciences
	Dicamba	Clarity	56.8	Liquid	BASF Agricultural Products

[a] Abbreviations: ALS, acetolactate synthase; HPPD, 4-hydroxyphenyl pyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PSII, photosystem II.

determine treatment means and least significant difference at the 0.05 level of probability. For the multiple–herbicide rate studies, a nonlinear regression procedure[10] was used to analyze five herbicide rates, with the rate range overlapping the 50% inhibition level. Herbicide rate response was analyzed with a logistic rate–response equation (Streibig et al. 1993), in which the biological response y to herbicide rate x was

$$y = C + ([D - C]/\{1 + \exp[b(\log x - \log \mathrm{GR}_{50})]\}) \quad [1]$$

where C is the lower response limit or no inhibition, D the upper response limit or 100% inhibition, b the slope, and GR_{50} the rate that gives 50% inhibition. The enzyme assays were analyzed similarly to generate the concentration producing 50% inhibition (IC_{50}) and response curves. The resistance ratio is the ratio of the 50% inhibition rate of the positive and negative isolines.

Results and Discussion

Response to 28 ALS-Inhibiting Herbicides. If the *hra* transgene makes corn highly resistant to ALS-inhibiting herbicides, then it has the potential to enable new weed management options. To evaluate how resistant, corn isolines with and without the *hra* gene were treated preemergence and postemergence with a high rate of 28 ALS herbicides from five chemical classes (sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinylthiobenzoate, and sulfonylamino–carbonyl–triazolinone). The 200 g ha^{-1} rate is a much higher than generally recommended rates for ALS herbicides. Still, the *hra* gene significantly increased resistance to all 28 ALS herbicides (Tables 2 and 3).

More tolerance to ALS-inhibiting herbicides with soil residual activity would be an advantage for corn growers. However, corn is currently not tolerant preemergence to most commercial ALS herbicides because of limited metabolic detoxification in roots (Koeppe et al. 2000). To evaluate for this utility, a high rate of 28 ALS-inhibiting herbicides was applied preemergence to the positive and negative isolines of 98140. The positive isoline was highly resistant to 200 g ha^{-1} of the ALS-inhibiting herbicides—even those that severely injured the negative isolines (Table 2). Specifically, 21 of the 28 ALS herbicides that were tested inhibited the negative isoline more than 90%, whereas 18 of the 28 did not

Table 2. Preemergence response to 200 g ha^{-1} of 28 acetolactate synthase (ALS)–inhibiting herbicides on 98140 hybrid corn isolines with and without a single copy of the *gat* and *hra* transgenes.

ALS herbicide class	Herbicide	Positive isoline	Negative isoline
		% Growth inhibition	
Imidazolinone	Imazethapyr	15	98
	Imazapic	24	99
	Imazapyr	14	100
	Imazaquin	6	98
Pyrimidinylthiobenzoate	Pyrithiobac	6	100
Sulfonylamino–carbonyl–triazolinone	Flucarbazone	7	100
Sulfonylurea	Amidosulfuron	11	98
	Azimsulfuron	16	100
	Bensulfuron	3	57
	Chlorimuron	4	100
	Chlorsulfuron	11	100
	Ethametsulfuron	8	100
	Flupyrsulfuron	5	91
	Iodosulfuron	25	100
	Metsulfuron	66	100
	Nicosulfuron	3	37
	Primisulfuron	2	85
	Prosulfuron	22	77
	Rimsulfuron	4	72
	Sulfometuron	20	100
	Sulfosulfuron	0	99
	Thifensulfuron	4	98
	Triasulfuron	29	98
	Tribenuron	10	24
	Trifloxysulfuron	21	100
	Triflusulfuron	0	94
Triazolopyrimidine	Chloransulam	1	100
	Flumetsulam	0	79
Treatment LSD (0.05)		13	

Table 3. Postemergence response to 200 g ha^{-1} of 28 acetolactate synthase (ALS)–inhibiting herbicides on 98140 hybrid corn isolines with and without a single copy of the *gat* and *hra* transgenes.

ALS herbicide class	Herbicide	Positive isoline	Negative isoline
		% Growth inhibition	
Imidazolinone	Imazethapyr	31	97
	Imazapic	26	98
	Imazapyr	33	99
	Imazaquin	9	96
Pyrimidinylthiobenzoate	Pyrithiobac	16	99
Sulfonylamino–carbonyl–triazolinone	Flucarbazone	4	99
Sulfonylurea	Amidosulfuron	10	92
	Azimsulfuron	7	99
	Bensulfuron	1	91
	Chlorimuron	8	98
	Chlorsulfuron	8	99
	Ethametsulfuron	3	97
	Flupyrsulfuron	5	9
	Iodosulfuron	13	96
	Metsulfuron	52	100
	Nicosulfuron	7	35
	Primisulfuron	7	71
	Prosulfuron	10	57
	Rimsulfuron	0	52
	Sulfometuron	72	100
	Sulfosulfuron	5	98
	Thifensulfuron	9	96
	Triasulfuron	1	90
	Tribenuron	15	99
	Trifloxysulfuron	65	100
	Triflusulfuron	5	97
Triazolopyrimidine	Chloransulam	2	95
	Flumetsulam	0	51
Treatment LSD (0.05)		11	

Table 4. Postemergence activity of glyphosate and 12 ALS-inhibiting herbicides on 98140 hybrid corn isolines with and without a single copy of the *gat* and *hra* transgenes. The resistance ratio is a ratio of the 50% growth inhibition rate (GR_{50}) of the positive and negative isoline.

		Positive isoline		Negative isoline	
Herbicide	Resistance ratio	GR_{50}	95% Confidence interval	GR_{50}	95% Confidence interval
		g ha^{-1}		g ha^{-1}	
Azimsulfuron	> 30,000	> 10,000		0.31	0.25–0.37
Bensulfuron	> 1,200	> 10,000		8.3	4.5–12.1
Chlorimuron	55,200	5,520	4,520–6,820	0.10	0.08–0.12
Chlorsulfuron	14,400	8,350	4,100–12,600	0.58	0.46–0.71
Ethametsulfuron	> 28,000	> 10,000		0.36	0.29–0.43
Glyphosate	> 1,100	> 32,000		29	24–34
Metsulfuron	364	215	168–261	0.59	0.43–0.75
Nicosulfuron	> 65	> 10,000		153	87–222
Pyrithiobac	18,200	5,830	3,820–7,840	0.32	0.21–0.43
Rimsulfuron	> 120	> 10,000		84	43–126
Sulfometuron	4,125	165	123–207	0.04	0.03–0.06
Thifensulfuron	> 625	> 10,000		16	12–20
Tribenuron	> 2,425	7,520	4,640–10,400	31	2.6–3.6

significantly inhibit the positive isoline (less than 14% inhibition). Of the herbicides tested, only metsulfuron inhibited the positive 98140 isoline more than 30%. Because 200 g ha^{-1} is potentially 50 times higher than the use rate of metsulfuron, all the ALS herbicides tested have some potential for use on 98140 corn.

Similarly, 98140 corn is highly resistant to postemergence applications of a high rate of a wide range of ALS-inhibiting herbicides, even those without any natural tolerance (Table 3). At 200 g ha^{-1} with a potent adjuvant system, most ALS herbicides severely injured the negative isolines and did not injure the positive isolines. Specifically, 22 of the 28 ALS herbicides that were tested inhibited the negative isoline at least 90%, whereas 19 of the 28 did not significantly inhibit the positive isoline (less than 12% inhibition). Several others slightly inhibited the positive isoline (less than 20%). Even the ALS herbicides that inhibited 98140 corn likely have a safe use rate. For example, metsulfuron and sulfometuron inhibited the positive isoline the most with 52 and 72% ratings, respectively; however, the 200 g ha^{-1} rate is 50 to 100 times the rate needed for these herbicides to control key weeds.

Rate Response. After the single-rate experiments, multiple rate studies were done with glyphosate and 12 postemergence ALS-inhibiting herbicides to better quantify the effect of the *gat4621* and *hra* genes (Table 4; Figure 1). To ensure highest activity, the herbicides were applied with a potent adjuvant system (Green and Cahill 2003). In all cases, the positive 98140 isoline with the *gat4621* and *hra* genes showed high resistance to glyphosate and the 12 ALS herbicides.

On a rate basis, the positive 98140 isoline increased resistance to glyphosate over 1,000-fold with a 50% inhibition rate over 32,000 g ha^{-1}, the highest rate tested, compared with 29 g ha^{-1} for the negative isoline. Similarly, the positive isoline with *hra* is highly resistant to all the ALS-inhibiting herbicides. When ALS herbicides were not naturally tolerant and had very low 50% inhibition rates, the ratio of the positive and negative 50% inhibition rates was more than 1,000-fold. The highest resistance ratios were more than 28,000 for chlorimuron, azimsulfuron, and ethametsulfuron. The ALS herbicides that most strongly inhibited the positive

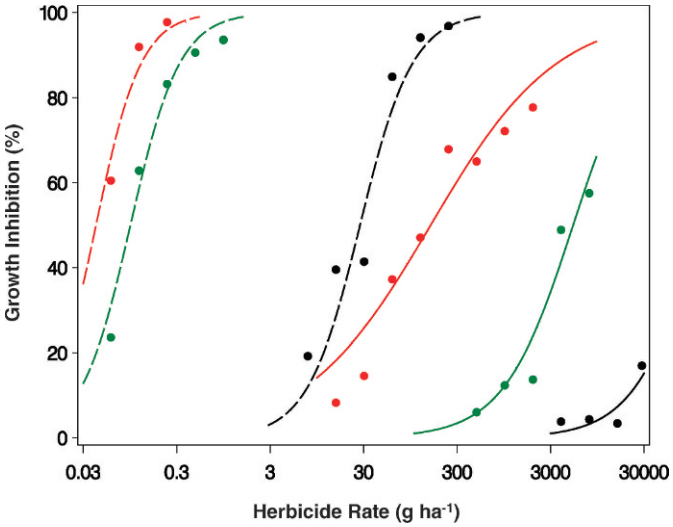

Figure 1. Postemergence response of sulfometuron (red), chlorimuron (green), and glyphosate (black) on hemizygous positive (— — —) and negative (——) 98140 corn isolines at the two- to three-leaf growth stage. The transgenes are *gat4621* and *hra*.

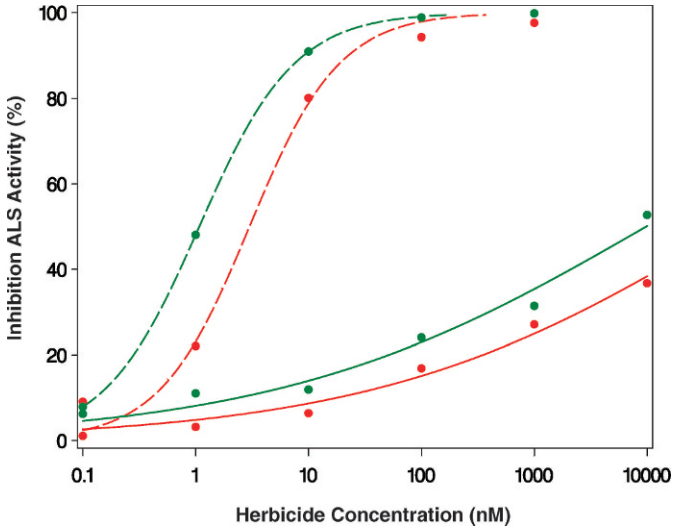

Figure 2. Inhibition of ALS enzyme from hemizygous positive (— — —) with *hra* transgene and negative (——) 98140 corn isolines at the two- to three-leaf growth stage by sulfometuron (red) and chlorimuron (green).

98140 isoline were metsulfuron and sulfometuron, with 50% inhibition rates of 215 and 165 g ha^{-1}, respectively. Even these herbicides could have some utility because the 50% rates were more than 350-fold higher than the corresponding rates on the negative isolines and nearly 100-fold higher than the rate needed to control broadleaf weeds (Russell et al. 2002).

The *hra* transgene was also effective at increasing the resistance to the ALS-inhibiting herbicides with natural tolerance. For example, the 50% inhibition rates for nicosulfuruon, rimsulfuron, and thifensulfuron were greater than 10,000 g ha^{-1}, or the highest rate tested. The resistance ratio is not as high for these herbicides because the negative isoline without *hra* is not as sensitive.

Enzyme Dose Response. Two sulfonylurea herbicides were used to evaluate the sensitivity of ALS enzyme extracted from positive and negative 98140 isolines (Figure 2). Because corn does not metabolize either of these herbicides, crop safety is dependent solely on the insensitivity of the target site, and some correlation between the ALS enzyme and whole-plant results was expected. Indeed, the increased resistance in the positive isoline with the *hra* transgene was more than 1,000-fold to both sulfonylurea herbicides at the whole-plant and enzyme level. Such increases in resistance have been observed with HRA from other plant species (Bedbrook et al. 1995), but these are the first data that confirm similar efficacy in corn with the corn HRA (ZM-HRA).

Growth Time Course. Metabolic tolerance to high rates of herbicides, even when the tolerance is very high, is typically associated with some transient stunting and yellowing after application (Green 1998). The transient response is due to the time needed to metabolically inactivate all the herbicide after it enters the crop. A key question for 98140 corn with metabolic tolerance based on the *gat4621* transgene is whether it will show similar transient responses to high rates of glyphosate. To evaluate this, an extensiometer measured corn leaf growth very precisely before and after application of a high rate of glyphosate (Figure 3). The sensitive corn isoline grew normally for 3 or 4 h after glyphosate application, and then growth rapidly stopped. In contrast, the positive isoline did not show any inhibition after glyphosate application. The extensiometer measurements clearly showed that the positive 98140 isoline grew the same if it was untreated or treated with 3,360 g ha^{-1} glyphosate. In addition, no transient yellowing was associated with the glyphosate application. These whole-plant results support previously published enzyme results that showed very high effectiveness of the GAT4621 metabolic system (Siehl et al. 2007).

Response to Other Modes of Action. Weed scientists generally agree that one of the principal causes for the evolution of herbicide-resistant weeds is the overreliance on a single mode of action to control weeds. Growers need to use herbicides with different modes of action, as well as mechanical and cultural methods to delay the evolution of resistant weeds. The *gat4621* and *hra* transgenes will enable new ALS-inhibiting herbicide options not currently safe enough for corn. Because the transgenes should not change natural tolerance to currently available selective herbicides, the total number of herbicide options will increase. To confirm that the transgenes did not change the natural tolerance of 98140 corn, a range of selective corn herbicides with diverse chemical structures and modes of actions was evaluated preemergence and postemergence on positive and negative isolines (Tables 5 and 6).

Seven herbicides with three modes of action were evaluated preemergence on positive and negative 98140 isolines. Both isolines responded similarly to preemergence application of various selective corn herbicides (Table 5). For example, the standard application rate of 170 g ha^{-1} mesotrione, an inhibitor of 4-hydroxyphenyl pyruvate dioxygenase [HPPD; EC 1.13.11.27], was applied to both the positive and negative isolines. Both isolines were highly tolerant to mesotrione; no statistically significant fresh weight reduction or visual injury was observed. Similarly, herbicides that inhibit mitosis or photosystem II (PSII) caused no to slight injury and the isolines responded similarly. Since both the positive and negative isolines of 98140 are equivalent in their tolerance to a

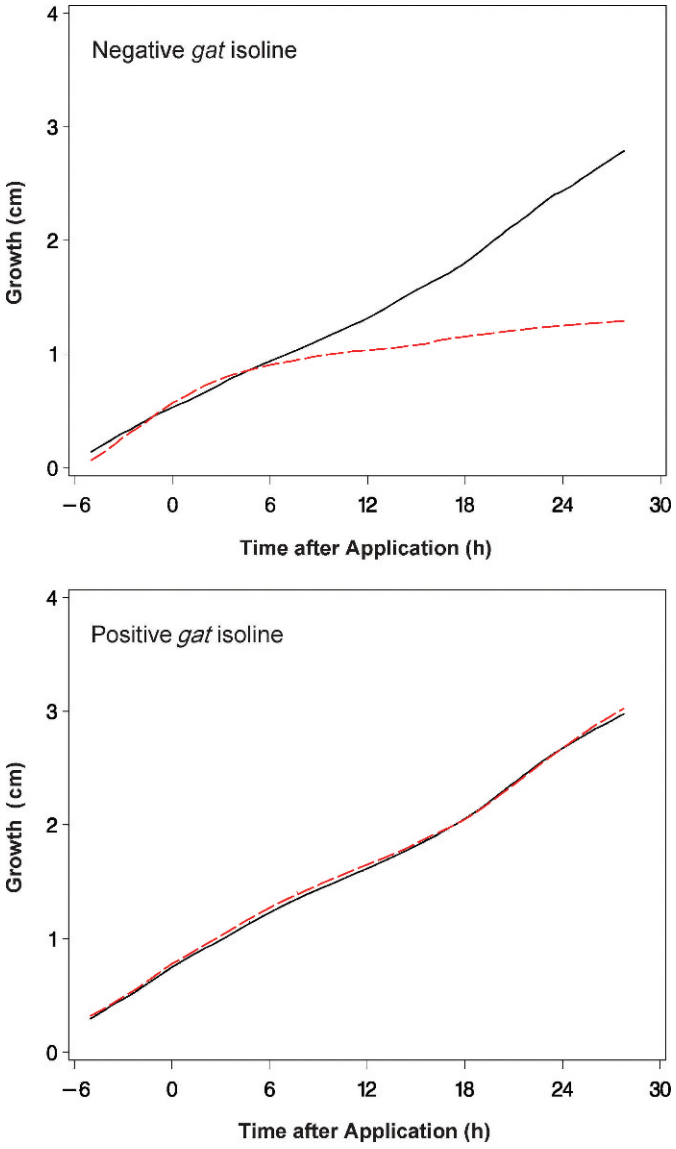

Figure 3. Time course for the growth of 98140 corn isolines before and after glyphosate application. Glyphosate was applied at time 0. The mean of five growth measurements are indicated with the black line when untreated and with the red dashed line when treated with 3,360 g ha^{-1} glyphosate.

Table 5. Preemergence response of naturally selective corn herbicides on 98140 hybrid isolines with and without a single copy of the *gat* and *hra* transgenes.

Mode of action[a]	Herbicide	Rate	Positive isoline	Negative isoline	Positive isoline	Negative isoline
		g ha^{-1}	% Growth inhibition		% Visual injury	
HPPD inhibitor	Mesotrione	170	0	1	4	4
	Isoxaflutole	158	19	10	9	7
PSII inhibitor	Atrazine	2,240	0	7	4	7
Mitosis inhibitors	Acetochlor	1,120	3	5	1	3
	Alachlor	3,060	13	19	11	7
	Dimethenamid	1,680	32	39	25	23
	Pendimethalin	1,120	0	0	4	3
Treatment LSD (0.05)			14		6	

[a] Abbreviations: HPPD, 4-hydroxyphenyl pyruvate dioxygenase; PSII, photosystem II.

Table 6. Postemergence response of naturally selective corn herbicides on 98140 hybrid isolines with and without a single copy of the *gat* and *hra* transgenes.

Mode of action[a]	Herbicide	Rate	Positive isoline	Negative isoline	Positive isoline	Negative isoline
		g ha^{-1}	% Growth inhibition		% Visual injury	
HPPD inhibitor	Mesotrione	105	8	2	1	1
	Topramezone	18	10	1	10	10
PSII inhibitor	Atrazine	2,240	11	11	1	1
	Bentazon	1,120	4	4	5	3
	Bromoxynil	210	0	0	4	4
PPO inhibitor	Carfentrazone	8	13	17	9	9
	Flumiclorac	30	0	7	4	8
Synthetic auxin	Dicamba	280	5	7	2	5
	Clopyralid	140	6	5	7	3
	2,4-D	35	8	8	5	4
Treatment LSD (0.05)			11		6	

[a] Abbreviations: HPPD, 4-hydroxyphenyl pyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PSII, photosystem II.

range of preemergence selective herbicides, no currently available herbicide options will need to be restricted and 98140-based hybrids will increase preemergence herbicide options.

Ten herbicides with four modes of action were evaluated postemergence on positive and negative isolines of 98140. Both isolines responded similarly to all the selective postemergence herbicides tested (Table 6). For example, the standard application rate of 105 g ha^{-1} mesotrione, an HPPD-inhibitor, did not significantly inhibit either of the isolines. Topramezone, three synthetic auxins, three PSII inhibitors, and the protoporphyrinogen oxidase (PPO; EC 1.3.3.4) inhibiting herbicide flumiclorac gave similar results. Because both the positive and negative isolines of 98140 are equivalent in their tolerance to a range of postemergence selective herbicides, no currently available herbicide options need to be restricted, and 98140-based hybrids will increase postemergence herbicide options.

In summary, 98140 corn with the *gat4621* and *hra* transgenes has a very high level of resistance to both glyphosate and ALS-inhibiting herbicides. Hybrids with these genes will expand the utility of existing ALS herbicides that are not currently safe enough to use on corn. The *gat4621* and *hra* transgenes will not change the natural tolerance of corn to selective herbicides, so new 98140 hybrids will give growers more options for herbicide mixtures with multiple modes of action to manage weed spectrum shifts and delay the evolution of herbicide-resistant weeds.

Sources of Materials

[1] Glyphosate- and ALS-inhibiting herbicide-resistant corn, trade name Optimum® GAT®, Pioneer Hi-Bred International, Inc., 7100 NW 62nd Avenue, Johnston, Iowa 50131.

[2] Redi-Earth® potting media, The Scotts Company, Marysville, OH 43041.

[3] Peters® Professional® Water Soluble Fertilizer 20–20–20, The Scotts Company, Marysville, OH 43041.

[4] Sprint® 330 iron chelate, Becker Underwood Inc., Ames, IA 50010.

[5] Champion® 15.5–0–0 calcium nitrate, The Scotts Company, Marysville, OH 43041.

[6] Dosatron D1 injector, Dosatron International Inc., Clearwater, FL 33765.

[7] TeeJet® nozzle, Spraying Systems Co., Wheaton, IL 60188.

[8] Ethomeen® T/25 surfactant, 100% tallow amine ethoxylate with 15 mol of ethylene oxide (TAE-15), Akzo Nobel Surface Chemistry, 5 Livingstone Avenue, Dobbs Ferry, NY 10522.

[9] Agri-Dex® paraffin-based petroleum oil concentrate with 83% heavy range, paraffinic petroleum hydrocarbons and 17% surfactant emulsifiers (polyoxyethylene sorbitan fatty acid esters), Helena Chemical Co., Memphis, TN 38119.

[10] SAS® statistical software, SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513.

[11] Series 240 Transducer model 0244-0000, Trans-Tek Incorporated, Route 83, Ellington, CT 06029.

[12] Industrial chart recorder model 4103M. Eurotherm Recorders Limited, Dominion Way, Worthing, West Sussex BN14 8QL, UK.

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