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Vacuolar Glyphosate-Sequestration Correlates with Glyphosate Resistance in Ryegrass (*Lolium* spp.) from Australia, South America, and Europe: A ^{31}P NMR Investigation

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Supporting Information

ABSTRACT: *Lolium* spp., ryegrass, variants from Australia, Brazil, Chile, and Italy showing differing levels of glyphosate resistance were examined by ^{31}P NMR. Extents of glyphosate (i) resistance (LD_{50}), (ii) inhibition of 5-enopyruvyl-shikimate-3-phosphate synthase (EPSPS) activity (IC_{50}), and (iii) translocation were quantified for glyphosate-resistant (GR) and glyphosate-sensitive (GS) *Lolium multiflorum* Lam. variants from Chile and Brazil. For comparison, LD_{50} and IC_{50} data for *Lolium rigidum* Gaudin variants from Italy were also analyzed. All variants showed similar cellular uptake of glyphosate by ^{31}P NMR. All GR variants showed glyphosate sequestration within the cell vacuole, whereas there was minimal or no vacuole sequestration in the GS variants. The extent of vacuole sequestration correlated qualitatively with the level of resistance. Previous ^{31}P NMR studies of horseweed (*Conyza canadensis* (L.) Cronquist) revealed that glyphosate sequestration imparted glyphosate resistance. Data presented herein suggest that glyphosate vacuolar sequestration is strongly contributing, if not the major contributing, resistance mechanism in ryegrass as well.

KEYWORDS: *glyphosate, glyphosate resistance, in vivo ^{31}P NMR, Lolium spp., glyphosate tonoplast transporter*

INTRODUCTION

Glyphosate is the world's most important and widely used herbicide.[1] The introduction of glyphosate-resistant crops in 1996 extended its global use.[2] Glyphosate inhibits the plastidic enzyme 5-enopyruvyl-shikimate-3-phosphate synthase (EPSPS) located in cell chloroplast.[3] EPSPS is critical to the shikimate biosynthesis pathway, which is the source for up to 30% of the complex carbon-containing moieties in higher plants.[4] Inhibition of EPSPS curtails this critical biosynthetic pathway and ultimately results in plant death. It follows that for glyphosate to be effective, it must be taken up by the plant cell and must further enter the chloroplast to inhibit plastidic EPSPS. Glyphosate is translocated symplastically by the phloem from photosynthetically active source leaves to highly sensitive sink tissues, leading to whole plant death.[5−7]

After over 35 years of continuous glyphosate usage, selective weed species have evolved glyphosate-resistant (GR) populations.[8,9] Today 21 GR weed species have been discovered worldwide.[10] Common agricultural delivery of glyphosate is at the millimolar level with a fraction taken up and a smaller fraction of this amount translocated to sink-tissue chloroplasts.[11] Although chloroplast glyphosate concentrations at the submicromolar level are sufficient to effectively inhibit EPSPS function,[12−14] anything that compromises chloroplast glyphosate delivery could play an important role in establishing herbicide resistance.[7,15]

Ryegrass species are economically important in several regions of the world.[16] There are three well-recognized cross-pollinated species within the *Lolium* genus: *L. rigidum* Gaudin (Stiff darnel, Wimmera ryegrass), *L. multiflorum* Lam. (Italian ryegrass), and *L. perenne* L. (perennial ryegrass). These three *Lolium* species freely cross-pollinate, and the hybrids are highly fertile.[17] Glyphosate-resistant *L. rigidum* was discovered in Australia in 1996.[11,18,19] Today, *L. rigidum* ranks in the top 10 most important herbicide-resistant species. Both EPSPS mutation (target-site) and reduced translocation (non-target-site) have been suggested to play a role, but non-target-site resistance may be the major GR mechanism in rigid ryegrass.[20,21]

Millimolar concentrations of glyphosate in the plant cell can be readily monitored by ^{31}P NMR in vivo,[22,23] a spectroscopic technique that can be advantageously combined with a pulse-chase protocol to quantify glyphosate partitioning by plants.[22] In this protocol, perfused plant tissue is exposed to herbicide in the perfusate for a fixed time period (the pulse phase). Switching to a glyphosate-free perfusion medium at the conclusion of the pulse phase washes unincorporated herbicide from the plant surface and apoplast (the wash phase). Following the

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wash phase, continued perfusion with glyphosate-free media (the chase phase) allows glyphosate inside the cell to be noninvasively monitored along with other phosphorus-containing metabolites, for example, adenosine triphosphate (ATP), inorganic phosphate (P_i), uridine diphosphoglucose (UDPG), sugar phosphates, and shikimate-3-phosphate (S3P). The pulse-chase method monitors plant vacuolar sequestration and bioenergetic competence (e.g., ATP content, intracellular pH), in real time, typically over a 24−48 h time period.

By applying this in vivo ^{31}P NMR protocol to horseweed (*Conyza canadensis* (L.) Cronquist), rapid vacuolar sequestration was documented as the cause of reduced translocation in the GR biotype and was proposed as the major GR mechanism.[22,23] Vacuole sequestration is consistent with a major dominant trait as determined in inheritance studies.[24]

It is thus of interest that reduced translocation has been reported as the GR mechanism in ryegrass populations in the United States (Mississippi,[25] Oregon[26]), Chile,[27,28] and other regions.[29−31] The current ^{31}P NMR study examines whether vacuole sequestration of glyphosate occurs in resistant ryegrass variants from Australia, Brazil, Chile, and Italy, a finding that would account for reduced translocation and suggest the mechanism of resistance. There also have been reports of a mutation in the EPSPS enzyme that could contribute to the GR mechanism through decreased glyphosate binding affinity.[26,32,33] Thus, in addition to ^{31}P NMR analysis, this study examines the extent of glyphosate (i) resistance (LD_{50}), (ii) inhibition of EPSPS enzyme activity (IC_{50}), and (iii) translocation for GR and GS *L. multiflorum* variants from Chile and Brazil. For comparison, LD_{50} and IC_{50} data for GR and GS *L. rigidum* variants from Italy are also examined using the same analysis procedure.

■ MATERIALS AND METHODS

Ryegrass Plants for ^{31}P NMR Studies. *L. rigidum* lines were obtained from Italy, GR (R332, R336) and GS (S328),[34] and from Australia, GS (SLR4) and GR (NLR71).[35] The origin and characterization of these lines have been discussed in the literature.[31−35] Lines from Brazil, confirmed *L. multiflorum* by DNA testing at UNESP University of Sao Paulo, were received from Monsanto Brazil. Lines from Chile were received in 2003 from Monsanto Chile and confirmed to be *L. multiflorum* by Dr. Gerrit Davidse of the Missouri Botanical Garden in St. Louis, MO. GR and GS variants of the plant lines from each locale were grown side by side from seed in Readi-Earth (Osmocote 14-14-14) soil in 11.4 cm square pots under controlled climate conditions in a greenhouse (28/20 °C day/night temperature, 14/10 h photoperiod, 700−900 μE m^{-2} s^{-1}). Plants were watered daily as needed. Ryegrass was harvested for study when plants were >20 cm in height.

LD_{50} Greenhouse Measurements. Seeds of *L. multiflorum* variants from Brazil and Chile were planted in Metromix 350 soil in 11 cm deep pots, later thinned to 10 plants, and were grown out and cut back three times to 8 cm to ensure the plants were mature with sufficient root mass. Plants had regrown to 30 cm and were beginning to flower at the time of herbicide treatment. Seven glyphosate doses were tested: 0.25*X*, 0.50*X*, 0.75*X*, 1*X*, 1.5*X*, 3*X*, 6*X*, where *X* is 0.84 kg ae ha^{-1}. Monsanto-branded Roundup UltraMAX was diluted to the lowest dose (0.25*X*), and unformulated glyphosate with no surfactant was added as required to achieve the specified ae dose. Plants were scored alive or dead 21 days after treatment (DAT). Greenhouse growth conditions were 28/20 °C day/night temperature, 14/10 h photoperiod, and 700−900 μE m^{-2} s^{-1}. Published LD_{50} greenhouse data for *L. rigidum* variants from Italy[34] are reanalyzed here to allow direct comparison of results from Bayesian modeling of log−logistic LD_{50} data across ryegrass lines from Italy, Chile, and Brazil (vide infra). LD_{50} field data for *L. rigidum* variants from Australia have been reported.[35] Our greenhouse conditions are identical to those mentioned above for the growth of plants for ^{31}P NMR studies. The growth conditions for published LD_{50} studies of *L. rigidum* variants from Australia and Italy employed 12 h photoperiods with day/night temperatures roughly 5 °C lower than used in this study; scoring (alive or dead) of *L. rigidum* variants from Australia and Italy occurred 21 and 28 DAT, respectively.

IC_{50} Measurements (EPSPS Enzyme Assays). EPSPS enzyme activity was determined by the phosphate-release method[36] for *L. multiflorum* from Chile and Brazil and for *L. rigidum* from Italy. Bayesian modeling provides direct comparison of log−logistic IC_{50} analysis across ryegrass lines from Italy, Chile, and Brazil (vide infra). IC_{50} values for *L. rigidum* variants from Australia (obtained by HPLC measurement of the reaction product EPSP) have been published.[29]

Glyphosate Translocation Studies. Translocation studies were carried out employing the method reported by Feng.[11] Seeds from GR and GS *L. multiflorum* from Chile and Brazil were greenhouse germinated and transplanted into 5 cm square pots. When plant leaves were fully expanded, 5 μL of a solution containing ^{14}C-glyphosate in ~20× diluted Roundup UltraMAX was spotted across the middle of the leaf in eight separate plants for each variant (32 plants total). The glyphosate solution concentration was 13.3 mM with a ^{14}C-glyphosate specific activity of 39.37 mCi/mmol (New England Nuclear). The average amount of ^{14}C-glyphosate delivered to each leaf corresponds to 0.0306 μCi. Plants were moved to a growth chamber maintained at 27 °C, 70% humidity, and 14 h light cycle at 800 μE m^{-2} s^{-1}. Three days after treatment, the treated leaf was washed thoroughly with water to remove nonabsorbed, surface-bound ^{14}C-glyphosate and cut into three sections corresponding to the leaf tip, treated section, and leaf base. Each leaf part was extracted with 0.5 mL of 0.25 N sulfuric acid and analyzed for ^{14}C-glyphosate content. The remainder of the plant (shoot and roots) was also extracted and analyzed for ^{14}C-glyphosate content. ^{14}C-glyphosate content was assessed from radiation counts measured from 100 μL of extract aliquots via a Packard Scintillation counter. Counting periods were extended to include two standard deviations of confidence for all measurements. Quenching was not observed at this low acid concentration. A mass balance of content from extracted tissue and the amount removed from the leaf surface during the wash phase accounted for 87% of all ^{14}C delivered to the plant.

^{31}P NMR Studies. An Agilent/Varian INOVA-500 (11.74 T, 500 MHz ^{1}H resonance frequency) NMR spectrometer equipped with a 10 mm broad-band probe (Nalorac Corp.) was used for ^{31}P NMR data collection. A laboratory-constructed perfusion apparatus[22] was employed to physiologically support plant leaf tissue contained within standard, 18 cm long, 10 mm outer diameter glass NMR tubes (Wilmad-LabGlass). A peristaltic mini-pump (VWR International, LLC) enabled buffer recirculation.

For the pulse-chase ^{31}P NMR studies, mature source-leaf tissues were harvested from healthy GR and GS ryegrass variants (taken from the greenhouse) and cut to roughly 5.0 cm long segments. For all investigations, care was taken to ensure that the sample within the sensitive region of the NMR detector (radiofrequency transceiver coil) was made up of leaf tissue harvested equally from top, middle, and bottom leaf sections. The bottom section was harvested above the ligule. The top section was harvested from the upper leaf, but did not include the uppermost leaf tip. The leaves (~1.2 g) were degassed and vacuum infiltrated with buffer A (12 mM MES, 50 mM sucrose, and 10 mM glyphosate in 10% D_2O, pH 5.0) and then were gently packed (leaves oriented vertically) into a 10 mm NMR tube (along with a capillary concentration-reference containing 20 mM methylene diphophosphonate in D_2O). Removal of air from the leaves during the vacuum infiltration period reduced magnetic field inhomogeneities caused by the differing magnetic susceptibility of water versus air.[22] The sample was then mated to a perfusion system in which a peristaltic pump recirculated O_2-saturated buffer A, placed into the NMR detector probe, and sited in the magnet (202.3 MHz ^{31}P resonance frequency). The tissue was perfused with buffer A at a flow rate of 4 mL/min at 20 ± 1 °C. The buffer pH was chosen to be 5.0 for these studies to shift the perfusate's glyphosate ^{31}P resonance to higher

frequency/lower shielding (9.5 ppm) compared to cell vacuole (8.6 ppm) and cytosol (8.0 ppm) glyphosate resonances. This enabled more accurate quantification of glyphosate compartmental partitioning. The sample was perfused with buffer A for 10 h (pulse phase), and then the perfusion medium was switched to buffer B, which, except for being glyphosate-free, was of the same composition as buffer A. The intense glyphosate signal from buffer A obscures the much weaker vacuole glyphosate signal (Figure S1 in the Supporting Information), thus the rationale for switching to glyphosate-free buffer B following the pulse phase. The first 2 h of buffer B perfusion constituted the wash phase. The first 100−125 mL of buffer B to pass through (perfuse) the plant tissue was not recirculated but was discarded, substantially eliminating extracellular glyphosate from the system.

^{31}P NMR data were collected and averaged over successive 2 h data periods for 24 h. The deuterium (D, ^{2}H) resonance of D_2O served to provide an internal field/frequency lock signal during NMR studies. A simple pulse-and-acquire pulse sequence was employed (67.5° flip angle, 1.1 s relaxation delay) over the 24 h of ^{31}P NMR data acquisition. Relative glyphosate partitioning (cytoplasm and vacuole) was established from chemical-shift resolved signal amplitudes, which are proportional to content, using signal analysis software based on Bayesian probability theory.[37] The Bayesian signal analysis toolbox, *Bayesian Analysis of Common NMR Problems*, is available for free download at URL http://bayesiananalysis.wustl.edu/. The ^{31}P chemical shifts are given relative to an external 85% phosphoric acid reference assigned as 0.0 ppm.

Bayesian Parameter Estimation and Model Selection (LD_{50} and IC_{50}). Ryegrass mortality and EPSPS enzyme activity data were modeled with the four parameter log−logistic equation[38]

$$y = \text{fn}(x) = C + \{(D - C)/[1 + (x/A)^B]\} \quad (1)$$

Eqwhere x is the glyphosate dose (independent variable), y is the measured response (dependent variable; plant mortality in the case of LD_{50} data, enzymatic rate in the case of IC_{50} data, A is either LD_{50} or IC_{50} as appropriate for the data set, C is the response at $x = \infty$, and D is the response at $x = 0$; when y is plotted against $\log(x)$, B gives the slope (response rate) near $x = A$.

Algorithms based on Bayesian probability theory in conjunction with Markov chain Monte Carlo methods were used to derive probability distributions for all parameters (A, B, C, D) independent of each other. Parameter estimates are reported as the mean ± 1 standard deviation of the corresponding probability distribution. When data sets were compared to ascertain whether parameter values were different or equivalent (e.g., IC_{50} for GR vs GS ryegrass from Brazil), Bayesian model selection methods were used to derive the probability for each competing model (e.g., model 1, IC_{50} values are different, vs model 2, IC_{50} values are equivalent). The reader is referred to the URL given above for additional details regarding the use of Bayesian methods for parameter estimation and model selection.

RESULTS

^{31}P NMR Data. ^{31}P NMR protocols were carried out on a total of nine *Lolium* biotypes (Australia, 1 GS and 1 GR; Brazil, 1 GR and 1 GS; Chile, 1 GR and 1 GS; Italy, 2 GR and 1 GS). At least one repeat pulse-chase protocol was carried out with each ryegrass biotype. The three lines of *L. rigidum* from Italy show different susceptibilities to glyphosate: sensitive, moderately resistant, and strongly resistant (vide infra).

Representative ^{31}P NMR spectra from *L. rigidum* are shown in Figure 1. Because of the significant pH difference between the cytoplasm (approximately 6.8) and vacuole (approximately 5.2−5.5), the phosphonate group on glyphosate shows a ^{31}P chemical shift difference of about 0.6 ppm between these two environments.[22] Thus, the glyphosate occupancy of these compartments can be established simultaneously. For example, consider the spectra shown in Figure 1B (GR variant R332) and in Figure 1C (GR variant R336). Glyphosate's presence in

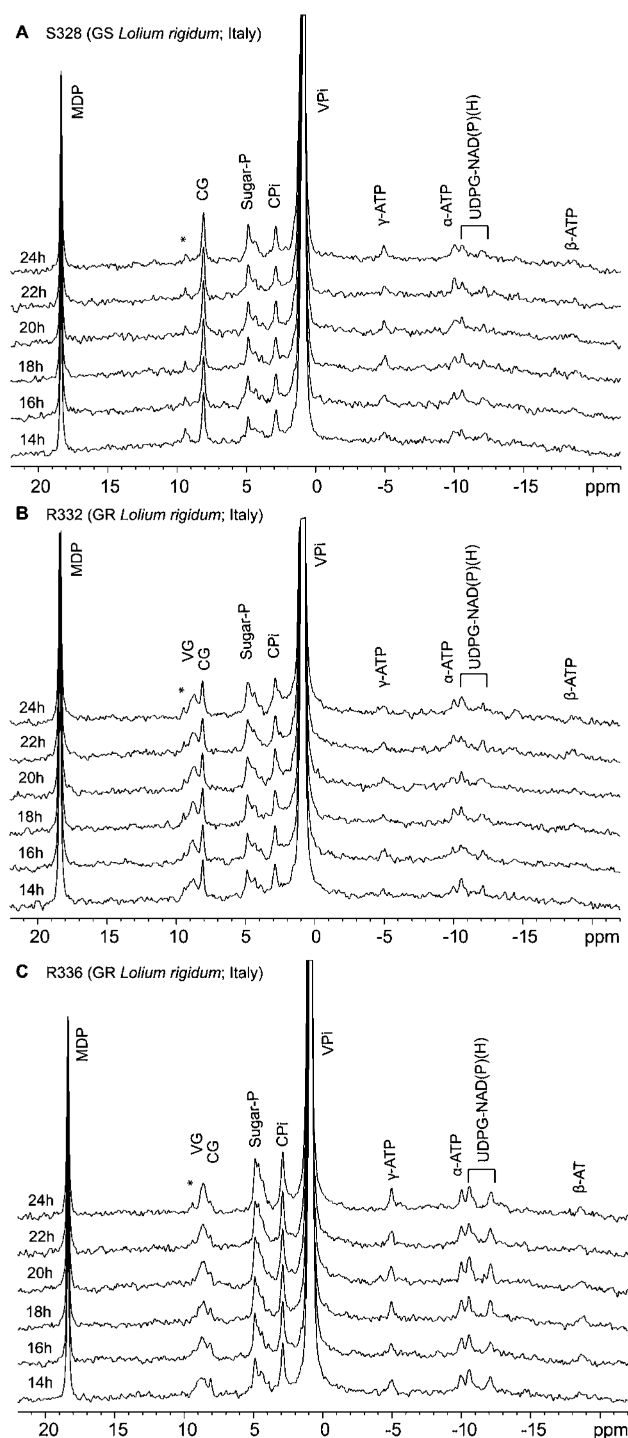

Figure 1. ^{31}P NMR spectra of *Lolium rigidum* (Italy) source leaves in vivo: (A) sensitive variant (S328); (B) moderately resistant variant (R332); (C) strongly resistant variant (R336). Stacked spectra show data averaged over progressive 2 h periods of the chase phase following a 10 h pulse phase with perfusion media containing 10 mM glyphosate (buffer A). The asterisk (*) indicates signals arising from residual glyphosate remaining in the perfusate buffer following the 2 h wash phase. Chemical shifts are reported relative to an external sample of 85% phosphoric acid (0.00 ppm; determined in separate experiments). Resonance assignments are as follows: MDP, methylene diphosphonate, an external concentration reference; VG, vacuolar glyphosate; CG, cytoplasmic glyphosate; VPi, vacuolar phosphate (truncated amplitude); CPi, cytoplasmic phosphate; UDPG, uridine 5′-diphosphoglucose; α-, β-, γ-ATP, corresponding phosphate groups of ATP; Sugar-P, sugar phosphates.

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both vacuole and cytoplasm is clearly observed for each GR variant at the 12−14 h time block of the chase phase, where it is clear a significant loading of the vacuole has occurred during the pulse phase and that it remains so during the chase phase. However, the spectra shown in Figure 1A (GS variant S328) show glyphosate's presence throughout the entire chase phase is limited to cytoplasm only.

The average cellular glyphosate component fractions (vacuole, cytoplasm) throughout the chase phase for each *L. rigidum* variant from pulse-chase repeats with different samples ($n = 3$) are presented in Figure 2. Considering the strongly resistant line (R336), a far greater fraction of glyphosate is vacuole sequestered at 14 h, 69%, and at 24 h, 90%, than is seen for the moderately resistant line (R332), 54 and 62%, respectively. The strongly resistant line sequestered glyphosate into the vacuole more avidly during the pulse phase compared to the moderately resistant line and showed evidence of continued loading during the chase phase. As noted earlier, the GS 328 line gave no evidence of vacuole occupancy and the cytoplasm glyphosate content remained essentially unchanged throughout the chase phase, clearly demonstrating that glyphosate once taken up does not readily leave the cell, also observed for horseweed previously.[22] Finally, the total glyphosate uptake was greater for those biotypes that employed vacuole sequestration; that is, total relative glyphosate content at the second 2 h time block of the chase phase (hours 12−14) was 100, 86, and 73 for R336, R332, and S328 variants, respectively.

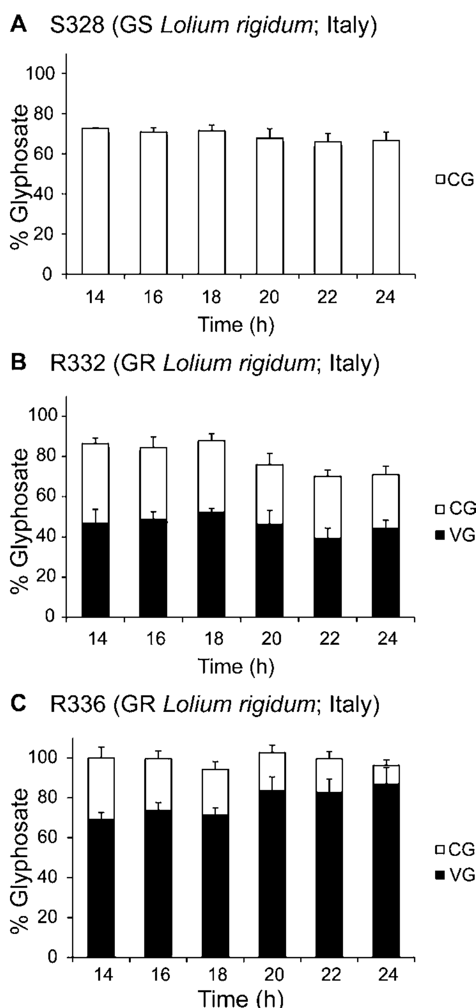

Figure 2. ^{31}P NMR measured, chase-phase glyphosate content in *Lolium rigidum*. Data are the mean of three separate perfusion experiments. Uncertainty bars indicate one standard deviation above the mean. Abscissa values are normalized to mean total glyphosate content ($n = 3$) of the strongly resistant variant (R336) during the 12−14 h signal-averaging period. (A) Glyphosate is present only in the cytosol of the sensitive variant (S328). (B) A significant fraction (~50%) of glyphosate is found in the vacuole of the moderately sensitive variant (R332). (C) A greater fraction (~75−90%) of glyphosate is sequestered in the vacuole of the strongly resistant variant (R336).

Figure 3 shows analogous vacuole and cytosol occupancy data obtained from ^{31}P NMR studies of *Lolium* from Australia (*rigidum*) and Brazil and Chile (*multiflorum*). The GR variants from Australia and Brazil showed significant vacuole occupancy (45 and 55% of total, respectively) from glyphosate sequestration. The GR variant from Chile showed a more moderate vacuole component (20% of total) under similar treatment conditions. The greater degree of glyphosate vacuole sequestration in the variant from Brazil versus the variant from Chile is in keeping with the greater glyphosate LD_{50} of the ryegrass from Brazil versus Chile, as measured herein (vide infra). In contrast, the GS variants from all four countries showed negligible vacuole sequestration of glyphosate.

LD_{50} Greenhouse Data. Survival of *Lolium* variants from Brazil, Chile, and Italy as a function of glyphosate treatment level are quantified by LD_{50} in Table 1. Data for the *L. rigidum* variants from Italy have been published previously[34] but are reanalyzed here using Bayesian methods. All GS variants have similar, relatively low, LD_{50} values. The GR *L. multiflorum* variant from Brazil has an LD_{50} 14 times greater than the GS variant from Brazil and 2-fold greater (more resistant) than its GR *multiflorum* counterpart from Chile. *L. rigidum* GR variants R332 and R336 from Italy have LD_{50} values 6.9 and 8.5 times greater, respectively, than GS variant S204L. The LD_{50} values derived from field data for *Lolium* variants from Australia and Italy, as determined by others,[35] are included in the Table 1 footnote.

EPSPS Enzyme Activity Data. The EPSPS enzyme activity assays regarding protein extracted from *L. multiflorum* variants from Brazil and Chile and *L. rigidum* variants from Italy are quantified by IC_{50} in Table 2.

Considering GS versus GR *L. multiflorum* variants from Brazil, Bayesian preference (68% probability) is for same-valued IC_{50}, 6 μM. Considering GS versus GR *L. multiflorum* variants from Chile, Bayesian preference (60% probability) was for different-valued IC_{50}. The GR variant IC_{50} is 2-fold greater than that of the GS variant, 10 versus 5 μM, respectively. Considering *L. rigidum* from Italy and comparing GS variant (S204L) and a moderately resistant (R332) variant, Bayesian preference (58% probability) was for different-valued IC_{50}. However, the R332 variant IC_{50} is only 20% greater than that of the S204 variant, 14 versus 17 μM, respectively. The strongly resistant *L. rigidum* variant from Italy (R336) was compared to its S204L and R332 *L. rigidum* counterparts from Italy. For both comparisons, Bayesian preference (~100%) was for different-valued IC_{50}. The R336 variant IC_{50} (42 μM) was 3−4-fold greater than the IC_{50} of either the S204L or R332 variants. (Note that GS variant S204L is different from the GS variant examined by ^{31}P NMR.)

^{14}C-Glyphosate Translocation Data. Translocation data from ^{14}C labeling studies of *L. multiflorum* variants from Chile

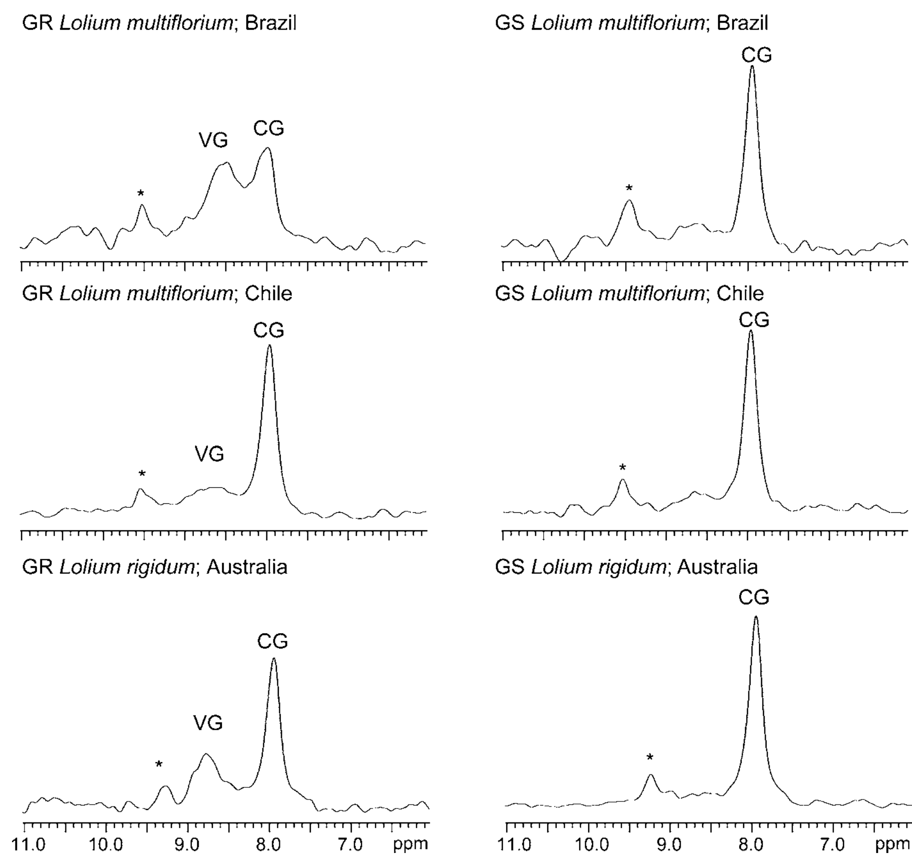

Figure 3. ^{31}P NMR glyphosate region of *Lolium* from Australia, Brazil, and Chile. Signal averaging occurred late in the chase phase, during hours 18-24 h following the initial 10 h pulse period. Vacuole glyphosate occupancy is measured to be roughly 55, 20, and 45% for Brazilian, Chilean, and Australian GR variants, respectively. The asterisk (∗) indicates residual buffer glyphosate remaining after the 2 h wash period. CG, cytosolic glyphosate; VG, vacuolar glyphosate.

and Brazil are summarized in Table 3. Data are given in percent total counts and have been normalized to total 100%. Data from Table 3 show translocation differences between the GR and GS plant variants consistent with observations of glyphosate response in the field. For example, the GR *L. multiflorum* variant from Brazil retained a 3-fold greater fraction of glyphosate in the treated zone while conversely transporting a smaller fraction of glyphosate to sink tissues. The GR Chile variant, on the other hand, retained roughly 1.7-fold as much glyphosate as the GS biotype and also delivered less glyphosate to sink tissues. These basic proportions conform to the differences observed for glyphosate sequestration by NMR and for glyphosate sensitivity in the greenhouse.

■ DISCUSSION

^{31}P NMR monitoring shows that glyphosate enters the cell readily and at roughly comparable levels for all ryegrass variants investigated under the perfusion conditions employed herein. Glyphosate uptake in ryegrass was roughly 70% of that found in horseweed under identical conditions.[22] In all cases, 2 h postinitiation of perfusion with media containing glyphosate, the pulse phase, the cytosolic glyphosate ^{31}P resonance is observed and increases in amplitude (content) throughout the pulse phase (data not shown). Thus, glyphosate uptake appears to be facile in both GR and GS ryegrass lines.

Following loading during the pulse phase, glyphosate efflux from the cell during the chase phase is limited (Figure 2B) or does not occur (Figure 2A,C). Furthermore, vacuole glyphosate efflux during the chase phase is minimal (Figure 2B). Indeed, for the highly resistant *L. rigidum* variant from Italy (R336), vacuole sequestration of glyphosate continues during the chase phase (Figure 2C) in the face of decreasing cytosolic glyphosate levels, suggestive of active transport across the tonoplast. As long as tissue is maintained viable, glyphosate uptake into the cell and sequestration into the vacuole appear to be unidirectional, findings consistent with active (nondiffusive) processes.

Figures 1 and 3 show, furthermore, that vacuole sequestration in GR ryegrass variants occurs on a time scale of hours as substantial sequestration is evident following the 10 h pulse phase. This suggests[22] that there is a tonoplast glyphosate transporter present in the GR variants. Conversely, as no vacuole sequestration is observed over the entire 24 h pulse-chase protocol in GS populations; these variants either do not have a tonoplast transporter or, if it is present, it is at far lower concentration than in the GR variants.

In the case of the three *L. rigidum* variants from Italy, the extent of vacuole sequestration follows the ordering of resistance level. This suggests that vacuolar sequestration is playing a major, if not dominant, role in resistance. Consistent with this hypothesis, the GR variant from Chile has a similar LD_{50} and extent of vacuole sequestration compared to the moderately GR variant (R332) from Italy, whereas the strongly GR variant (R336) from Italy has substantially greater LD_{50} and extent of vacuole sequestration compared to the (moderately) GR variants from Brazil and Chile, as well as Italy (R332).

Table 1. Glyphosate LD_{50} Greenhouse Data Analysis of *Lolium* Variants[a]

log–logistic, eq 1	B	C	D	LD_{50} (kg ae ha^{-1})
		(A) Bayesian Parameter Estimation		
		L. multiflorum		
GS; Brazil	2.2 (0.6)	5 (2)	99 (7)	0.19 (0.03)
GR; Brazil	2.3 (0.9)	16 (9)	92 (6)	2.7 (0.6)
GS; Chile	3.1 (0.7)	4 (2)	98 (8)	0.27 (0.03)
GR; Chile	3 (1)	17 (7)	101 (6)	1.3 (0.2)
		L. rigidum[34]		
S204L; Italy	4.3 (0.8)	4.6 (3)	98 (6)	0.26 (0.02)
R332; Italy	3.2 (0.8)	7.5 (4)	94 (5)	1.8 (0.2)
R336: Italy	4.1(0.9)	36 (3)	99 (2)	2.2 (0.1)
		(B) Bayesian Model Selection		
		L. multiflorum		
GS; Brazil	2.0 (0.5)	3 (2)	94 (4)	0.19 (0.03)
GR; Brazil	same valued	15 (9)	same valued	2.7 (0.6)
GS; Chile	3.1 (0.6)	3 (2)	100 (4)	0.27 (0.03)
GR; Chile	same valued	17 (7)	same valued	1.3 (0.2)
		L. rigidum[34]		
S204L; Italy	4.1 (0.7)	3 (2)	94 (3)	0.26 (0.02)
R332; Italy	same valued	8 (5)	same valued	1.8 (0.2)
S204; Italy	4.4 (0.6)	3 (2)	99 (2)	0.26 (0.01)
R336; Italy	same valued	36 (2)	same valued	2.2 (0.1)
R332; Italy	3.5 (0.7)	8 (5)	99 (2)	1.7 (0.2)
R336; Italy	same valued	34 (3)	same valued	2.2 (0.1)

[a](A) The four parameters of the log-logistic equation, eq 1, were estimated by Bayesian methods for each *Lolium* variant. Values are cited as the mean and standard deviation (SD is enclosed in parentheses) of each parameter's probability distribution, independent of the other parameters. (B) Parameters estimated from the most probable log-logistic model, as derived by Bayesian model selection methods. Three models were compared: model 1, all four parameters were same valued comparing GR vs GS variants from a given country; model 2, parameters *B* and *D* were same valued but *C* and LD_{50} were different valued comparing GR vs GS variants from a given country; model 3, all four parameters were different valued comparing GR vs GS variants from a given country. In all cases, model 2 was strongly preferred (100% probability). Note that the bottom two data row entries compare moderately GR and strongly GR *rigidum* variants from Italy. Previously reported LD_{50} values from field data for *L. rigidum* variants SLR4 and NLR71 from Australia are 0.17–0.23 and 0.44–1.2 kg ae ha^{-1}, respectively,[35] and for variants S204L, S328, R332, and R336 from Italy are 0.34, 0.43, 1.61, and 6.98 kg ae ha^{-1}, respectively.[34]

If EPSPS enzyme point mutations are present for the GR variants, the enzyme inhibition assays certainly do not support a significant contribution to resistance for the *L. multiflorum* variant from Brazil. Here, Bayesian model selection analysis argues for the same IC_{50} value, 6 μM (68% probability), for both GS and GR variants despite LD_{50} values that are 14-fold different (0.19 vs 2.7 kg ae ha^{-1}). The GR *L. multiflorum* variant from Chile has an IC_{50} twice that of its GS counterpart, 10 versus 5 μM, suggesting a possible point mutation contribution to resistance (LD_{50} values of 1.3 vs 0.27 kg ae ha^{-1}). However, comparing IC_{50} versus LD_{50} assay results argues against enzyme point mutations as the dominant cause of resistance. The GR variant from Chile has an LD_{50} (resistance) less than the GR variant from Brazil (1.3 vs 2.7 kg ae ha^{-1}), yet the IC_{50} value of the GR variant from Chile is greater than the GR variant from Brazil (10 vs 6 μM), the opposite of what would be expected of point mutation dominated resistance.

The *L. rigidum* lines from Italy[34] also provide evidence of, at most, modest point mutation contributions to resistance. Bayesian model selection IC_{50} analysis finds GS (S204L) and moderately GR (R332) variants from Italy to have similar values (14 vs 17 μM, respectively) despite a 5-fold difference in LD_{50} (0.26 vs 1.8 kg ae ha^{-1}). The strongly GR variant (R336) from Italy, 2.2 kg ae ha^{-1} LD_{50}, has been shown to have an amino acid substitution at position 106, consistent with the most commonly observed EPSPS enzyme point mutation.[34] Interestingly, this variant has an IC_{50} (42 μM) markedly greater than that found via pairwise modeling for its GS and moderately GR counterparts (12 μM), suggesting a possible substantive point mutation contribution to resistance. However, the degree of resistance imparted via this target site mechanism relative to that imparted by the enhanced vacuole sequestration seen with this biotype is not known. It is likely that the resistance of variant R-336 has contributions from both the EPSPS mutation and vacuolar sequestration.

The translocation data presented in Table 3 are consistent with ^{31}P NMR observation of glyphosate sequestration in GR *Lolium* variants from Australia, Brazil, Chile, and Italy and the hypothesis that vacuole sequestration of glyphosate is causative for resistance. Vacuole sequestration of glyphosate would be expected to increase glyphosate retention in treated zone (source) tissue, thus reducing the amount available for phloem access and translocation to sink tissue. This predicted outcome occurs and is clearly shown by the translocation data for *L. multiflorum* from Brazil and Chile.

More quantitative comparisons between glyphosate vacuole sequestration ^{31}P NMR analysis and ^{14}C-glyphosate translocation data are complicated by marked differences in administered glyphosate dosages and light conditions. In the ^{31}P NMR protocol, ryegrass was globally exposed to 10 mM glyphosate in flowing, O_2-saturated, perfusion media for 10 h in the dark. ^{31}P NMR monitoring then continued for another 14 h, during which ryegrass perfusion continued (absent glyphosate) in the dark. If, as hypothesized, glyphosate is transported into the vacuole via a tonoplast ATP binding cassette pump, it is possible or likely that the high cell loading of glyphosate under 10 mM perfusion conditions saturates pump activity. In contrast, the ^{14}C-labeled glyphosate translocation studies employed a low, sublethal dose of glyphosate, and the ryegrass was maintained under normal greenhouse conditions (light/dark cycle) for 3 days post treatment with glyphosate. Nevertheless, the qualitative findings are consistent: GR variants sequester glyphosate in source tissue vacuoles, reducing the amount translocated in the phloem to sink tissue. Previous ^{31}P NMR studies with horseweed[22] showed that sink tissue also sequesters glyphosate, yet further reducing the amount accessible to sink tissue chloroplasts.

In summary, glyphosate movement has been examined in GR and GS ryegrass lines from four different countries: Australia, Brazil, Chile, and Italy. GS and GR ryegrass biotypes take up glyphosate to a similar, although not identical, extent. Vacuole sequestration of glyphosate occurs in all GR ryegrass lines, but the corresponding GS variants show no measurable vacuole sequestration. The extent of glyphosate vacuole sequestration qualitatively correlates with the level of glyphosate resistance. It is likely that vacuole sequestration plays a major, if not the responsible, role in ryegrass glyphosate resistance.

Table 2. Glyphosate IC_{50} Analysis of *Lolium* EPSPS Activity[a]

log–logistic, eq 1	*B*	*C*	*D*	IC_{50} (μM)
(A) Bayesian Parameter Estimation				
L. multiflorum				
GS; Brazil	1.4 (0.4)	5 (3)	92 (5)	9 (2)
GR; Brazil	2.0 (0.6)	7 (2)	99 (3)	5 (1)
GS; Chile	1.0 (0.2)	8 (3)	96 (4)	5 (1)
GR; Chile	1.4 (0.4)	12 (4)	102 (4)	9 (2)
L. rigidum				
S204L; Italy	0.7 (0.1)	8 (4)	96 (4)	13 (4)
R332; Italy	1.2 (0.2)	6 (3)	97 (3)	16 (2)
R336: Italy	0.9 (0.1)	7 (3)	101 (3)	40 (4)

log–logistic, eq 1	*B*	*C*	*D*	IC_{50} (μM)
(B) Bayesian Model Selection				
L. multiflorum				
GS; GR; Brazil	1.4 (0.2)	4 (2)	97 (3)	6 (1)
GS; Chile	1.1 (0.2)	9 (3)	100 (3)	5 (1)
GR; Chile	same valued	same valued	same valued	10 (2)
L. rigidum				
S204L; Italy	0.6 (0.1)	4 (3)	97 (2)	14 (3)
R332; Italy	1.1 (0.1)	same valued	same valued	17 (2)
S204L; Italy	0.6 (0.1)	6 (3)	99 (2)	12 (2)
R336; Italy	0.9 (0.1)	same valued	same valued	42 (4)
R332; Italy	0.8 (0.1)	6 (3)	99 (3)	12 (2)
R336; Italy	same valued	same valued	same valued	42 (4)

[a](A) The four parameters of the log-logistic equation, eq 1, were estimated by Bayesian methods. Values cited are the mean and standard deviation (SD is enclosed in parentheses) of each parameter's probability distribution, independent of the other parameters. (B) Parameters estimated from the most probable log-logistic model, as derived by Bayesian model selection methods. Six models were compared: model 1, all four parameters were same valued for GR and GS variants from a given country; model 2, parameters *B*, *C*, and *D* were same valued but IC_{50} was different valued for GR and GS variants from a given country; model 3, parameters *C* and *D* were same valued but *B* and IC_{50} were different valued comparing GR vs GS variants from a given country; model 4, parameters *B* and *D* were same valued but *C* and IC_{50} were different valued for GR and GS variants from a given country; model 5, parameter *D* was same valued but parameters *B*, *C*, and IC_{50} were different valued for GR and GS variants from a given country; model 6, all four parameters were different valued for GR and GS variants from a given country. GS vs GR *L. multiflorum* from Brazil EPSPS activity data were best represented by model 1 (all parameters same valued); probabilities were model 1, 68%, and model 2, 32%. GS vs GR *L. multiflorum* from Chile EPSPS activity data were best represented by model 2 (only IC_{50} different valued); probabilities were model 2, 60%, model 1, 26%, and model 3, 12%. In general, models 3–6 showed small to insignificant probabilities comparing GS vs GR EPSPS activity data sets for *L. multiflorum* variants from Chile or Brazil. Considering *L. rigidum* from Italy and comparing the GS variant (S204L) vs the moderately resistant variant (R332), EPSPS activity data were best represented by model 3 (58% probability) followed closely by model 1 (42% probability). Comparing the GS variant (S204L) vs the strongly resistant variant (R336), EPSPS activity data were best represented by model 3 followed by model 2, both of which support different valued IC_{50}; probabilities were model 3, 70%, and model 2, 28%. Comparing the strongly resistant variant (R336) vs the moderately resistant variant (R332), EPSPS activity data were best represented by model 2 followed by model 3, both of which support different valued IC_{50}; probabilities were model 2, 88%, and model 3, 12%. Models 4–6 showed insignificant probabilities comparing GS vs GR EPSPS activity data sets for *L. rigidum* variants from Italy. Previously reported IC_{50} values for *L. rigidum* variants VLR4 and NLR70 from Australia, GS and GR, respectively, are equivalent, 1.2 μM.[29] Note that these are different Australian variants from those examined herein by ^{31}P NMR. (An IC_{50} valued for *L. rigidum* variant S328 from Italy is not available.)

Table 3. Glyphosate Fractional (Percent) Distribution in *Lolium multiflorum* Variants from Chile and Brazil[a]

		treated zone				sink tissue		
L. multiflorum	wash	treated mature leaf	tip	base	sum	roots	shoot	sum
GS; Brazil	40.6 (1.9)	2.1 (0.2)	8.2 (1.6)	1.2 (0.2)	11.5	19.7 (2.4)	28.3 (2.7)	48.0
GR; Brazil	35.9 (3.4)	14.6 (2.8)	19.3 (3.6)	0.9 (0.1)	34.8	13.7 (1.7)	15.5 (1.6)	29.2
GS; Chile	37.7 (2.5)	2.3 (0.3)	18.9 (3.3)	1.8 (0.2)	23.0	19.4 (1.3)	19.9 (2.2)	39.3
GR; Chile	37.6 (1.7)	3.3 (0.7)	34.0 (3.7)	0.7 (0.1)	38.0	11.5 (1.3)	12.8 (1.0)	24.3

[a]Sublethal levels of glyphosate (trace ^{14}C glyphosate in 0.5% Roundup UltraMax) was spotted in the middle of the third leaf (from top of plant). Plants were harvested at 3 DAT, washed extensively, and counted for radioactivity. Data are normalized to sum to 100%, and uncertainties (in parentheses) are expressed as SEM (n = 8).

■ ASSOCIATED CONTENT

Supporting Information

In vivo ^{31}P NMR stacked spectra of GR *Lolium rigidum* (R332), Italy source leaves. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

GR, glyphosate-resistant; GS, glyphosate-sensitive; MDP, methylene diphosphonate; VG, vacuolar glyphosate; CG, cytoplasmic glyphosate; VPi, vacuolar phosphate (truncated amplitude); CPi, cytoplasmic phosphate; UDPG, uridine 5′-diphosphoglucose; α-, β-, γ-ATP, corresponding phosphate groups of ATP; Sugar-P, sugar phosphates.

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