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ORIGINAL ARTICLE

Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype

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Abstract Glyphosate is the world's most widely used herbicide. A potential substitute for glyphosate in some use patterns is the herbicide paraquat. Following many years of successful use, neither glyphosate nor paraquat could control a biotype of the widespread annual ryegrass (*Lolium rigidum*), and here the world's first case of multiple resistance to glyphosate and paraquat is confirmed. Dose–response experiments established that the glyphosate rate causing 50% mortality (LD_{50}) for the resistant (R) biotype is 14 times greater than for the susceptible (S) biotype. Similarly, the paraquat LD_{50} for the R biotype is 32 times greater than for the S biotype. Thus, based on the LD_{50} R/S ratio, this R biotype of *L. rigidum* is 14-fold resistant to glyphosate and 32-fold resistant to paraquat. This R biotype also has evolved resistance to the acetyl-coenzyme A carboxylase (ACCase) inhibiting herbicides. The mechanism of paraquat resistance in this biotype was determined as restricted paraquat translocation. Resistance to ACCase-inhibiting herbicides was determined as due to an insensitive ACCase. Two mechanisms endowing glyphosate resistance were established: firstly, a point mutation in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, resulting in an amino acid substitution of proline to alanine at position 106; secondly, reduced glyphosate translocation was found in this R biotype, indicating a co-occurrence of two distinct glyphosate resistance mechanisms within the R population. In total, this R biotype displays at least four co-existing resistance mechanisms, endowing multiple resistance to glyphosate, paraquat and ACCase herbicides. This alarming case in the history of herbicide resistance evolution represents a serious challenge for the sustainable use of the precious agrochemical resources such as glyphosate and paraquat.

Keywords ACCase · EPSP synthase mutation · Glyphosate · Herbicide resistance · *L. rigidum* · Paraquat · Translocation

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Introduction

Glyphosate is the world's most widely used herbicide (Baylis 2000). Glyphosate is versatile, controls a broad spectrum of annual and perennial weeds (non-selective) under varied agricultural, industrial and domestic situations, and has low mammalian toxicity and little soil activity. Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19), resulting in shikimate accumulation and reduced production of aromatic amino acids (Steinrücken and Amrhein 1980; Schönburnn et al. 2001). Paraquat is also a non-selective herbicide with a broad-spectrum and rapid-action. Paraquat is toxic because it diverts photosynthetic electron transport to oxygen to produce free radicals that cause lipid peroxidation and membrane damage.

In world cropping, glyphosate and paraquat have helped enable minimum/zero tillage, providing productivity and soil conservation benefits. Already high

usage of glyphosate dramatically increased from 1995 onwards with the introduction of transgenic glyphosate resistant soybean, cotton, oilseed rape and maize. This innovation allowed glyphosate to be used as a selective herbicide in transgenic crops (Shaner 2000). Now, transgenic glyphosate resistant crops dominate much of North and South American cropping.

Among the attributes of glyphosate has been the absence of evolved glyphosate resistance in weed species (reviewed by Dyer 1994; Bradshaw et al. 1997). However, evolved glyphosate resistance, first reported in *Lolium rigidum* (Pratley et al 1996, 1999; Powles et al. 1998), is now reported in at least eight weed species. (Heap website: http://www.weedscience.org; reviewed by Powles and Preston 2006). In the limited biochemical studies thus far conducted on evolved glyphosate resistant weeds, two different mechanisms have been shown to confer glyphosate resistance, i.e. mutations of the EPSPS gene, or reduced glyphosate translocation (reviewed by Powles and Preston 2006).

Biotypes of 23 weed species worldwide have evolved paraquat resistance and recently we have identified the instance of field-evolved paraquat resistance in *L. rigidum* (Yu et al. 2004a). Most evidence has implicated a paraquat resistance mechanism of reduced translocation/or sequestration, although enhanced oxygen radical detoxification has been proposed as the mechanism in a few biotypes (reviewed by Hart and DiTomaso 1994; Preston 1994; Szigeti and Lehoczki 2003). Despite much effort, the precise mechanisms endowing paraquat resistance are only partially understood.

Lolium rigidum is a very resistance prone weed species with extensive resistance to numerous herbicides (Powles and Matthews 1992; Preston et al. 1996). Until this study, there were no weed species known to possess multiple resistance to both glyphosate and paraquat. Here, we confirm the first case of field-evolved multiple resistance to glyphosate and paraquat (plus resistance to acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides). We investigate the resistance mechanisms in this biotype and reveal at least four co-existing mechanisms endowing multiple resistance across three very different herbicide modes of action.

Materials and methods

Plant material

Seeds of the putative resistant (R) population of *L. rigidum* (termed AFLR2) were originally collected from a farm in the Tulbagh Valley, South Africa, where glyphosate had been used over 25 years, paraquat for over 40 years, and ACCase inhibiting herbicides for only about 3 years. Seedlings of AFLR2 were initially screened by sequentially spraying glyphosate (Roundup Power Max®) and paraquat (Gramoxone®) at commercial rates. Survivors were grown to maturity and seeds obtained from bulk crosses within the population were used in subsequent experiments (*L. rigidum* is obligate cross-pollinated). A known herbicide susceptible (S) *L. rigidum* biotype (VLR1) from Australia was used as a control. Seeds stocks were maintained at Western Australian Herbicide Resistance Initiative. Seeds of the R and S biotypes were germinated in 16-cm diameter plastic pots containing potting soil and seedlings (20–30 per pot) grown in a glasshouse at 20/15°C day/night temperature under natural sunlight. Seedlings were well watered and fertilized and were herbicide treated at the 2–3 leaf stage. For radiolabeled herbicide translocation experiments, plants were thinned to 2–3 seedlings per pot and moved to a growth chamber with 20/15°C day/night temperature, 12 h/12 h day/night photoperiod, and a photon flux density of 250 μmol quanta m^{-2} s^{-1}.

Dose response to herbicides

In dose–response experiments, R and S plants were sprayed with rates of glyphosate (0, 0.028, 0.056, 0.112, 0.224, and 0.45 kg ha^{-1} for S plants, 0, 0.45, 0.9, 1.8, 3.6, 7.2 kg ha^{-1} for R plants), paraquat (0, 0.0125, 0.025, 0.05, 0.1, and 0.2 kg ha^{-1} for S plants; 0, 0.2, 0.4, 0.8, 1.6, and 3.2 kg ha^{-1} for R plants), diclofop-methyl (0, 0.25, 0.5, 1, 2 and 4 kg ha^{-1}), fluazifop (0, 25, 50, 100, and 200 g ha^{-1}), haloxyfop (0, 13, 26, 52, 104, and 208 g ha^{-1}), propaquizafop (0, 12.5, 25, 50, 100, and 200 g ha^{-1}), sethoxydim (0, 25, 50, 100, 200, and 400 g ha^{-1}) and tralkoxydim (0, 38, 76, 152, 304, and 608 g ha^{-1}). In single-dose experiments, R and S plants were sprayed at the following herbicide doses known to control S plants: 100 g ha^{-1} chlorsulfuron, 100 g ha^{-1} triasulfuron, 15 g ha^{-1} sulfometuron, 20 g ha^{-1} imazapyr, 72 g ha^{-1} imazethapyr, 1,000 g ha^{-1} atrazine, 1,800 g ha^{-1} diuron, 500 g ha^{-1} trifluralin, and 500 g ha^{-1} metolachlor. Herbicides were applied as commercial formulations plus adjuvant as required (either as 0.1% v/v BS1000 or 0.1% v/v Hasten) using a cabinet sprayer delivering 106 l ha^{-1} water at a pressure of 200 kPa. Plants were returned to the glasshouse after treatment, and the mortality and shoot dry mass (oven-dried at 70°C for 2 days) were recorded 21 days after herbicide application. Plants were recorded as alive if they had strongly tillered since herbicide application. For application of the soil applied trifluralin and metolachlor herbicides,

100 seeds were placed on the soil surface, covered with 0.5 cm of soil, watered and left for 1 day to imbibe before herbicide treatment. After herbicide treatment, 1 cm of untreated soil was placed on the soil surface. The number of emerged seedlings was recorded 21 days after herbicide spraying. Dose–response experiments were repeated during April and October in 2005 and each treatment contained 3–4 replicates.

Leaf uptake and translocation of herbicides

In order to simulate commercial agricultural herbicide treatment, and follow the movement of the radiolabeled herbicides, plants at the 2 or 3 leaf stage were sprayed with commercial herbicides (450 g ha^{-1} glyphosate, or 50 g ha^{-1} paraquat, respectively) 20 min prior to the application of radiolabeled herbicides. A 1 µl drop of radiolabeled herbicide treatment solution (25 mM glyphosate containing 1.4 kBq methyl-labeled [^{14}C]-glyphosate made up in Roundup Power Max and 0.06% (v/v) non-ionic surfactant BS1000, or 3.5 mM paraquat containing 1.1 kBq methyl-labeled [^{14}C]-paraquat made up in commercial formulation of Gramoxone and 0.06% BS1000) was then applied to the midpoint of the youngest fully expanded leaf. Plants were carefully washed out of soil 1 day (paraquat treated), 2 and 4 days (glyphosate treated) after treatment and sectioned into untreated leaves, treated leaves with stems, and roots. The treated leaf of each plant was rinsed with 10 ml 0.1% (v/v) Triton X−100, and the radioactivity present in the rinse solution was quantified by liquid scintillation to determine un-absorbed radioactivity. Herbicide leaf uptake was calculated from the total applied radioactivity minus the activity present in the rinse solution. Plant sections were oven-dried at 70°C and combusted in a Biological Sample Oxidizer (RJ Harvey Instrument Corporation, Hillsadale, NJ, USA). The $^{14}CO_2$ evolved was trapped in the cocktail solution and radioactivity was measured by liquid scintillation spectrometry as described by Lorraine-Colwill et al. (2003). Herbicide translocation was expressed as percentage of total applied radioactivity. The experiment was repeated for each herbicide and each harvest had six to eight individual replicates.

Phosphor imaging

Visualization of herbicide translocation was achieved using a phosphor imager (BS 2500, FujiFilm, Japan). Plants used for phosphor imaging were treated with respective unlabeled and radiolabeled herbicide as described for the uptake and translocation experiment. At harvest, whole plants were gently washed out of the soil, rinsed in 0.1% Triton X-100, blotted dry, pressed and oven-dried at 70°C for 48 h, then exposed to a phosphor imager plate (24 h) before scanning for radioactivity. The experiment was repeated at least two times for each herbicide and each harvest had 10–12 individual replicates.

EPSPS gene sequencing

The R biotype plants were sprayed with 900 g ha^{-1} glyphosate at the 2–3 leaf stage and the survivors were used for RNA preparation. Total RNA was extracted from shoot tissue of the R and S biotypes using the Plant RNeasy Mini Kit (Qiagen, Pty Ltd., Doncaster VIC, Australia). Oligo(dT)-primed first-strand cDNA was prepared from 2 µg total RNA using Omniscript Reverse Transcription system (Qiagen, Pty Ltd., Doncaster VIC, Australia). A pair of primers was designed based on homologous regions of EPSPS cDNA sequences of *L. rigidum* (GenBank accession number AJ310166 and AF349754) and *L. multiflorum* (DQ153168) to amplify a highly conserved region in which point mutations conferring glyphosate resistance in plants and bacteria have been found (Comai et al. 1983; Stalker et al. 1985; Padgette et al. 1991, 1996; Baerson et al. 2002; Ng et al. 2003, 2004; Zhou et al. 2006). A forward primer 5′-TTGAAAAGGATGCC AAGGAG-3′ at position 71 and a reverse primer 5′-C AGCTTAACCTTGCCACCAG-3′ at position 336 were used to amplify a 266 bp cDNA fragment containing the potential mutation sites. Polymerase chain reaction (PCR) was conducted in a 50 µl volume. The reaction mixture consisted of 5 µl of first-strand cDNA, 0.5 µM of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, 1 unit of *Taq* DNA polymerase (Promega Co., Madison, WI USA). The PCR was run in a Mastercycler (Eppendorf, Germany) with the following profile: 94°C for 4 min, 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, followed by a final extension step of 10 min at 72°C. The PCR product was purified from agarose gel with Wizard® SV Gel and PCR Clean-up System (Promeaga Co., Madison, WI, USA) and sequenced from both ends. Three independent RNA extractions from each biotype were analyzed and the sequence results were aligned and compared.

Inhibition of ACCase activity by herbicides

Shoot tissue of the R and S biotypes was harvested at the 2 to 3 leaf stage, snap-frozen in liquid nitrogen and stored at −20°C. ACCase extraction and purification,

enzyme assay, and inhibition by ACCase herbicides were performed as described (Yu et al. 2004b).

Statistical analysis

The herbicide rate causing 50% mortality (LD_{50}), or growth reduction (GR_{50}) of plants and herbicide concentration causing 50% inhibition of enzyme activity (I_{50}) were calculated by non-linear regression analysis using Sigma Plot® software (version 8.02, SPSS Inc. 233 South Wacker Drive, Chicago, IL). Data sets from repeated experiments were pooled and analyzed by ANOVA. When the variance between repeated experiments was not significant, pooled data were used for subsequent analysis. The data were fitted to the log-logistic model $y = C + [(D - C)/[1 + (X/I_{50})^b]]$ (Seefeldt et al. 1995), where C = lower limit, D = upper limit, b = slope, and I_{50} = dose giving 50% response. Significance of difference between the two biotypes in glyphosate and paraquat translocation was analyzed by t test.

Results

Dose response to herbicides

Glyphosate

As expected, the glyphosate dose–response study established 100% mortality of a known susceptible (S) *L. rigidum* biotype at rates of 0.45 kg ha^{-1}, or higher (Fig. 1a). In contrast, the putative resistant (R) biotype was markedly less affected by glyphosate, requiring high rates (>3.6 kg ha^{-1}) for substantial mortality. The glyphosate rate causing 50% mortality (LD_{50}) for the R biotype was 2.3 kg ha^{-1} versus 0.16 kg ha^{-1} for the S biotype (Table 1). On the basis of the R/S ratio of LD_{50}, the R biotype is confirmed to be 14-fold resistant to glyphosate. The growth–response curve showed that glyphosate at 0.45 kg ha^{-1} dramatically reduced shoot dry mass production in the S biotype and reduced growth in the R plants (Fig. 1b). The glyphosate rate causing 50% reduction of growth (GR_{50}) for the R biotype was found to be 0.85 kg ha^{-1}, ninefold greater than for the S biotype (0.096 kg ha^{-1}) (Table 2).

Paraquat

As expected, the S biotype was killed at the recommended paraquat field dose of 0.2 kg ha^{-1} (Fig. 2a), with a LD_{50} of 0.041 kg ha^{-1} (Table 1). However, there

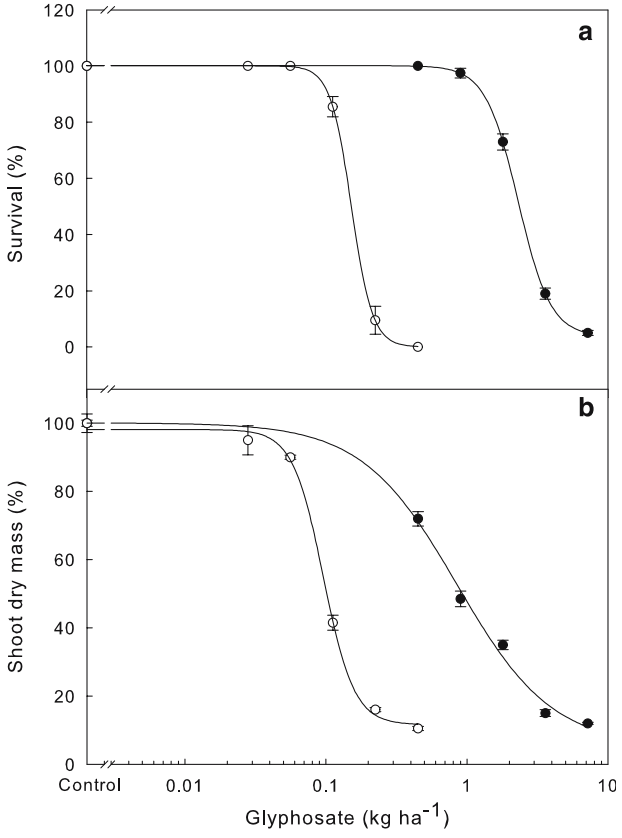

Fig. 1 Glyphosate dose response for survival (**a**) and shoot dry mass (**b**) of the known susceptible (S) biotype VLR1 (*open circle*) and the putative resistant (R) biotype AFLR2 (*filled circle*). Plants were sprayed at the 2–3 leaf stage and harvested 21 days after treatment. Each data point is mean ± standard error of six replicates from two experiments

Table 1 Parameter estimates for log-logistic analysis of glyphosate and paraquat dose–response survival data (pooled from two experiments) for susceptible (S) biotype VLR1 and resistant biotype (R) AFLR2

Biotype	C	D	b	$I_{50} = LD_{50}$ (kg ha^{-1})	r^2 (coefficient)	R/S ratio of LD_{50}
Glyphosate dose response						
AFLR2 (R)	3.69 (0.14)	100 (0.75)	3.76 (0.02)	2.31 (0.005)	0.999	14
VLR1 (S)	0.00 (0.23)	100 (0.13)	5.81 (0.006)	0.16 (0.001)	0.999	
Paraquat dose response						
AFLR2 (R)	5.90 (5.0)	102 (3.4)	2.47 (0.62)	1.28 (0.17)	0.99	32
VLR1 (S)	0.00 (0.8)	101 (0.79)	3.40 (0.15)	0.041 (0.001)	0.999	

Standard errors are in parentheses

Table 2 Parameter estimates for log-logistic analysis of glyphosate and paraquat dose–response biomass data (pooled from two experiments) for susceptible (S) biotype VLR1 and resistant biotype (R) AFLR2

Biotype	*C*	*D*	*b*	$I_{50} = GR_{50}$ (kg ha^{-1})	r^2 (coefficient)	R/S ratio of GR_{50}
Glyphosate dose response						
AFLR2 (R)	5.17 (7.15)	100 (3.88)	1.29 (0.28)	0.85 (0.15)	0.99	9
VLR1 (S)	11.59 (2.24)	98.2 (2.05)	3.99 (0.61)	0.096 (0.004)	0.99	
Paraquat dose response						
AFLR2 (R)	1.13 (3.88)	99.9 (2.64)	1.77 (0.21)	0.54 (0.04)	0.99	24
VLR1 (S)	3.88 (2.1)	99.4 (2.49)	2.35 (0.25)	0.023 (0.001)	0.99	

Standard errors are in parentheses

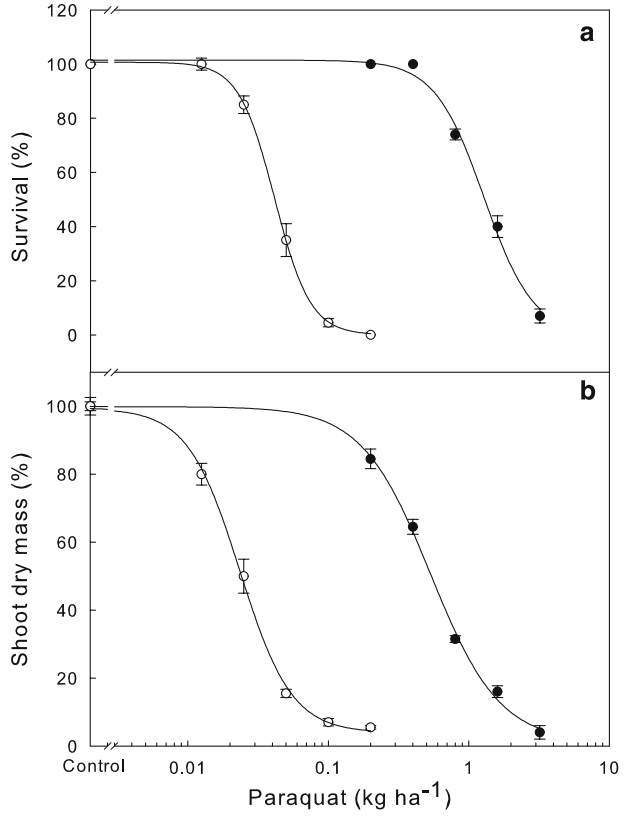

Fig. 2 Paraquat dose response for survival (**a**) and shoot dry mass (**b**) of the known susceptible (S) biotype VLR1 (*open circle*) and the putative resistant (R) biotype AFLR2 (*filled circle*). Plants were sprayed at the 2–3 leaf stage and harvested 21 days after treatment. Each data point is mean ± standard error of six replicates from two experiments

was no mortality at this dose for the R biotype, with the LD_{50} value being 1.28 kg ha^{-1} (Table 1). On the basis of the R/S ratio, the R biotype is 32-fold resistant to paraquat. While the R plants are able to survive high paraquat doses, there is severe foliar damage to older leaves and local chlorosis in some young leaves and reduced growth (Fig. 2b). The GR_{50} for the R biotype was 0.54 kg ha^{-1}, 24-fold greater than for the S biotype (0.023 kg ha^{-1}) (Table 2).

Therefore, the R biotype is clearly resistant to both glyphosate and paraquat.

ACCase-inhibiting herbicides

In addition to its confirmed resistance to glyphosate and paraquat (Figs. 1, 2, Tables 1, 2), the R biotype has also evolved high-level resistance to the ACCase-inhibiting herbicides diclofop, haloxyfop, fluazifop, propaquizafop, sethoxydim and tralkoxydim. There was a clear difference in dose response to these herbicides between R and S plants based on both survivorship (Fig. 3) and shoot dry mass (data not shown).

Susceptibility to other herbicides

A single-dose experiment was conducted to determine the susceptibility of the R biotype to a number of other herbicides to which this biotype has not been exposed in the field. It was established that the R biotype remains susceptible to the ACCase-inhibiting herbicide clethodim, and a number of other herbicides. These herbicides include the acetolactate synthase (ALS)-inhibiting herbicides, the photosynthesis-inhibiting herbicides, a mitosis-inhibiting herbicide, and a microtubule-inhibiting herbicide. The overall resistance status of this biotype is summarized in Table 3.

Leaf uptake and translocation of herbicides

Glyphosate

There were no visible symptoms of glyphosate damage in either S or R plants 2 days after treatment, whereas 4 days after treatment, visual chlorotic leaf damage became evident in S plants, while R plants remained green and healthy. After 6 days, all S plants had died whereas all R plants survived with no symptoms of damage (data not shown).

The leaf uptake of [^{14}C]-glyphosate was the same in both the S and R biotypes ($S = 74\% \pm 2.5$, $R = 73\% \pm 2.2$ of applied glyphosate, 2 days after treatment; $S = 76\% \pm 2.3$, $R = 74\% \pm 2.3$, 4 days) as reported for other glyphosate resistant *L. rigidum* biotypes

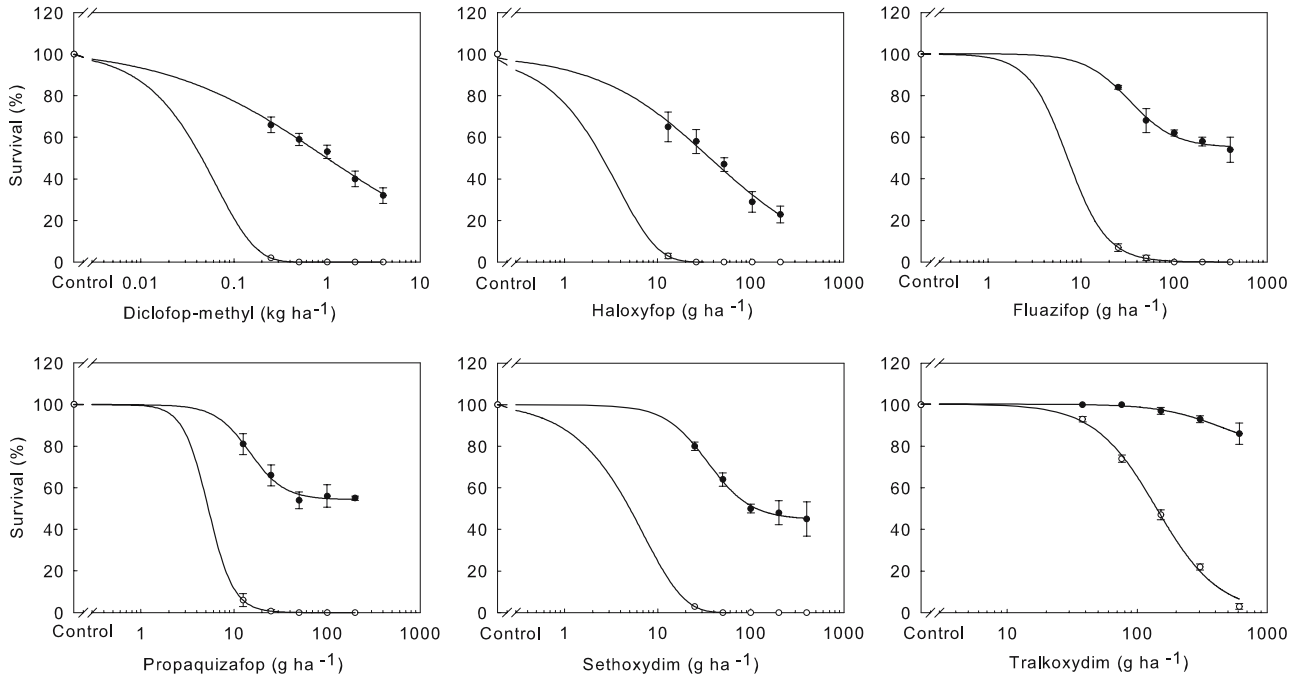

Fig. 3 Dose response to six ACCase-inhibiting herbicides of the known susceptible (S) biotype VLR1 (*open circle*) and the putative resistant (R) biotype AFLR2 (*filled circle*). Plants were sprayed with the respective herbicide at the 2–3 leaf stage and harvested 21 days after treatment. Each data point is mean ± standard error of three replicates from a single dose–response experiment

Table 3 Summary of resistance status across herbicide chemistries for the resistant (R) *L. rigidum* biotype AFLR2

Herbicide chemical class	Herbicide mode of action	Active ingredient	Resistance status
Glycine	Inhibition of EPSPS	Glyphosate	R
Bipyridyl	Inhibition of photosystem I	Paraquat	R
AOPP	Inhibition of ACCase	Diclofop	R
		Fluazifop	R
		Haloxyfop	R
		Propaquizafop	R
CHD	Inhibition of ACCase	Sethoxydim	R
		Tralkoxydim	R
		Clethodim	S
Sulfonylurea	Inhibition of ALS	Chlorsulfuron	S
		Sulfometuron	S
Imidazolinone	Inhibition of ALS	Imazepyr	S
		Imazethapyr	S
Dinitroaniline	Inhibition of tubulin formation	Trifluralin	S
Triazine	Inhibition of photosystem II	Atrazine	S
Urea	Inhibition of photosystem II	Diuron	S
Amide	Inhibition of mitosis	Metolachlor	S

Resistance was confirmed with full dose–response experiments (Figs. 1, 2, 3) or a single dose which obtained full control of the susceptible (S) *L. rigidum* biotype VLR1

AOPP aryloxyphenoxypropionate, *CHD* cyclohexanedione, *ACCase* acetyl-coenzyme A carboxylase, *ALS* acetolactate synthase, *EPSPS* 5-enolpyruvylshikimate-3-phosphate synthase

(Feng et al. 1999; Lorraine-Colwill et al. 2003). Although leaf uptake was the same, there was significantly less [^{14}C]-glyphosate translocated to the young leaves of R compared to S plants, ranging from 1.6- to 2.0-fold (Table 4). This was due to more [^{14}C]-glyphosate remaining in treated leaves plus stem of R plants. Translocation of [^{14}C]-glyphosate to roots of both biotypes did not significantly differ (Table 4). The difference in the translocation of [^{14}C]-glyphosate to untreated leaves, although relatively small, was significant

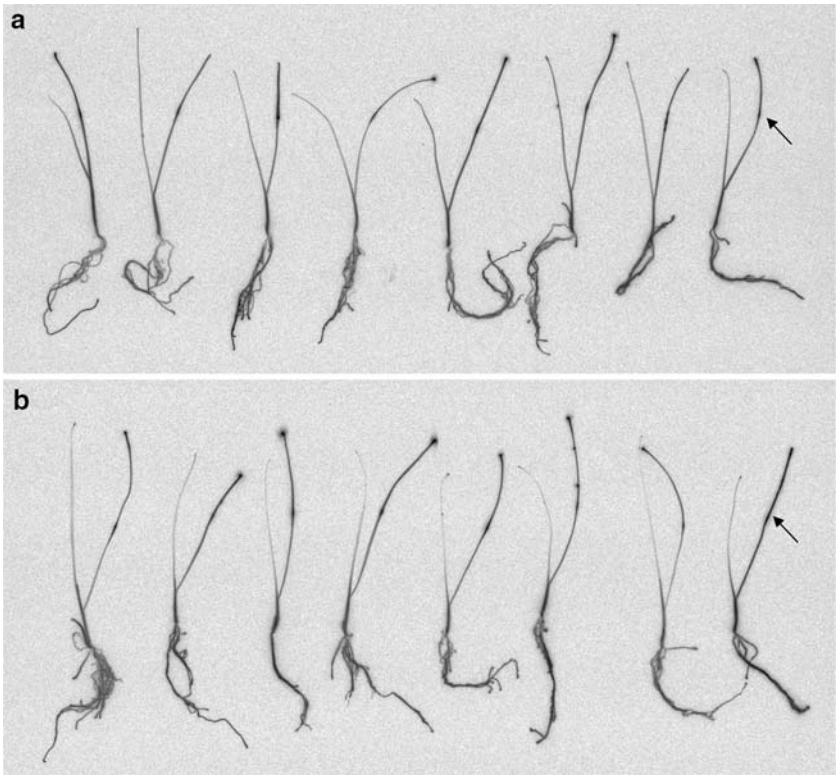

Fig. 4 Phosphor imaging comparing the translocation pattern of [^{14}C]-glyphosate between the susceptible biotype VLR1 (**a**) and the resistant biotype AFLR2 (**b**). Plants at the 2 leaf stage were sprayed with 450 g ha^{-1} glyphosate 20 min prior to the application of radiolabeled herbicide. [^{14}C]-glyphosate treatment solution was applied as a 1 µl droplet to the midpoint (*arrowed*) of the first leaf of each plant, and plants were harvested 2 and 4 days after treatment. The representative image is from plants 4 days after treatment

Table 4 Quantification of [^{14}C]-glyphosate translocation from a single leaf to other parts of the plant in the susceptible (S) biotype VLR1 and resistant (R) biotype AFLR2

Biotype	Applied ^{14}C-glyphosate (%)			
	Untreated leaves	Roots	Treated leaves and stem	Leaf wash
2 days after treatment				
VLR1 (S)	3.41 ± 0.34	27.9 ± 5.60	31.8 ± 4.42	26.0 ± 1.81
AFLR2 (R)	2.13 ± 0.28	26.1 ± 3.22	35.8 ± 3.45	27.1 ± 2.04
P value	0.01	>0.05	>0.05	>0.05
4 days after treatment				
VLR1 (S)	4.33 ± 0.62	29.7 ± 4.15	30.9 ± 2.21	24.0 ± 2.10
AFLR2 (R)	2.15 ± 0.48	22.5 ± 7.41	40.0 ± 3.08	26.3 ± 1.73
P value	0.01	>0.05	0.05	>0.05

The data (2 and 4 days after treatment) are the means ± standard error of 12 replicates from two experiments. Total recovery of applied radioactivity was 91% ± 3.3 and 89% ± 4.1 for R and S biotypes, respectively

and reproducible, and this result was also confirmed by phosphor imaging. As shown in Fig. 4, less [^{14}C]-glyphosate translocated from treated leaves (arrowed) to untreated leaves of R (Fig. 4b) than S (Fig. 4a) plants, 2 and 4 days after treatment (2 days data not shown).

Paraquat

About 95% of the applied paraquat was absorbed by leaves of R and S plants within 24 h after treatment. Because paraquat adheres to various surfaces, this figure may reflect both leaf surface adherence and uptake. There was no difference in paraquat total leaf uptake between R and S plants. In the S plants, 24 h after treatment, visible paraquat damage (leaf wilting) was evident in both treated and untreated leaves (indicating translocation), while in R plants only the upper part of treated leaves wilted, with untreated leaves showing no visual damage. Quantification and phosphor-imaging visualization of paraquat translocation revealed a significant difference between S and R plants in the distribution of [^{14}C]-paraquat (Table 5, Fig. 5a, b). About 6.0% [^{14}C]-paraquat translocated into untreated leaves of the S plants, while only 0.68% translocated in R plants. The amount of [^{14}C]-paraquat translocated to roots of R plants was less than half of

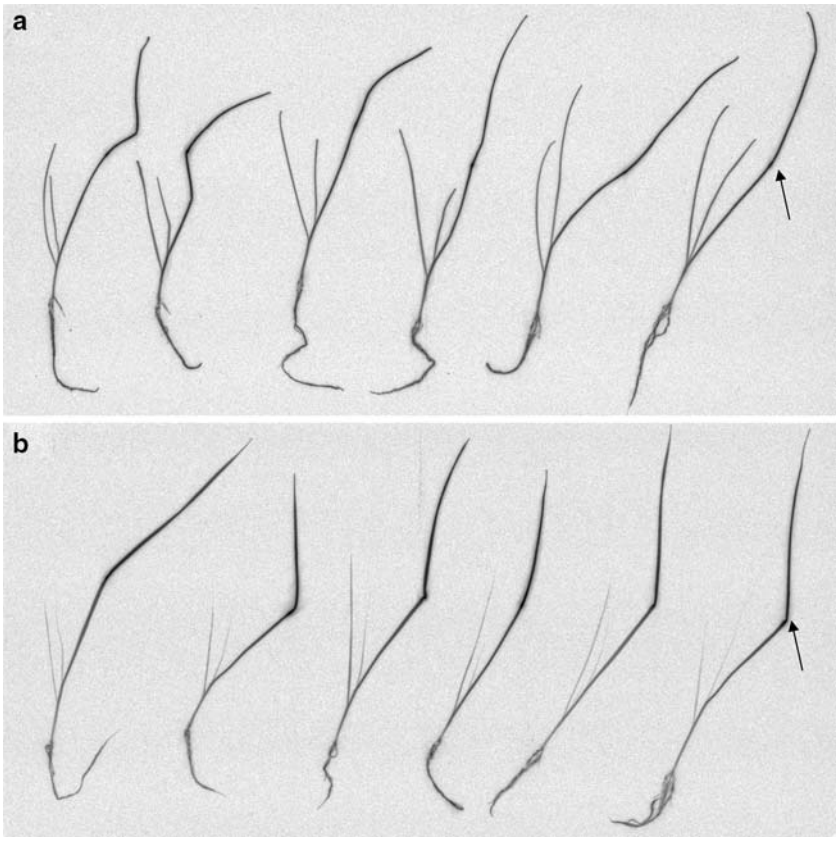

Fig. 5 Phosphor imaging comparing translocation pattern of [^{14}C]-paraquat between the susceptible biotype VLR1 (**a**) and the resistant (R) biotype AFLR2 (**b**). Plants at the 3 leaf stage were sprayed with 50 g ha^{-1} paraquat 20 min prior to the application of radiolabeled herbicide. [^{14}C]-paraquat treatment solution was applied as a 1 µl droplet to the midpoint (*arrowed*) of the second leaf of each plant, and plants were harvested 24 h after treatment

Table 5 Quantification of [^{14}C]-paraquat translocation from a single leaf to other parts of the plant in the susceptible (S) biotype VLR1 and resistant (R) biotype AFLR2

Biotype	Applied ^{14}C-paraquat (%)			
	Untreated leaves	Roots	Treated leaves and stem	Leaf wash
VLR1 (S)	5.70 ± 0.21	14.3 ± 2.81	62.9 ± 3.53	6.03 ± 1.47
AFLR2 (R)	0.64 ± 0.14	6.08 ± 0.82	80.3 ± 4.90	4.92 ± 1.87
P value	<0.01	0.01	0.01	>0.05

The data (24 h after treatment) are the means ± standard error of 12 replicates from two experiments. Total recovery of applied radioactivity was 92% ± 4.5 and 89% ± 5.0 for R and S biotypes, respectively

that in S plants. In contrast, there was more [^{14}C]-paraquat remaining in treated leaves plus stem of R than S plants. Clearly, much less paraquat was translocated (ninefold) into untreated leaves and roots (twofold) of the R, relative to the S plants.

EPSPS gene sequencing

To examine the molecular basis for differential response to glyphosate in the S and R biotypes, partial EPSPS cDNA (266 bp) was amplified and sequenced (Fig. 6). This sequenced region includes the highly conserved EPSPS gene region in which mutation sites conferring glyphosate resistance in plants and bacteria have been found (Comai et al. 1983; Stalker et al. 1985; Padgette et al. 1991, 1996; Baerson et al. 2002; Ng et al. 2003, 2004; Zhou et al. 2006). The nucleotide sequence of the sequenced region from the S and R biotypes showed 98 and 97% homology to the EPSPS gene of *L. rigidum* (AJ310166), respectively. Six single nucleotide polymorphisms were identified between the S and R biotypes at position 63, 74, 101, 173, 215, and 224 (Fig. 6). However, only the nucleotide change at position 63 is a codon mutation resulting in an amino acid substitution, while the other five changes are silent mutations. Nucleotide sequences and the deduced amino acid sequences of the highly conserved region of EPSPS from the S and R biotypes are compared in

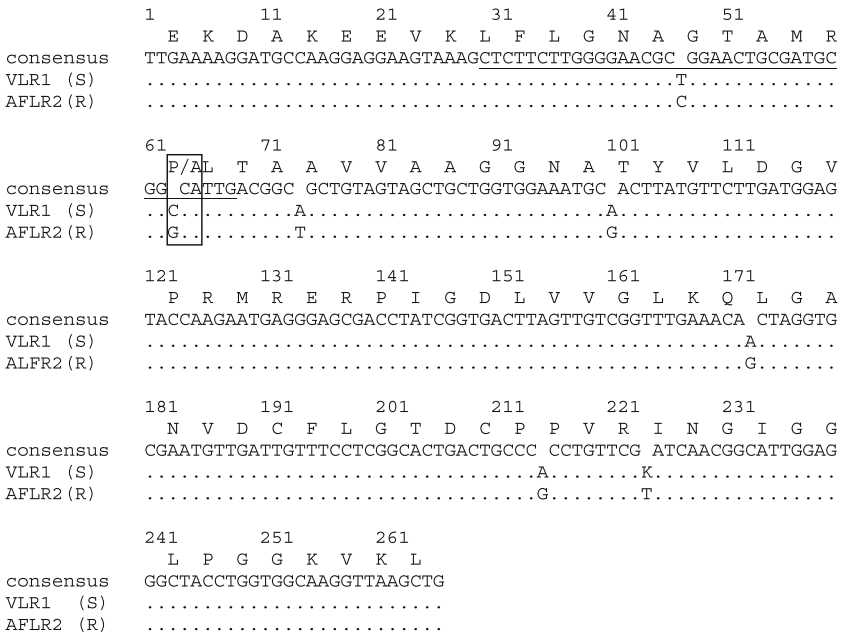

Fig. 6 Sequence comparison of the amplified 266 bp fragment of EPSPS cDNA from susceptible (S) biotype VLR1 and resistant (R) biotype ALFR2. The deduced amino acid sequence (single-letter code) is included. Highly conserved region is *underlined* in which point mutations conferring glyphosate resistance in plants and bacteria have been found. The homology regions are indicated by *dots*. The *boxed* codon indicates a non-synonymous mutation resulting in an amino acid substitution

Table 6. Two single-nucleotide differences were detected in this region. One of the nucleotide changes, T (S biotype) to C (R biotype) in the codon for Ala-100, is a silent mutation without causing an amino acid substitution. However, the nucleotide change, C (S biotype) to G (R biotype) in the codon for Pro-106, replaces Pro with Ala in the R biotype. As different substitutions at this same site have been reported to confer glyphosate resistance in other glyphosate resistant weed species (Baerson et al. 2002; Ng et al. 2003; Perez-Jones et al. 2005a, b; Wakelin and Preston 2006), this point mutation and substitution in EPSPS of the R biotype very likely confers glyphosate resistance.

In vitro inhibition of ACCase activity by herbicides

To determine if resistance to ACCase inhibiting herbicides in this multiple R biotype is due to an insensitive herbicide target enzyme, ACCase activity was assayed on partially purified shoot extracts from the S and R biotypes, and the herbicide concentration causing 50% inhibition of enzyme activity (I_{50}) values were determined for ACCase-inhibiting aryloxyphenoxypropanoate (AOPP) and cyclohexanedione (CHD) herbicides (Table 7). ACCase preparations from the R biotype were found to be three- to fourfold less inhibited by the AOPP herbicides diclofop acid, haloxyfop acid and fluzifop acid, relative to the S biotype. ACCase from the R biotype was found to be highly insensitive to the CHD herbicides tralkoxydim and sethoxydim (Table 7). Thus, resistance to ACCase herbicides in the R biotype is associated with a mutation of the ACCase gene (mutation not identified) that reduces the sensitivity of ACCase to these herbicides.

Discussion

Resistance profile

Unlike resistance to some herbicides (e.g., ALS or ACCase herbicides) which evolves rapidly, usually it takes many years for weed species to develop resistance to glyphosate or paraquat (see Heap website http://www.weedscience.org). Thus, glyphosate or paraquat resistance alleles are likely to be rare. Despite the rarity of glyphosate and paraquat resistance alleles, over-reliance on these herbicides results in the evolution of resistance. Biotypes of glyphosate resistant *Lolium* from several countries are known (Powles et al. 1998; Pratley et al. 1999; Perez and Kogan 2003; Simarmata et al. 2003; Perez-Jones et al. 2005a; recently reviewed by Powles and Preston 2006), and paraquat resistant *L. rigidum* has evolved (Yu et al. 2004a). However, this is the first report of multiple resistance to both glyphosate and paraquat (as well as to ACCase-inhibiting herbicides). This has occurred after more than 25 years of successful use of glyphosate and paraquat. Although this is unsurprising it is nevertheless an alarming example of the evolutionary potential for herbicide resistance. It is not surprising that this has occurred first in *L. rigidum* as this cross-pollinated, genetically diverse weed species is a very resistance-prone weed species. Glyphosate and paraquat are

often used to manage this weed. Multiple resistance to glyphosate, paraquat and ACCase herbicides is evident in *L. rigidum* on about 10–15 farms in the Tulbagh Valley, South Africa. Multiple resistance across major herbicides greatly reduces control options.

The appearance of multiple glyphosate and paraquat resistance in *Lolium* indicates the likelihood of such evolution in other major weed species. The evolution of glyphosate and paraquat resistance in this area is obviously due to insufficient diversity in the agroecosystem and persistent use of these herbicides. The accumulation of multiple resistance within the population is either as a result of sequential selection or due to cross pollination between individuals with different resistance mechanisms. Obligatory cross-pollination of *L. rigidum* aids in the rapid accumulation of multiple resistance.

Glyphosate resistance mechanisms

The active site of EPSPS has been probed using site-directed mutagenesis and inhibitor binding techniques (Padgette et al. 1991). The highly conserved region with a consensus amino acid sequence of LXLG-NAG^{101} TAMRP^{106}L (refer to plant numbering system, Padgette et al. 1996) in most plants and bacteria is a critical part of the EPSPS active site. This active site is crucial for the binding of phosphoenolpyruvate (PEP) or its competitive inhibitor glyphosate.

Either a Gly to Ala substitution at position 101 (G101A), or Pro to Ser at position 106 (P106S) has resulted in a glyphosate-resistant EPSPS in *Escherichia coli, Petunia* and *Salmonella typhimurium* (reviewed by Padgette et al. 1996). Similarly, a glyphosate resistant mutant of *Oryza sativa* with a Pro-106 to Leu-106 (P106L) substitution of EPSPS gene has been selected and characterized using a directed evolution strategy (Zhou et al. 2006). Likewise, with field evolved glyphosate resistance, the molecular basis of the reduced sensitivity of EPSPS to glyphosate in an evolved resistant *Eleusine indica* biotype (goosegrass) was revealed as an amino acid change from Pro-106 to Ser-106 (Baerson et al. 2002). In addition, a Pro-106 to Thr-106 (P106T) substitution was also identified to endow glyphosate resistance in other resistant biotypes of *E. indica* (Ng et al. 2003, 2004).

We expect that the mutations of Pro-106 in EPSPS found in glyphosate resistant *E. indica* would occur in other weed species. Indeed, glyphosate resistant *L. rigidum* biotypes have been shown to posses a P106T substitution (Wakelin and Preston 2006), and a P106S substitution has also been identified in a glyphosate resistant *L. multiforum* (Perez-Jones et al. 2005a, b). In the present study with this *L. rigidum* biotype, alanine substitutes for Pro-106 and very likely confers resistance to glyphosate (Table 6, Fig. 6).

Proline is the only cyclic amino acid. When proline is in a peptide bond, it does not have a hydrogen bond to stabilize an α-helix or a ß-sheet. Usually proline cannot exist in an α-helix. When proline is found in a α-helix, the helix will have a slight bend due to lack of hydrogen bond. In EPSPS, Pro-106 is not directly involved in glyphosate binding but located in an α-helix and bends this helix slightly (Zhou et al. 2006). While sharing some properties with the aliphatic amino acid alanine, proline has conformational constraints due to the cyclic nature imposed by its pyrrolidine side group, among other differences. Therefore, the change from Pro to Ala will affect the conformation of the α-helix, and consequently affect glyphosate binding to the enzyme, leading to glyphosate resistance. The effect of Pro-106 substitution by Ala-106 (P106A) on EPSPS could be further verified by examining EPSPS sensitivity to glyphosate in vitro or determining kinetic characterization of variant EPSPS enzyme using an *E. coli* expression system.

The occurrence of diverse resistant alleles in weed species suggests an independent selection event for glyphosate resistance, largely depending on initial spontaneous mutations, selection conditions and ecological fitness. Mutations that contribute to plant survival can be selected for and enriched in the populations under herbicide selection. The high fre-

Table 6 Comparison of nucleotide sequence and deduced amino acid sequence of highly conserved region of the EPSPS enzyme from susceptible (S) biotype VLR1 and resistant (R) biotype ALFR2

Amino acid number[a]	95	96	97	98	99	100	101	102	103	104	105	106	107
Amino acid	Leu	Phe	Leu	Gly	Asn	Ala	Gly	Thr	Ala	Met	Arg	Pro	Leu
Consensus sequence	CTC	TTC	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG
VLR1 (S)	-	-	-	-	-	-	-	-	-	-	-	-	-
AFLR2 (R)	-	-	-	-	-	GCC	-	-	-	-	-	GCA Ala	-

[a] Amino acids are numbered according to the plant EPSPS numbering system used by Padgette et al. (1996)

- Indicates identical codon to the consensus sequence

quency of the Pro-106 substitutions occurring in bacterial or plant EPSPS (Stalker et al. 1985; Baerson et al. 2002; Ng et al. 2003; Perez-Jones et al. 2005b; Zhou et al. 2006; Wakelin and Preston 2006; and this study) confirms that the Pro-106 site is important for glyphosate resistance and that this site is prone to mutation. Diverse mutations and substitutions at this site (P106S, P106T, P106L, and P106A) reflect the flexibility of the herbicide binding site of EPSPS.

Comparison of the kinetic parameters of *E. coli*-expressed EPSPS variants P106S, P106L and G101A with wild-type EPSPS revealed that a G101A mutant had a greater Km (PEP) and a lower V_{max} than P-106 mutants, resulting in a greater reduction in PEP binding capacity and in EPSPS reaction rate (Zhou et al. 2006). Such results indicate a less severe impact of Pro-106 substitutions on EPSPS catalytic capacity. Site-directed mutagenesis studies also indicated that Gly-101 is directly involved in PEP and glyphosate binding (Padgette et al. 1991, 1996). In addition, the crystal structure of the ternary EPSPS-S3P-glyphosate complex clearly reveals that Gly-101, but not Pro-106, is directly involved in glyphosate and PEP binding by forming a hydrogen bond (Schönburnn et al. 2001). Since the Pro-106 mutations do not occur at the binding site of the EPSPS enzyme, the impact on the enzyme and whole plant performance may be less severe than Gly-101 mutations. The fitness of glyphosate resistant biotypes with specific EPSPS mutations needs to be evaluated.

One of the important features of glyphosate is its systemic action. Glyphosate has considerable xylem and phloem mobility within plants (Franz et al. 1997), and this rapid and widespread glyphosate translocation is important in achieving herbicide efficacy. Meristems, such as roots and shoot apices, are sensitive sites for glyphosate action. Therefore, the ability to reduce glyphosate translocation towards developing tissue could confer resistance in plants. In this study, we consistently observed in R plants less glyphosate translocating to young leaves (Table 4, Fig. 4b). Altered glyphosate translocation pattern has been found in several glyphosate resistant weed species. Glyphosate resistance in other *L. rigidum* biotypes has been determined to be associated with decreased translocation to roots (threefold) or shoot meristematic zones (1.6-fold) due to increased glyphosate translocation to leaf tips (Lorraine-Colwill et al. 2003; Wakelin et al. 2004). In glyphosate resistant biotypes of *Conyza canadensis*, translocation of glyphosate to roots was twofold less and overall translocation 1.4- to 1.9-fold lower than in S biotypes, and export of glyphosate out of the treated leaf was impaired resulting in accumulation of glyphosate in the treated R leaves (Feng et al. 2004; Koger and Reddy 2005). We consistently observed a 1.6- to 2.0-fold reduced glyphosate translocation to the young leaves of R relative to S plants (Table 4), and the magnitude of the difference in glyphosate translocation is of the same magnitude to that mentioned above. Therefore, a similar glyphosate resistance mechanism, involving reduced glyphosate translocation to growing tissues, is evident in this R biotype of *L. rigidum*. The biochemical and molecular basis of this reduced glyphosate translocation resistance mechanism needs to be elucidated.

Thus, in the limited studies so far on plants with evolved glyphosate resistance, it is evident that at least two different mechanisms can confer glyphosate resistance, i.e. mutation of the EPSPS gene and/or reduced glyphosate translocation. In this study, both mechanisms are present in the one R biotype. As *L. rigidum* is an obligatory cross-pollinated species, different resistance mechanisms are easily accumulated in individuals due to cross-pollination among resistant survivors. The relative contribution of this reduced glyphosate translocation to the overall level of glyphosate resistance in this R biotype is unknown, but it is likely that reduced glyphosate translocation would be additive to the level of glyphosate resistance conferred by EPSPS mutation.

Paraquat resistance mechanism

In the present study, quantification and phosphor imaging of paraquat translocation clearly revealed a different pattern of [^{14}C]-paraquat translocation between S and R plants (Table 5, Fig. 5a, b). The S plants translocated ninefold more paraquat to untreated leaves and twofold more paraquat to roots, while the R plants largely confined paraquat within treated leaves. Correspondingly, wilting/desiccation of both treated and untreated leaves (including young leaves) were observed in the S but not in the R plants. Reduced paraquat movement in paraquat resistant plants has been demonstrated in *Erigeron philadelphicus* and *E. canadenisis* (Tanaka et al. 1986), *C. bonariensis* (Fuerst et al. 1985), *Hordeum glaucum* (Bishop et al. 1987; Preston et al. 1992; Purba et al. 1995), *A. calendula* (Soar et al. 2003) and *L. rigidum* (Yu et al. 2004a). Unlike glyphosate, paraquat is a contact herbicide, moving upwards largely in the apoplast with transpiration. Significant basipetal translocation does not occur until leaf damage occurs (Baldwin 1963). This is in line with the observations that differences in paraquat translocation between resistant and susceptible biotypes of *A. calendula* were only detected when treated plants were exposed to light (Soar et al. 2003).

Therefore, it is postulated that paraquat translocation depends on light-mediated leaf damage.

As paraquat induced damage/inhibition of photosynthesis is usually limited, delayed or temporary in leaves of resistant plants (Shaaltiel and Gressel 1987; Lehoczki et al. 1992; Preston et al. 1992), reduced translocation of paraquat in the resistant plants could be the consequence of restricted mobility of paraquat within individual cells. Intact leaf photosynthetic fluorescence imaging showed limited diquat movement in the leaves of a resistant *A. calendula* biotype, compared to a susceptible biotype (Preston et al. 1994). It has been suggested that the restricted mobility of paraquat in R biotypes is primarily due to sequestration into metabolically inactive compartments, which in turn results in reduced penetration to chloroplasts (reviewed by Hart and DiTomaso 1994; Preston 1994). However, despite much effort, there is no direct evidence to reveal where and how paraquat is sequestered. There is only indirect evidence for vacuolar sequestration of paraquat in the roots of resistant *H. glaucum* using compartmentation analysis (Hart and DiTomaso 1994; Lasat et al. 1997). Direct measurement of paraquat in leaf protoplasts indicates that vacuolar paraquat sequestration most likely occurs in the paraquat resistant biotype of *L. rigidum* AFLR1 (Q. Yu and S.B. Powles, unpublished).

As the paraquat translocation pattern in this *L. rigidum* biotype AFLR2 appears similar to AFLR1 (Yu et al. 2004a), we propose that reduced paraquat translocation in this R biotype is primarily due to increased sequestration of paraquat into vacuoles of the cells, reducing paraquat damage and therefore overall paraquat translocation. The observation that paraquat treated R plants suffer local leaf chlorosis as well as tip burn supports the vacuole sequestration hypothesis. The biochemical and molecular basis of paraquat sequestration remains to be determined.

Glyphosate and paraquat are different in molecular structure, electron charge and mobility within plants (Baldwin 1963; Franz et al. 1997). It is unlikely that a single mechanism is responsible for both reduced glyphosate and paraquat translocation. However, difference in molecular structure may not be enough to preclude some transporters which have broad substrate specificity, such as some ABC transporters (especially multidrug resistance transporters or multidrug resistance associated protein) (reviewed by Chang 2003). Notwithstanding, *L. rigidum* biotypes resistant to glyphosate due to reduced glyphosate translocation are not cross resistant to paraquat (Lorraine-Colwill et al. 2003; Wakelin et al. 2004). Equally, *L. rigidum* biotypes resistant to paraquat due to reduced paraquat translocation are not cross resistant to glyphosate (Yu et al. 2004a). This indicates that the reduced translocation mechanisms for glyphosate and paraquat resistance are unrelated. This can be tested by a genetic study.

In this study with this particular R biotype, we did not examine glyphosate or paraquat metabolism. A large body of data indicates that glyphosate metabolism in most whole plants is very slow or nonexistent (reviewed by Franz et al. 1997). Especially, in herbicide resistance studies, it has been demonstrated that there was little to no metabolism of glyphosate in glyphosate resistant biotypes of *L. rigidum* (Feng et al. 1999; Lorraine-Colwill et al. 2003), *E. indica* (Tran et al. 1999) and *C. canadensis* (Feng et al. 2004). However, glyphosate degradation was found in plant cell cultures of soybean cells under sterile conditions. (Komoßa et al. 1992). Therefore, the possibility of metabolic detoxification of glyphosate is slight but cannot be completely ruled out in this glyphosate and paraquat resistant *L. rigidum* biotype AFLR2.

Similarly, no metabolism of paraquat has yet been detected for any higher plant (Summers 1980). Metabolic detoxification of paraquat was not detected for paraquat resistant biotypes of *C. bonariensis* (Norman et al. 1993) and paraquat tolerant biotypes of *L. perenne* (Harvey et al. 1978). In our study on paraquat resistant *L. rigidum* biotype AFLR1, we found no paraquat metabolites in this biotype using TLC analysis (Q. Yu et al., unpublished). As the paraquat translocation pattern previously studied in AFLR1 (Yu et al. 2004a) appears similar to this *L. rigidum* biotype AFLR2, we consider metabolic degradation of paraquat is unlikely in this glyphosate and paraquat resistant biotype AFLR2.

ACCase herbicide resistance mechanism

In vitro ACCase inhibition assay has revealed that ACCase from the R biotype is moderately (three- to fourfold) insensitive to AOPP herbicides diclofop, haloxyfop and fluazifop, but highly insensitive to CHD herbicides tralkoxydim (11-fold) and sethoxydim (> 16-fold) (Table 7). It is clear that there is resistance across both chemical classes of ACCase inhibiting herbicides (AOPP and CHD). Many grass weed species, especially *L. rigidum*, have developed resistance to ACCase herbicides (Powles and Matthews 1992; Preston et al. 1996), and this resistance is therefore unremarkable. However, in this case, resistance to ACCase herbicides is accompanied by resistance to glyphosate

Table 7 Parameter estimates for log-logistic analysis of in vitro inhibition of ACCase enzyme activity by various ACCase inhibiting herbicides for susceptible (S) biotype VLR1 and resistant biotype (R) AFLR2

Herbicide	Biotype	*C*	*D*	*b*	I_{50} (μM)	r^2 (coefficient)	R/S ratio of I_{50}
AOPP							
Diclofop acid	AFLR (R)	14 (4.7)	91 (2.32)	0.87 (0.21)	11.89 (2.88)	0.99	3.1
	VLR1 (S)	9.7 (1.67)	92 (0.90)	0.89 (0.055)	3.8 (0.32)	0.999	
Haloxyfop acid	AFLR (R)	14 (3.0)	97 (2.19)	0.65 (0.15)	11.49 (3.6)	0.99	3.3
	VLR1 (S)	14 (5.5)	96 (2.59)	1.05 (0.22)	3.44 (0.97)	0.999	
Fluazifop acid	AFLR (R)	18.69 (6.5)	101 (2.6)	0.56 (0.12)	31.27 (6.8)	0.99	4.5
	VLR1 (S)	18 (3.11)	98 (1.97)	0.78 (0.11)	6.84 (1.32)	0.999	
CHD							
Tralkoxydim	AFLR (R)	9.7 (0.56)	96 (4.88)	0.51 (0.11)	3.08 (0.27)	0.99	11
	VLR1 (S)	16.1 (2.89)	95 (5.34)	0.85 (0.17)	0.28 (0.08)	0.999	
Sethoxydim	AFLR (R)	44.6 (1.38)	100 (1.45)	0.71 (0.08)	>10	0.99	>16
	VLR1 (S)	22.7 (0.56)	100 (0.76)	0.89 (0.04)	0.64 (0.03)	0.999	

Standard errors are in parentheses. Data are pooled from two experiments and each contained three extractions

and paraquat. This means that three very different herbicide modes of action are ineffective on this R biotype.

Currently, there are five amino acid substitutions identified in the plant plastidic ACCase isoforms (carboxyl transfer domain) that endow resistance to ACCase inhibiting herbicides (reviewed by Delye 2005). An Ile to Leu substitution at position 1781 (refers to *A. myosuroides* plastidic ACCase AJ310767) generally confers high-level resistance to the CHD herbicide sethoxydim, but low-level resistance to other CHD or AOPP herbicides. Considering the cross resistance pattern revealed in this study for this *L. rigidum* R biotype (Table 7), an Ile to Leu substitution is most likely expected.

In summary, we have confirmed the first case of glyphosate and paraquat (and ACCase) multiple resistance. Glyphosate resistance in this R biotype is due to a proline to alanine substitution at amino acid position 106 of the EPSPS gene, as well as reduced glyphosate translocation to young leaves. Paraquat resistance is due to reduced paraquat movement (presumably the consequence of increased sequestration) to young leaves. Resistance to ACCase herbicides is caused by an insensitive ACCase. Therefore, this multiple resistant *L. rigidum* possesses at least four distinct herbicide resistance mechanisms endowing resistance to glyphosate, paraquat and ACCase herbicides. The evolution of multiple resistance, especially to glyphosate and paraquat, in this resistance-prone weed species indicates a further threat to the sustainability of the world's most widely used herbicide glyphosate, and its alternative paraquat.

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