Research Article

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EPSPS gene amplification in glyphosate-resistant *Bromus diandrus*

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Abstract

BACKGROUND: Glyphosate is the most widely used herbicide in the world and has been intensively used to control *B. diandrus*, a problematic weed of crops and pastures in southern Australia.

RESULTS: Resistance to glyphosate was identified in two populations of *B. diandrus* that were nearly fivefold more resistant to glyphosate than wild-type plants. Both populations contained *EPSPS* gene amplification, with resistant plants having an average of around 20-fold the number of copies of *EPSPS* compared with susceptible plants. *EPSPS* expression was also increased in resistant plants of both populations; however, expression levels were not correlated with the number of *EPSPS* copies. Amplification of only one of the four *EPSPS* genes present in *B. diandus* was detected. Investigation into the inheritance of glyphosate resistance found no segregation in the F_2 generation. Every individual in the F_2 populations contained between three and 30 copies of *EPSPS*; however, on average they contained fewer copies compared with the parent resistant population.

CONCLUSIONS: Glyphosate resistance in *B. diandrus* is due to *EPSPS* gene amplification. Resistance is heritable but complex.

Keywords: *EPSPS*; gene amplification; glyphosate resistance; inheritance

1 INTRODUCTION

Glyphosate [*N*-(phosphonomethyl) glycine] is the most widely used and versatile herbicide in the world. It has numerous uses in the management of chemical fallows, controlling vegetation prior to sowing crops and controlling weeds under trees and vines, and in non-agricultural uses, such as along road and rail rights-of-way, around buildings, along fence lines and in home gardens. Since 1996, glyphosate has also been used to control weeds in glyphosate-resistant crops. The large number of weed species controlled by glyphosate, its lack of soil residual activity and reductions in cost have resulted in great reliance on glyphosate for weed control.[1]

One consequence of the widespread reliance on glyphosate for weed control has been the evolution of glyphosate-resistant weeds. To date, glyphosate resistance has evolved in 31 weed species.[2] While glyphosate-resistant crops have been a key driver for the evolution of glyphosate resistance in weed species, glyphosate resistance has evolved in many of the other situations where this herbicide is used. In Australia, where relatively few glyphosate-resistant crops are grown, resistance to glyphosate has still occurred in ten weed species.[3] The largest resistance problem in Australia is with *Lolium rigidum*, with almost 600 resistance sites known.[3]

The genetic basis of glyphosate resistance in many weed species remains unknown, but those studied in detail have shown that resistance can be the result of a number of different mechanisms. Early work with *L. rigidum* identified a lack of translocation as the mechanism of resistance to glyphosate.[4] This mechanism has since been identified in *Conzya canadensis*[5] and *L. multiflorum*[6] and been shown to result from sequestration of glyphosate in the leaf vacuoles.[7,8] Modification of the target enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) was observed in glyphosate-resistant *Eleusine indica*[9] and has since been identified in several other species, including *L. rigidum*,[10] *L. multiflorum*[6] and *Echinochloa colona*.[11] In 2010, glyphosate resistance in *Amaranthus palmeri* was shown to be caused by extensive amplification of the *EPSPS* gene.[12] This mechanism has since been identified in a number of other species, including *Amaranthus tuberculatus*,[13,14] *Amaranthus spinosus*,[15] *Kochia scoparia*[16] and *Lolium multiflorum*.[17]

Understanding the inheritance pattern of herbicide resistance can be useful in predicting the likely evolution and spread of resistance, which in turn can inform resistance management strategies. Traits inherited as recessive alleles, for example, can be managed by using a high dose with refuge strategy, while such a strategy will not be effective where resistance is the result of a single dominant allele.[18,19] Where resistance is the result of multiple alleles,

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resistance is less likely to evolve unless low rates are used.[19,20] Resistance due to reduced translocation and target-site mutations are inherited as single incompletely dominant alleles.[21–23] However, the inheritance of gene amplification appears to be more complex and does not fit a single-gene model.[24,25]

In 2011, the first cases of glyphosate-resistant *Bromus diandrus* were identified in Australia. *B. diandrus* is a winter annual weed occurring in both crops and pastures across the southern Australian cereal belt. It is an aggressive competitor of cereals and can cause significant yield losses.[26] *B. diandrus* is prolific in no-till crops, as seeds germinate upon shallow burial by the sowing process, prompting a large in-crop flush.[27] Increased adoption of reduced tillage in grain production systems has increased the reliance on herbicides as the main tool for *B. diandrus* control. There are, however, few selective in-crop herbicides that are effective against *B. diandrus* in cereals,[28] making glyphosate an important management tool, threatened by the evolution of resistance.

The aim of this study was to identify the mechanism and mode of inheritance of glyphosate resistance in glyphosate-resistant *B. diandrus* populations.

2 EXPERIMENTAL METHODS

2.1 Plant material

The resistant populations occurred in cropping fields near Arthurton, South Australia (BdSaR), and Ouyen, Victoria (BdVicR), where old fence lines had been removed from fields and cropped over. The resistant weeds were noticed when they were not controlled by a presowing application of glyphosate. The susceptible population (BdS) was collected from the same field as BdSaR, 20 m from the old fence line. The plants were tested for resistance using the Quick-Test method.[29] Resistant plants, surviving a commercially relevant rate of glyphosate (570 g a.e. ha^{-1} of Roundup Attack; Nufarm, Laverton, Victoria, Australia), and untreated susceptible plants were retained and grown to maturity to produce bulked seeds for use in the subsequent experiments.

2.2 Generation of F_2 progeny

Crosses were made by emasculating flowers on susceptible plants and hand pollinating using pollen from resistant plants of BdSaR. Most of the florets were removed from the panicle of the susceptible plant, and 1–2 florets were hand pollinated. The panicle was bagged immediately after pollination and the seeds were allowed to mature. This process yielded two F_1 seeds. The F_1 seeds were sown individually into pots and allowed to self-pollinate to produce F_2 seeds, and these seeds were used for the inheritance studies.

2.3 Response of parents and F_2 progeny to glyphosate

Seeds were sown directly into soil, chilled in the dark for 7 days and allowed to germinate and emerge. At the one-leaf stage, seedlings were transplanted into 9.5 × 8.5 × 9.5 cm punnet pots (Masrac Plastics, South Australia, Australia) containing standard potting mix[28] with nine seedlings per pot and three replicates, and grown outdoors at the Waite Campus, University of Adelaide. At the 2–3-leaf stage, plants were treated with glyphosate (Roundup Attack, 570 g a.e. L^{-1}; Nufarm) at 0, 35.6, 71.3, 142.5, 285, 427.5, 570, 1140 and 2280 g a.e. ha^{-1}. Nonionic surfactant (Wetter TX®; Nufarm) was added to the spray solution at 0.2% (v/v). Glyphosate was applied using a laboratory moving boom pesticide applicator and applied at an equivalent of 109 L ha^{-1} of water at a pressure of 250 kPa and a speed of 1 m s^{-1} using Tee-Jet 001 nozzles (Tee-Jet 8001E; Spraying Systems Co., Wheaton, IL). Survival was assessed 21 days after herbicide treatment. Data from the susceptible and resistant populations were analysed by probit analysis.[30] The response of the F_2 populations was compared with that of the parents to identify segregation. A *G*-test with Williams' correction was used to compare the segregation pattern with one- and two-gene models.

2.4 *EPSPS* sequencing

Fresh leaf material was harvested from young leaves, snap frozen in liquid nitrogen and stored at −80 °C. Genomic DNA was extracted using the Isolate Plant DNA extraction kit (Bioline, Alexandria, New South Wales, Australia) according to the manufacturer's instructions. Total mRNA was extracted using the Dynabeads mRNA DIRECT purification kit (Bioline), and cDNA was synthesised using the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instruction. The concentration of nucleic acids was determined spectrophotometrically on a nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) at 260 nm.

For polymerase chain reaction (PCR) amplification, ~200 ng of gDNA or cDNA was added to a standard 25 µL PCR reaction mix containing 1× MyFi reaction buffer (containing 0.2 mM of dNTPs and 0.6 mM of $MgCl_2$), 0.4 µM of each gene-specific primer (Table 1) and 1 µL of MyFi DNA polymerase (Bioline). Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler Gradient; Eppendorf, Hamburg, Germany) with PCR conditions as follows: 3 min denaturing at 95 °C; 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, 45 s elongation at 72 °C and a final extension for 7 min at 72 °C.

PCR products were prepared with 1× Ficoll loading dye [15% (w/v) Ficoll 4000, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF] and visualised on SYBR Safe (Life Technologies,

Table 1. Primer name and sequence used in *EPSPS* gene sequencing, copy number determination and expression analysis

Primer name	Primer sequence 5′–3′
Mutation sequencing	
EPSPS-seqF	AACAGTGAGGAYGTYCACTACATGCT
EPSPS-seqR	CGAACAGGAGGGCAMTCAGTGCCAAG
Cloning	
EPSPS-cloneF	AAGTCGCTMTCCAAYCGRATCCT
EPSPS-cloneR	GGGAAGGTCTTNCGGGTGCA
QPCR	
EPSPS-qF	CCAAGAATGAGGGAGCGACCTAT
EPSPS-qR	CAGTGCCAAGGAAACAATCAACA
EPSPSProbe	TGGTGACTTAGTTGTCGGTTTGAAGCA + *FAM*
ALS-qF	GACCGCGTTACAGGGAAA
ALS-qR	GCCAATCTCTGCTGGATCAATG
ALSProbe	TTTGCAAGCAGGTCCAAGATTGTGC + *TET*
QPCR normalisation	
Actin-F	TGGCATCACACTTTCTACAAT
Actin-R	GCTGACACCATCACCAGAGTC
ELF-F	GGTACCTCCCAGGCTGACTGT
ELF-R	GTGGTGGCGTCCATCTTGTTA
Tubulin-F	ACCAACCTTGTGCCCTATCC
Tubulin-R	GGGCACCAGTCAACAAACTG
GAP-F	GTGAGGCTGGTGCTGATTACG
GAP-R	TGGTGCAGCTAGCATTTGAGAC

Mulgrave, Victoria, Australia) stained 1.5% agarose gels. Samples were electrophoresed in 1× TAE buffer (40 mM of Trizma base, 1 mM of Na_2EDTA, pH to 8 with glacial acetic acid) at 100 V and photographed under UV light (λ = 302 nm). DNA fragment sizes were estimated by comparing their mobility with bands of known sizes of a low-molecular-weight marker (EasyLadder I; Bioline). DNA sequencing was conducted by the Australian Genome Research Facility (AGRF) using the same primers as for amplification. Sequence data were analysed using ContigExpress from the Vector-NTI Suite 6 programs (Life Technologies), and all sequences were visually rechecked using the chromatogram files.

2.5 Absorption, translocation and root extrusion of glyphosate

Absorption and translocation of glyphosate were studied using the method described by Wakelin *et al.*,[31] with modifications. Husks were removed from the seeds, and the seeds were placed on agar for germination; 4–5 days in the dark at 4 °C until the first signs of emergence, followed by 3–4 days in a controlled-environment cabinet (20 °C, 12 h light period, 15 °C, 12 h dark period). Seedlings were then transplanted and grown hydroponically in nutrient solution[32] and black polypropylene beads. The plants were maintained in a growth room with a 12 h/20 °C light 12 h/15 °C dark period, until they reached the three-leaf stage. For the root extrusion experiments, seedlings were transplanted into individual 10 mL vials containing 10 mL of nutrient solution before herbicide application. The plants were then sprayed with 125 g a.e. ha^{-1} of glyphosate immediately prior to application of the radiolabelled herbicide. Radiolabelled ^{14}C-glyphosate solution made up in 125 g a.e. ha^{-1} commercial glyphosate formulation was applied to the middle of the second leaf of each seedling. The 0.5 µL aliquot applied to each leaf contained an average of 0.5 kBq of radioactivity and 0.0136 µmol of glyphosate (phosphonomethyl-^{14}C) (Sigma-Aldrich, Castle Hill, New South Wales, Australia).

For the translocation experiments, plants were harvested 1, 2 and 3 days after treatment (DAT) and sectioned into four parts: treated leaf, untreated leaves, leaf sheaths and roots. For the root exudation experiments, plants were harvested at 2, 3 and 5 DAT, and total leaf and root material were collected separately, as well as the 10 mL nutrient solution. The treated leaf was briefly washed in 5 mL of 0.1% Triton X-100 (BDH Chemicals, Merck, Melbourne, Victoria, Australia) to remove unabsorbed herbicide. Plant material was dried for 7 days at room temperature before combusting in a biological oxidider (R.J. Harvey Instrument Corporation, Hillsdale, NJ). Radioactive CO_2 was collected in a 14 mL carbon trap scintillation fluid mix [Carbo-Sorb E:Permaflour E+ 1:1 (v/v); PerkinElmer, Rowville, Victoria, Australia), and radioactivity was quantified using liquid scintillation spectroscopy (Packard Tri-Carb 2100 TR; PerkinElmer) as described by Lorraine-Colwill *et al.*[4] Radioactivity in the wash solutions as well as the nutrient solution from the root extrusion experiment was also quantified using liquid scintillation spectroscopy following the addition of a 1:1 (v/v) ratio of Ultima Gold XR scintillation fluid (PerkinElmer). The percentage of glyphosate absorbed was calculated as the sum of the amount in the various plant sections (treated leaf, roots and rest of plant) divided by the total amount recovered, including the treated leaf wash. The percentage of glyphosate in each plant part was calculated as the amount in that plant part divided by the amount of glyphosate absorbed. Absorption data were analysed by non-linear regression using an asympomatic regression function modified from Kniss *et al.*:[33]

$$\text{Absorption} = A_{max} \times [1 - \exp(-b \times t)]$$

Translocation data were analysed by two-way ANOVA.

2.6 *EPSPS* copy number and expression

Quantitative real-time PCR (QPCR) was used to analyse *EPSPS* expression and copy number. *EPSPS* expression was carried out as outlined in Burton *et al.*[34] Briefly, this involved HPLC purification, quantitation and sequence verification of QPCR products and measurement of gene expression with respect to a standard curve of the verified QPCR product. Gene expression was normalised as described in Vandesompele *et al.*[35] *EPSPS* and control gene primers are recorded in Table 1. The same cDNA as that used in the *EPSPS* sequencing described previously was used for expression analysis.

For *EPSPS* copy number analysis, parental genomic DNA used was the same as that used for *EPSPS* sequencing described previously, while F_2 progeny genomic DNA was extracted from 72 individual plants from each F_2 population as described previously. The gene copy number was determined by calculating the ratio of *EPSPS* to a control gene, *BdALS*. This was done by determining the amount of both genes with respect to the standard curves as used in expression analysis above. Dilution series of four concentrations for both QPCR products were prepared containing 10^7, 10^6, 10^5 and 10^4 copies μL^{-1}. A composite standard set was prepared for the two genes. They were combined to give a four-point standard curve for both genes but were assembled in opposite order, one gene from high copy number to low, the other from low to high. This was to ensure that the linearity of the two-gene assay standard curves could be assessed. The linearity was found to be satisfactory ($R^2 > 0.98$) for both standards. A Taqman-based assay with dual-labelled BHQ FRET probes (Bioresearch Technologies, Petaluma, CA) (Table 1) was used. The probes were designed with different fluorophores so the genes could be assayed independently in one QPCR reaction. QPCR reactions of 10 µL contained 5 µL of SsoFast Probe Supermix (Biorad, Gladesville, New South Wales, Australia), 1 µM of BdEPSPS and BdALS forward and reverse primers, 0.3 µM of BdEPSPS-FAM and BdALS-TET probe, and 2 µL of either DNA from the parent susceptible or resistant F_2 progeny or PCR product from the standard set. QPCR experiments were assembled by hand, in duplicate, and run on a RG3000 Rotor-Gene real-time thermal cycler with the following parameters: 3 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 16 s at 60 °C, acquiring at 510 nm (BdEPEPS-FAM) and at 555 nm (BdALS-TET).

Standard curves were used to calculate the amount of *EPSPS* and *ALS* in the parents and the F_2 population. The ratio of *EPSPS* to *ALS* was calculated for each QPCR, and the average and the standard deviation of the duplicate QPCRs were recorded for the parents and individuals in the population.

2.7 *EPSPS* cloning

A 1229 bp fragment of the *EPSPS* gene was amplified from one individual of BdSaR, BdVicR and BdS, using the same PCR protocol and cDNA as that for *EPSPS* gene sequencing and the primers presented in Table 1. Fragments were purified via gel extraction (QIAquick Gel Extraction kit; Qiagen, Valencia, CA, USA) before cloning using the Topo TA cloning kit (Life Technologies) according to the manufacturer's instructions. Colony PCR was performed to determine positive clones carrying the EPSPS fragment, using the

same PCR protocol as used for amplification, but replacing the template cDNA with a single clone colony and increasing the initial denaturing step to 10 min to aid cell lysis. Before addition into the PCR reaction, colonies were streaked onto standard LB/kan plates and plasmid DNA of positive clones: 26 from BdS, 52 from BdSaR and 44 from BdVicR, isolated (Qiagen Plasmid Mini kit) from the regrown streaked colonies. Plasmids were sequenced using the standard M13 vector primers.

The *EPSPS* sequences were aligned using AlignX from the Vector-NTI Suite 6 programs (Life Technologies) and single nucleotide polymorphisms (SNPs) identified. The SNP data were used to create a distance matrix based on the root-mean-square deviation (RMSD) coefficient using dendroUPGMA[36] and the phylogram viewed with Dendroscope.[37]

3 RESULTS

3.1 Response to glyphosate

The dose–response experiments confirmed glyphosate resistance in two populations of *B. diandrus*: BdSaR and BdVicR. Plants of the susceptible population BdS had 50% mortality due to glyphosate (LD_{50}) at 59 g a.e. ha^{-1}, while the LD_{50} values of the resistant populations BdSaR and BdVicR were 288 and 275 g a.e. ha^{-1} respectively, equating to 4.7–4.9-fold higher resistance than the susceptible population (Fig. 1). While both populations were resistant to glyphosate, they had relatively low levels of resistance. The highest glyphosate dose survived by any individual in BdSaR was 570 g a.e. ha^{-1}, and in BdVicR 428 g a.e. ha^{-1}. The amount of glyphosate required to control the susceptible population was also low, with complete control achieved with less than 150 g a.e. ha^{-1} glyphosate, well below the normal use rate of the herbicide in the field. The dose response experiments used healthy, well-watered plants maintained in shade houses, leading to high levels of glyphosate activity. In the field, where conditions are unlikely to be as optimal and plants would be under increased stress, glyphosate may be less effective, and modest levels of resistance would likely result in control failure.

3.2 *EPSPS* sequencing

To investigate the possibility of a resistance mechanism based on target-site mutation, a conserved region of the *EPSPS* gene covering the most common mutation site, proline106, was sequenced from genomic DNA and cDNA. A 422 bp fragment was amplified from genomic DNA of five resistant individuals that had survived 570 g a.e. glyphosate, from each of the two resistant populations, and their sequence was compared with that of the susceptible population. The same amplification using cDNA produced a 336 bp fragment owing to the presence of an intron in the genomic sequence. No nucleotide change that would lead to an amino acid substitution at proline106 was identified in the resistant individuals, nor anywhere else within the amplified fragment. While the possibility of a mutation somewhere else in the *EPSPS* gene cannot be ruled out, substitutions at proline106 are presently the only known naturally occurring mutations conferring glyphosate resistance, and therefore a resistance mechanism based on target-site mutation in *B. diandrus* is unlikely.

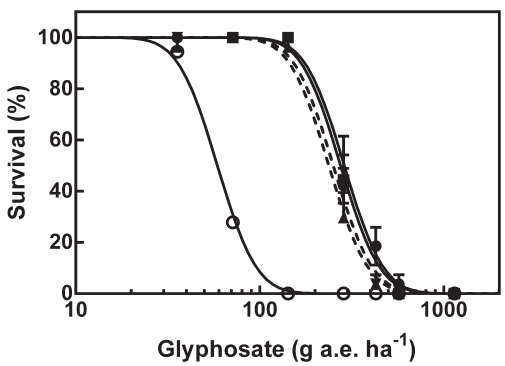

Figure 1. Response of susceptible (○), resistant BdSaR (●) and BdVicR (■) and two F_2 (F_2-1 ▲, F_2-2 ▼) populations of *B. diandrus* to glyphosate. Each point is the mean of three replicates ± SE. The lines are probit curves: BdS $y = -10.562 + 5.969x$ log(dose); BdSaR $y = -14.680 + 5.969x$ log(dose); BdVicR $y = -18.367 + 6.945x$ log(dose); F_2-1 $Y = -14.321 + 5.969x$ log(dose); F_2-2 $y = -14.178 + 5.969x$ log(dose), with probits back transformed to percentages.

3.3 Absorption, translocation and root extrusion of glyphosate

Absorption of glyphosate by leaves of resistant and susceptible plants of *B. diandrus* was examined using ^{14}C-glyphosate. The majority of absorption of ^{14}C-glyphosate occurred within 24 h after treatment (HAT). There was more ^{14}C-glyphosate absorbed in the second experiment with BdVicR (Fig. 2B) than in the first experiment with BdSaR (Fig. 2A); however, the amount absorbed by the susceptible plants was also proportionally increased, suggesting the increase is an artefact of experimental conditions. Maximum absorption was significantly higher in the susceptible population in both experiments, but the time to 90% absorption was not different (Table 2). However, this slightly decreased amount of glyphosate absorbed seems unlikely to explain completely the glyphosate resistance in the resistant populations.

In all populations, glyphosate rapidly translocated out of the treated leaves. By 24 HAT, less than 40% of the absorbed glyphosate remained in the treated leaves (Figs 2C and D). Over time, more glyphosate was translocated from the treated leaf in both experiments, resulting in a significant effect of time after treatment ($P = 0.0067$ in experiment 1; $P = 0.0001$ in experiment 2) on the amount of glyphosate retained in the treated leaf. There was also a difference between populations ($P = 0.0002$ in both experiments), with more translocation occurring in the resistant populations compared with the susceptible populations. Glyphosate was translocated to all other plant parts measured, but most notably to the roots. There was a significant interaction between population and time ($P = 0.031$) for translocation to the roots in experiment 2. In this experiment at 72 HAT there was more glyphosate present in roots of the resistant plants compared with susceptible plants (Fig. 2D). For the first experiment there was significantly greater ($P < 0.0001$) translocation of glyphosate to roots of the resistant plants (Fig. 2C). The translocation of glyphosate to the untreated leaf and shoot base material remained fairly constant over the 72 h ($P = 0.45$ and 0.77 for shoot base in experiments 1 and 2 respectively; $P = 0.14$ and 0.76 for untreated leaves in experiments 1 and 2 respectively). There were differences between populations where in susceptible plants more herbicide was translocated to the untreated leaves in experiment 1 ($P = 0.037$) and less herbicide to the shoot base in experiment 2 ($P = 0.011$). Other than translocation to the roots, the differences in translocation of glyphosate to other plant parts were not large.

In other weed species with reduced translocation as a mechanism of resistance, most glyphosate is retained at the site of application.[5,6,38] However, in *B. diandrus* there was less ^{14}C-glyphosate present in the treated leaf in the resistant

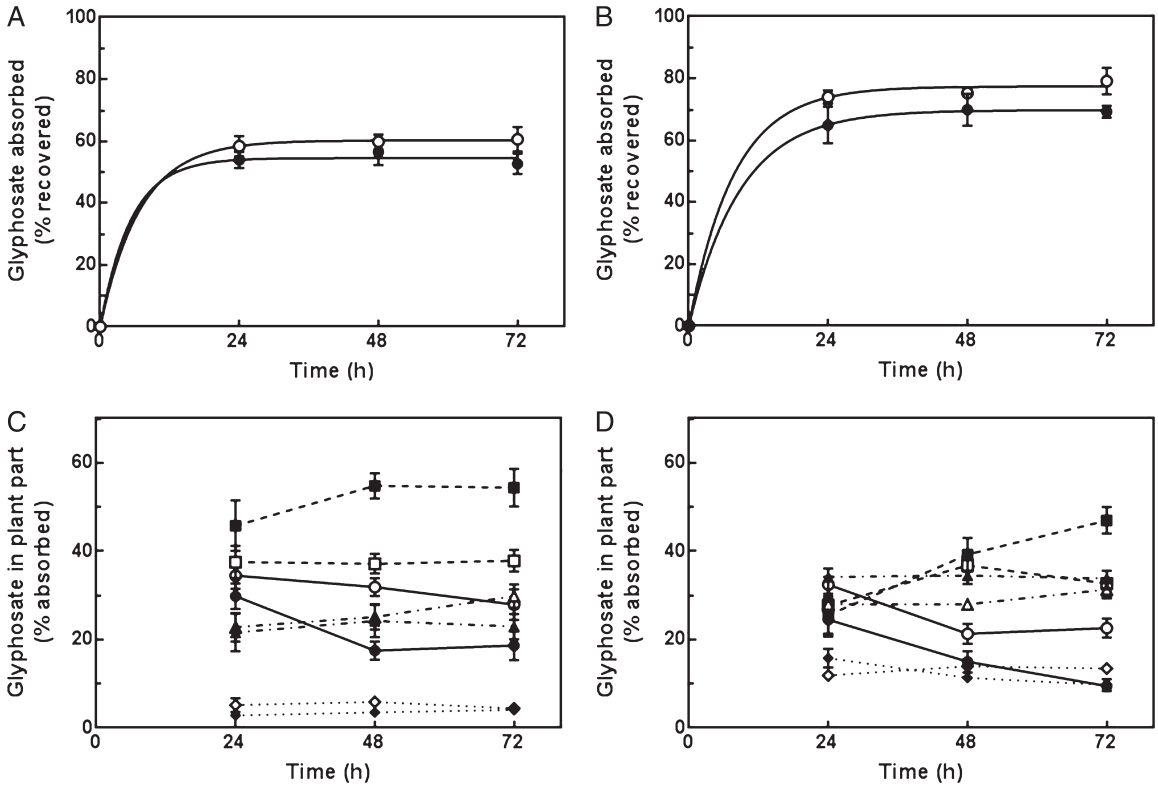

Figure 2. Percentage of ^{14}C-glyphosate absorbed by the resistant (●) populations (A) BdSaR and (B) BdVicR, compared with the susceptible BdS population (○), and the percentage of absorbed glyphosate present in the treated leaves (___) of susceptible (○) and resistant (●) populations, in the untreated leaves (. . .) of susceptible (◇) and resistant (◆) populations, in the shoot base (. _ .) of susceptible (△) and resistant (▲) populations and in the roots (_ _) of susceptible (□) and resistant (■) populations of (C) BdSaR and (D) BdVicR, 24, 48 and 72 h after treatment (HAT).

Table 2. Analysis of absorption data using asymptotic regression (Kniss *et al.*[33]), showing maximum absorption A_{max} and time to 90% absorption t_{90} (with standard errors)

Population	A_{max} (%) (± SE)	t_{90} (h) (+/− SE)
BdSaR	54.5(±2.06)	12.2 (7.65)
BdS	60.2(±1.97)	15.9 (3.53)
BdVicR	69.7(±2.65)	20.5 (3.38)
BdS	77.4(±1.78)	18.8 (2.16)

populations than in the susceptible populations. It may be possible that *B. diandrus* is resistant to glyphosate owing to altered translocation, and has a different translocation pattern to that of other species, namely increased translocation to the roots. Accumulation of glyphosate in the roots alone, however, would not explain resistance. Therefore, the possibility of glyphosate being exuded from the roots following translocation was investigated. Nutrient solution in which treated plants had been growing for up to 5 days after application of ^{14}C-glyphosate was tested for the presence of radiolabel. However, little to no radiolabelled glyphosate was found to be present (data not shown), suggesting that root extrusion of glyphosate was not occurring and that neither altered translocation nor root extrusion was the mechanism of resistance.

3.4 *EPSPS* copy number and expression

Gene amplification of *EPSPS* was assessed using QPCR. The susceptible individuals contained an average of 1.4 copies of *EPSPS* relative to the control gene acetolactate synthase (*ALS*), while the resistant individuals contained a much higher number of relative *EPSPS* copies, ranging from 10 to 36 (Table 3). The resistant population from South Australia (BdSaR) had a higher number of relative *EPSPS* copies than Victorian population (BdVicR), with an average of 29 and 13.5 copies respectively.

Table 3. *EPSPS* copy number relative to *ALS* copies, expression level and fold increase in expression in resistant compared with susceptible populations

Population		*EPSPS* copy number (*EPSPS*:*ALS*)	Expression level (copy number)	R/S expression ratio[a]
BdSaR	1	23.79	15 773	6.08
	2	27.70	10 476	4.04
	3	35.68	5227	2.01
BdVicR	1	9.85	7958	3.07
	2	14.57	22 881	8.81
	3	16.19	31 906	12.29
BdS	1	1.39	2748	
	2	1.45	2444	

[a] The average of the expression levels of two BdS individuals was used to calculate the R/S expression ratio.

EPSPS expression was also investigated using QPCR. The same primers as those employed for copy number analysis were used; however, expression level was compared with housekeeping control genes with constitutive expression. *EPSPS* expression was found to be increased in the resistant individuals, with expression

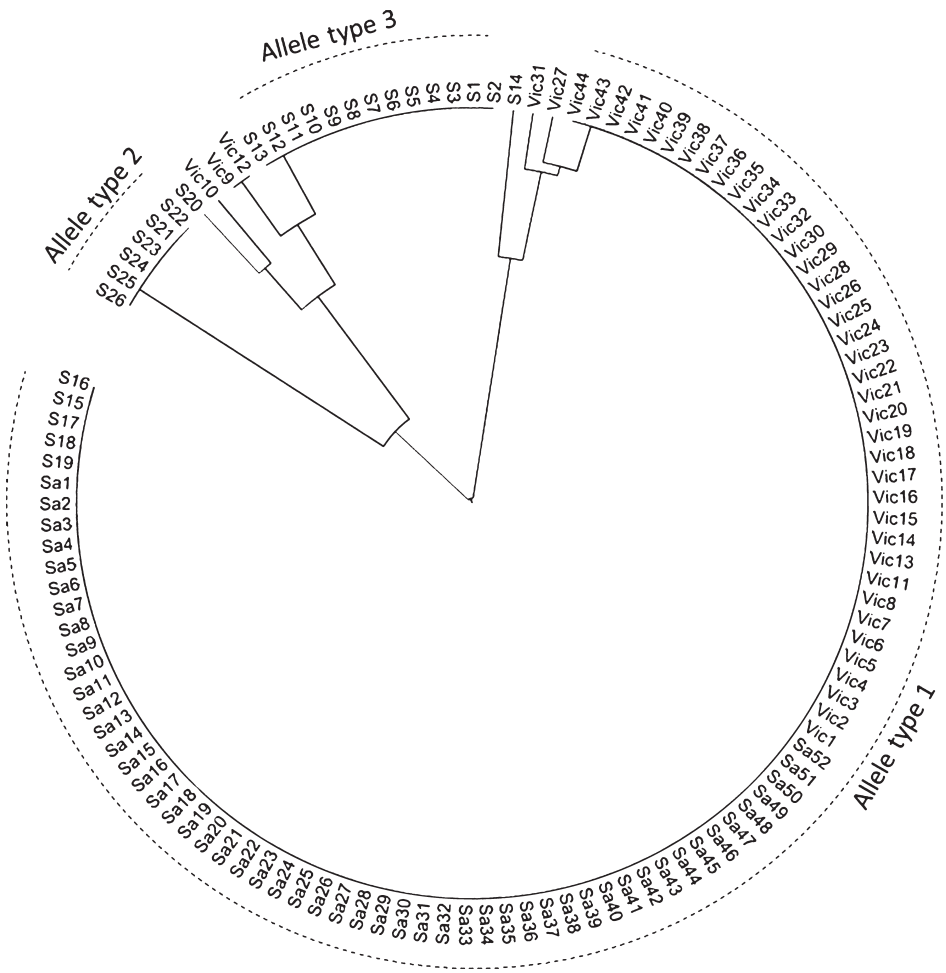

Figure 3. Circular phylogram based on polymorphic SNPs in *EPSPS* clones from BdS, BdSaR and BdVicR.

levels ranging from 2.0 to 12.3-fold that of the susceptible individuals (Table 3). However, this increased expression was not directly correlated with copy number. Lower-copy-number individuals displayed higher *EPSPS* expression levels than those with higher copy number, and BdVicR had higher average *EPSPS* expression levels than BdSaR, in spite of having lower copy numbers, suggesting that not all *EPSPS* copies may be being expressed.

3.5 *EPSPS* cloning

Cloning of expressed *EPSPS* RNA was used to determine the transcript profile of the *EPSPS* alleles. Using single nucleotide polymorphism (SNP) analysis, at least three different *EPSPS* alleles were detected in susceptible BdS. Two clones of BdS were not included in the three main groups (Fig. 3, Table 4). One clone only differed from allele type 1 by one SNP, suggesting that this may have just been a sequencing error; however, the second differed from the most closely related allele type, type 2, by nine SNPs, suggesting that it may represent a fourth allele type.

Only one *EPSPS* allele type was detected in resistant BdSaR, and this allele was the same as one of the three major alleles detected in BdS – allele type 1. This same allele was also present in a majority of BdVicR clones, suggesting that only one *EPSPS* locus was amplified in resistant individuals, and this allele was the same in both resistant populations. Two BdVicR clones only differed from the main allele type 1 by one SNP, and two differed from allele type 3 by two SNPs, again suggesting potential sequencing errors, or sequence differences due to the population being from a distant location and therefore potentially more genetically dissimilar, and indicating that they may likely belong to these allele-type groups. A fifth clone grouped with the ungrouped BdS clone, but differed by three SNPs, potentially supporting the hypothesis of a fourth allele type.

3.6 Inheritance of resistance

To determine whether the association between glyphosate resistance and increased *EPSPS* copy number was heritable, two F_2 populations (F_2-1 and F_2-2) were generated via hand-crossing of BdSaR and the susceptible BdS. Dose–response experiments were conducted on the F_2 progeny and the parental lines to investigate the inheritance pattern and level of dominance for glyphosate resistance. The dose response of the two F_2 populations was similar to that of the resistant parent and showed no obvious segregation for resistance (Fig. 4). At a rate of 142.5 g a.e. ha^{-1}, where all the susceptible plants were controlled by glyphosate, only one individual in the F_2-2 family was killed by glyphosate. The F_2 populations had a slightly decreased level of resistance to that of BdSaR, with LD_{50} values of 250 and 237 g a.e. ha^{-1} for F_2-1 and F_2-2 respectively. The segregation of glyphosate resistance at this herbicide rate fitted neither a one-gene ($G = 32.9$; $P < 0.001$) nor a two-gene ($G = 4.14$; $P = 0.04$) model.

The *EPSPS* copy number in 144 F_2 progeny was also assessed using QPCR, as in the parental lines, with quality copy number

Table 4. Groupings of identical *EPSPS* clones into allele type for 26 BdS (S1 to S26), 52 BdSaR (Sa1 to Sa52) and 44 BdVicR (Vic1 to Vic44) clones

	Allele type 1									Ungrouped					
BdS	BdSaR				BdVicR			Allele type 2	Allele type 3	1	2	3	4	5	6
S15	Sa1	Sa14	Sa27	Sa40	Vic1	Vic17	Vic32	S21	S1	Vic9	S20	Vic10	S14	Vic31	Vic27
S16	Sa2	Sa15	Sa28	Sa41	Vic2	Vic18	Vic33	S22	S2	Vic12					
S17	Sa3	Sa16	Sa29	Sa42	Vic3	Vic19	Vic34	S23	S3						
S18	Sa4	Sa17	Sa30	Sa43	Vic4	Vic20	Vic35	S24	S4						
S19	Sa5	Sa18	Sa31	Sa44	Vic5	Vic21	Vic36	S25	S5						
	Sa6	Sa19	Sa32	Sa45	Vic6	Vic22	Vic37	S26	S6						
	Sa7	Sa20	Sa33	Sa46	Vic7	Vic23	Vic38		S7						
	Sa8	Sa21	Sa34	Sa47	Vic8	Vic24	Vic39		S8						
	Sa9	Sa22	Sa35	Sa48	Vic11	Vic25	Vic40		S9						
	Sa10	Sa23	Sa36	Sa49	Vic13	Vic26	Vic41		S10						
	Sa11	Sa24	Sa37	Sa50	Vic14	Vic28	Vic42		S11						
	Sa12	Sa25	Sa38	Sa51	Vic15	Vic29	Vic43		S12						
	Sa13	Sa26	Sa39	Sa52	Vic16	Vic30	Vic44		S13						

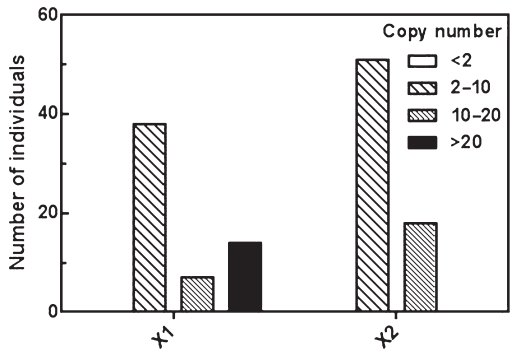

Figure 4. Number of individuals from each of two F_2 (F_2-1, F_2-2) populations of *B. diandrus* with different relative copy numbers of *EPSPS* compared with *ALS*.

data obtained for 59 individuals from F_2-1 and for 69 individuals from F_2-2. Every individual in the two F_2 populations contained more than threefold and up to 30-fold the number of copies of *EPSPS*. The F_2 populations tended to have lower numbers of copies of *EPSPS* compared with their resistant parents, although a small number were higher (Fig. 4).

4 DISCUSSION AND CONCLUSION

Glyphosate resistance in the two populations of *B. diandrus* appears to be conferred by *EPSPS* gene amplification and increased EPSPS expression. Glyphosate-resistant *B. diandrus* plants contained 10–36-fold more copies of *EPSPS* relative to susceptible plants, with the number of *EPSPS* copies found to be variable both between and within populations. This is consistent with findings from other species, with *A. palmeri* from Georgia found to have from five- to more than 160-fold the number of *EPSPS* copies of susceptible plants,[12] while plants of the same species from Carolina had a 22–63-fold increase[24] and from New Mexico only a 2–8-fold increase.[25] *EPSPS* gene amplification has also been identified in another *Amaranthus* species, *A. tuberculatus*, as well as in *K. scoparia*, at lower levels of 2–8- and 3–9-fold respectively.[13,16] The only grass weed species in which *EPSPS* gene amplification has been previously identified, *L. multiflorum*, was found to have a similar level of amplification to that seen here in *B. diandrus*, with resistant plants containing up to 25-fold the number of *EPSPS* copies than susceptible plants.[17]

B. diandrus populations from South Australia have been reported to be octaploid ($2n = 56$);[27] however, it appears that only one of the four *EPSPS* genes is amplified in the resistant populations. The same was found to occur in *A. palmeri* and *K. scoparia*, where resistance resulted from the amplification of only one of the two *EPSPS* loci.[16,39]

In *A. palmeri*, *EPSPS* copy number was found to be correlated with *EPSPS* expression level.[25,40] In *B. diandrus*, however, no strong correlation was observed. The BdSaR population with a higher average *EPSPS* copy number exhibited lower *EPSPS* expression levels than the BdVicR population with fewer average copies. This suggests that not all *EPSPS* copies may be being expressed, which could explain the lower level of resistance observed in *B. diandrus*, in spite of the relatively high *EPSPS* copy number. Glyphosate-resistant *B. diandrus* plants displayed only a low level resistance of around fivefold that of the susceptible plants, while resistance levels in other weed species with a similar amount of *EPSPS* gene amplification have been found to be higher.[17,24]

The lack of correlation between *EPSPS* gene copy number, *EPSPS* expression level and whole-plant resistance level could also indicate that *EPSPS* gene amplification may not be the sole mechanism of resistance present. In *L. multiflorum*, susceptible plants were found to contain increased copies of *EPSPS*.[17] However, neither a target-site mutation nor accumulation of glyphosate in the treated leaves was found to occur in the resistant *B. diandrus*, suggesting that *EPSPS* gene amplification is the major mechanism of resistance in both populations.

The inheritance pattern for the gene amplification mechanism has not been fully identified. In *A. palmeri*, F_2 populations segregate for both resistance and number of copies of *EPSPS*;[12] however, inheritance does not fit a single-gene pattern.[24,25] Variation in gene copy number in *A. palmeri* is high,[24,40] and this clearly affects the inheritance pattern. In *K. scoparia*, however, segregation of *EPSPS* copies followed single-locus inheritance. The different modes of inheritance in these two species may be due to the genomic organisation of the amplified *EPSPS* genes, with *EPSPS* copies dispersed randomly throughout the genome in *A. palmeri*,[12] while in *K. scoparia* they occur in tandem, localised on one pair of homologue chromosomes.[41] In *B. diandrus*, an unusual situation of no segregation in the F_2 was identified. Every F_2 individual tested was resistant to the herbicide, and all contained an elevated copy number for *EPSPS*. This suggests that, unlike the situation with glyphosate-resistant *K. scoparia*, the *EPSPS* copies in

B. diandrus are unlikely to be tandem repeats and more likely occur on several chromosomes. In spite of all individuals containing gene amplification of *EPSPS*, the F_2 populations were not as resistant as the parent population and on average contained fewer copies of *EPSPS*. This unusual inheritance pattern has some implications for management of this type of resistance. Firstly, it means that a high gene copy number will quickly move through populations, as all progeny from a cross with a susceptible plant will be resistant. This is less of an issue for a self-pollinated species, such as *B. diandrus*, but an outcrossing species with this type of resistance will prove problematic to manage.

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