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Non-target site mechanism of metribuzin tolerance in induced tolerant mutants of narrow-leafed lupin (*Lupinus angustifolius* L.)

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Abstract. Narrow-leafed lupin (*Lupinus angustifolius* L.) is an important grain legume crop in Australia. Metribuzin is an important herbicide used to control weeds in lupin crops. This study investigated metribuzin tolerance mechanism in narrow-leafed lupin by comparing two induced mutants (Tanjil-AZ-33 and Tanjil-AZ-55) of higher metribuzin tolerance with the susceptible wild type. Sequencing of the highly conserved region of the chloroplast *psb*A gene (target site) revealed that the sequences of the wild type and the mutants were identical and therefore metribuzin tolerance is not target site based. Photosynthetic activity was measured and the leaf photosynthesis of the two tolerant mutants was initially inhibited after metribuzin treatment, but recovered within 2.5 days whereas that of the susceptible plants remained inhibited. The photosynthetic measurements confirmed the target site chloroplast was susceptible and the tolerance mechanism is non-target site based. Investigation with known cytochrome P450 monooxygenase inhibitors (omethoate, malathion and phorate) showed that tolerance could be reversed in both mutants, indicating the tolerance mechanism in two tolerant mutants may involve cytochrome P450 enzymes. Interestingly, the inhibitor tridiphane reversed metribuzin tolerance of only one of the two tolerant mutants, indicating diversity in metribuzin tolerance mechanisms in narrow-leafed lupin. These results signify that further investigation of metribuzin metabolism in these plants is warranted. In conclusion, metribuzin tolerance mechanism in lupin mutants is non-target site based, likely involving P450-mediated metribuzin metabolism.

Additional keywords: cytochrome P450 inhibitor, *Lupinus angustifolius* L., metribuzin tolerance, photosynthesis, *psb*A gene sequence.

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Introduction

Weed control in world field crops is essential and mostly achieved with herbicides. Metribuzin, a triazinone herbicide, is used to control a range of monocot and dicot weeds in several crops (Simoneaux and Gould 2008). Metribuzin-tolerant cultivars in wheat (Kleemann and Gill 2007) and narrow-leafed lupin (*Lupinus angustifolius* L.) (Si *et al*. 2006) have been developed to allow selective use of metribuzin in these crops. Narrow-leafed lupin is grown on 400 000 ha in Australia and metribuzin is the main post-emergent herbicide used for selective control of the important yet difficult-to-control weed wild radish (*Raphanus raphanistrum* L.). However, lupin tolerance to metribuzin is marginal, therefore higher tolerance is preferred. A lupin mutagenesis program to improve metribuzin tolerance has yielded two mutants with higher tolerance than existing cultivars (Si *et al*. 2009). These mutants have been used as higher metribuzin tolerance sources in a lupin breeding program. However, the mechanism of metribuzin tolerance in these two mutants is unknown.

Plants can withstand herbicides due to target site-based (e.g. target site mutation or overexpression) or non-target site-based mechanisms (e.g. reduced herbicide uptake, translocation or enhanced metabolism) (Powles and Yu 2010). Metribuzin (asymmetrical triazine) inhibits photosynthesis at photosystem II (PSII) by competing with plastoquinone at the plastoquinone binding site on the D1 protein within the PSII complex. Specific point mutations in the target site chloroplast *psb*A gene encoding the D1 protein confer triazine resistance in many weeds (Powles and Yu 2010) and the triazine-tolerant canola crop (Reith and Straus 1987). It is known that *psb*A point mutations often result in a lower photosynthetic rate and fitness penalty (Vila-Aiub *et al*. 2009). Alternatively, metribuzin tolerance

can be non-target site based due to higher rates of metribuzin metabolism (Frear *et al*. 1983, 1985; Davis *et al*. 1991). Enhanced rates of herbicide metabolism, mediated by either cytochrome P450 monooxygenases (P450) or glutathione S-transferases (GST), are associated with metribuzin tolerance (Simoneaux and Gould 2008). P450 are a large family of enzymes that catalyse diverse reactions essential in many biosynthetic pathways and their involvement in herbicide metabolism in plants is known (Siminszky 2006; Powles and Yu 2010). Plant GST are another important enzyme family that catalyse the conjugation of glutathione or homoglutathione (in legumes) to various substrates to form a polar S-glutathionylated product (Yuan *et al*. 2006; Powles and Yu 2010). The present study investigates the basis of metribuzin tolerance in the lupin mutants.

Materials and methods

Plant materials

Two induced metribuzin-tolerant mutants Tanjil-AZ-33 and Tanjil-AZ-55 (hereinafter referred to as T) developed by Si *et al*. (2009) and the susceptible wild-type cv. Tanjil (S) were used in this study. Seedlings in 17.5 cm pots containing 5 kg standard potting soil were grown outdoors from September to November 2009 for P450 inhibitor experiments, or in a 20/12°C (day/night) glasshouse for the GST inhibitor experiment and net photosynthetic rate measurements. Plants were kept well watered and fertilised.

Genomic DNA extraction, PCR amplification and partial sequencing of psb*A gene*

A highly conserved region of the chloroplast *psb*A gene containing potential mutation sites was amplified, sequenced, and compared between the T and the S. Bulked shoot material obtained from the T and S genotypes without herbicide treatment was used for genomic DNA extraction using a Nucleon Phytopure DNA extraction kit (Amersham Biosciences, Piscataway, NJ, USA). A pair of primers was designed based on homologous regions of *psb*A sequences from *Arabidopsis* (GenBank accession number X79898.1), alfalfa (X04973.1), canola (M36720.1), soybean (X00152.1), and faba bean (X17694.2). The forward primer 5′-CGTGAGTGGGAACTTAGTTT-3′ and reverse primer 5′-TGAGCATTACGTTCATGCAT-3′ were used to amplify a 633-bp fragment encompassing the highly conserved region and potential resistance mutation sites of the *psb*A gene (Oettmeier 1999). The PCR was conducted in a 25-µL volume that consisted of ~300 ng of genomic DNA, 0.5 µM of each primer, and 12.5 µL of 2 × GoTag Green Master Mix (Promega, Madison, WI, USA). The PCR was run in a Mastercycler (Eppendorf, Hamburg, Germany) with the following profile: 94°C 4 min, 35 cycles of 94°C 30 s, 58°C 30 s, and 72°C 30 s, followed by a final extension step of 5 min at 72°C. The PCR product was purified from agarose gel with Wizard SV Gel and PCR Clean-up System (Promega) and sequenced from both ends with the AB-Big Dye Terminator system using a commercial sequencing service. Bulked DNA samples from each genotype were used for initial sequence analysis and subsequently three single plants from each genotype were analysed.

Photosynthesis measurement

Net photosynthetic rates were measured as carbon dioxide (CO_2) uptake on intact single leaflets of plants using a LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) before and after metribuzin treatments (0, 200, 400, and 800 g ha^{-1}) over a course of 6 days. Measurements occurred initially at 12 h after metribuzin treatment and then at 24-h intervals, and were always with the middle leaflet of the first- and second-treated leaf, at 1500 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by a red blue light source. Air containing 380 µmol mol^{-1} CO_2 was used at a flow rate of 500 µmol s^{-1}. Relative humidity of the air in the leaf chamber was controlled at 50–60% and leaf temperature at 22°C. The constant values of photosynthetic rate and intercellular CO_2 concentration of each sample leaf were recorded after 200 s. All measurements were performed between 0900 and 1200 hours.

Metribuzin treatment in combination with P450 inhibitors

Preliminary experiments established that the known P450 inhibitors malathion, omethoate and phorate when applied alone had no effect on lupin seedling growth or shoot dry weight at a rate of 1500, 1500, and 600 g ha^{-1}, respectively. These rates were therefore used to examine for interaction between P450 inhibitors and metribuzin. Tridiphane, an inhibitor for either GST (Gaul *et al*. 1995) or P450 pathways (Moreland *et al*. 1989), was selected as an inhibitor for this study at 50 g ha^{-1} based on the research of Gaul *et al*. (1995). Gaul *et al*. (1995) found that tridiphane inhibited the GST metabolic pathway for metribuzin in soybean. Seedlings of the T and S genotypes were treated at the four-leaf stage with the inhibitors 2 h before the metribuzin treatment (0, 200, 400, 800, 1600, 3200 g ha^{-1}). All inhibitor and metribuzin treatments were applied using a cabinet sprayer delivering 112 L ha^{-1} water at a pressure of 200 kPa. Plants were returned to the glasshouse in a completely randomised design with four replicates with 7–11 plants per replicate per herbicide treatment. Plant survival for each treatment was recorded 2 weeks after treatment.

Statistical analyses

Non-linear regression analysis was conducted for dose response experiments to estimate the herbicide rate causing 50% mortality (LD_{50}) of plants using SigmaPlot software (version 11.0, Chicago, IL, USA). Untransformed percentage data are presented in mortality dose response graphs. The data are fitted to the non-linear regression model (logistic four parameters):

$$Y = C + [(D - C)/(1 + (x/LD_{50})^{b})]$$

Where C is the lower limit, D is the upper limit, *b* is the slope and LD_{50} is the dose causing 50% mortality. Dose response curves are fitted with the logistic model or with an exponential decay model if the logistic model does not fit. The R^2 values for each LD_{50} were at least 0.999, indicating a very high degree of fit of the regression curves.

Results

PSII psb*A* gene sequencing revealed no known resistance endowing mutation

PCR with the aforementioned primer pair produced a single band of expected length of 633 bp in both the T and S genotypes. The nucleotide sequence of the clearly identified region (~620 bp) showed 96% homology with *psb*A genes from *Vicia faba*. This amplified sequence encompasses the known PSII resistance-conferring mutation sites (codon 211–266) of the chloroplast *psb*A gene. Sequence alignment revealed no difference between the two T mutants and the S wild type (data not shown). Thus the known mutations in the *psb*A gene conferring target site metribuzin resistance were not present in the two metribuzin-tolerant lupin mutants.

Photosynthetic rate revealed initial inhibition with recovery only in T mutants

In the absence of metribuzin, net photosynthetic rates measured as CO_2 uptake for the T mutants and S wild type were the same with values ~35 µmol CO_2 m^{-2} s^{-1} (Fig. 1). However, large differences between T and S plants were evident within 12 h of treatment with metribuzin, even at the lowest rate of 100 g ha^{-1} metribuzin (Fig. 1). Photosynthetic rates of the metribuzin-treated S plants had decreased to 2 µmol CO_2 m^{-2} s^{-1} and remained close to zero up to 5.5 days after treatment (Fig. 1*a*). Conversely, metribuzin treatment initially inhibited photosynthetic rate in the two T mutants but recovery commenced 1.5 days after treatment (Fig. 1*b*, *c*). At 100 g ha^{-1} metribuzin, by 2 days after treatment photosynthesis had fully recovered in the T mutants. The initial inhibition but then recovery in photosynthesis of the treated T mutants indicates a non-target site-based mechanism in metribuzin tolerance.

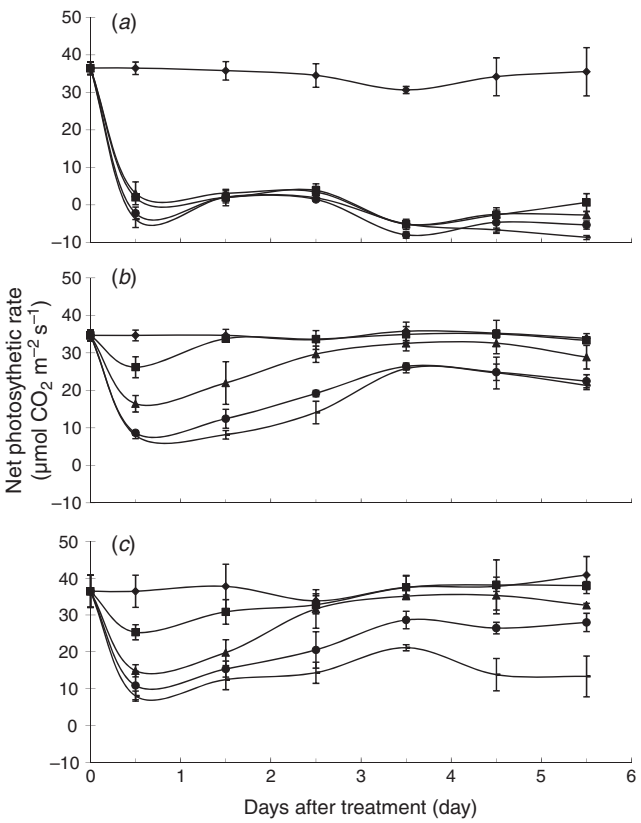

Fig. 1. Net photosynthetic rate of the treated leaves of narrow-leafed lupin at 0 (♦), 100 (■), 200 (▲), 400 (●) and 800 g ha^{-1} metribuzin (–); (*a*) the susceptible wild-type cv. Tanjil, (*b*) the tolerant mutant Tanjil-AZ-33 and (*c*) tolerant mutant Tanjil-AZ-55. Each data point represents the mean of three replicates and bars on the data points are standard errors.

Higher metribuzin rates had a more severe impact on photosynthesis with less CO_2 assimilation. At a high rate of 800 g ha^{-1} metribuzin, the two T mutants had only partially recovered by 5.5 days after treatment (Fig. 1*b*, *c*).

Chlorophyll contents were also measured for each point of the photosynthetic rates as above. No differences were observed between the T and the S at various rates of metribuzin (data not shown), confirming that metribuzin reduced photosynthetic rates, but did not cause chlorophyll destruction.

P450 inhibitors reversed tolerance in the two T mutants

The two T mutants were visually unaffected by metribuzin at 400 g ha^{-1} (Fig. 2*b*, *c*), whereas S plants were killed at this rate (Figs 2*a*, 3). However, when the known P450 inhibitor omethoate (1500 g ha^{-1}) was added to 400 g ha^{-1} metribuzin, only 5–10% T plants survived (Figs 2*b*, *c*, 3). The P450 inhibitor omethoate alone had no effects on lupin seedling growth (Fig. 3). Survival of the S plants was also reduced by the treatment of metribuzin plus omethoate. The reduction in metribuzin LD_{50} values in the presence of omethoate was 22.5-fold for T mutant Tanjil-AZ-33; 12.3-fold for T mutant Tanji-AZ-55 and 7.3-fold for the S Tanjil (Table 1). The metribuzin + omethoate treatment reduced the metribuzin LD_{50} of the T mutants to the level of the S plants in the absence of omethoate (Table 1). Hence, metribuzin plus the P450 inhibitor omethoate completely reversed metribuzin tolerance in the T mutants.

Similar results were obtained by the combination of metribuzin with either of two other P450 inhibitors, malathion or phorate (Table 1). Malathion increased metribuzin sensitivity of both the T and the S, with LD_{50} values decreased by 10-fold in the T Tanjil-AZ-33, by 5-fold in T Tanjil-AZ-55 and by 6-fold in the S Tanjil. Phorate also increased metribuzin sensitivity of the T. These consistent results of P450 inhibitor synergism with metribuzin indicate that metribuzin tolerance in the two T mutants is likely to be due to enhanced rates of metribuzin metabolism, perhaps mediated by enhanced cytochrome P450 enzymes.

Inhibitor tridiphane reversed tolerance in only one T mutant

Tridiphane when applied alone had no effect on plant survival of the T or the S lupins (Fig. 4). However, survival of the T Tanjil-AZ-55 decreased from 100 to 26% when treated with 400 g ha^{-1} metribuzin + 50 g ha^{-1} tridiphane (Fig. 4*c*). The survival of the other T mutant Tanjil-AZ-33 remained at ~90% regardless of metribuzin rates (200–800 g ha^{-1}) + tridiphane (Fig. 4*b*). The

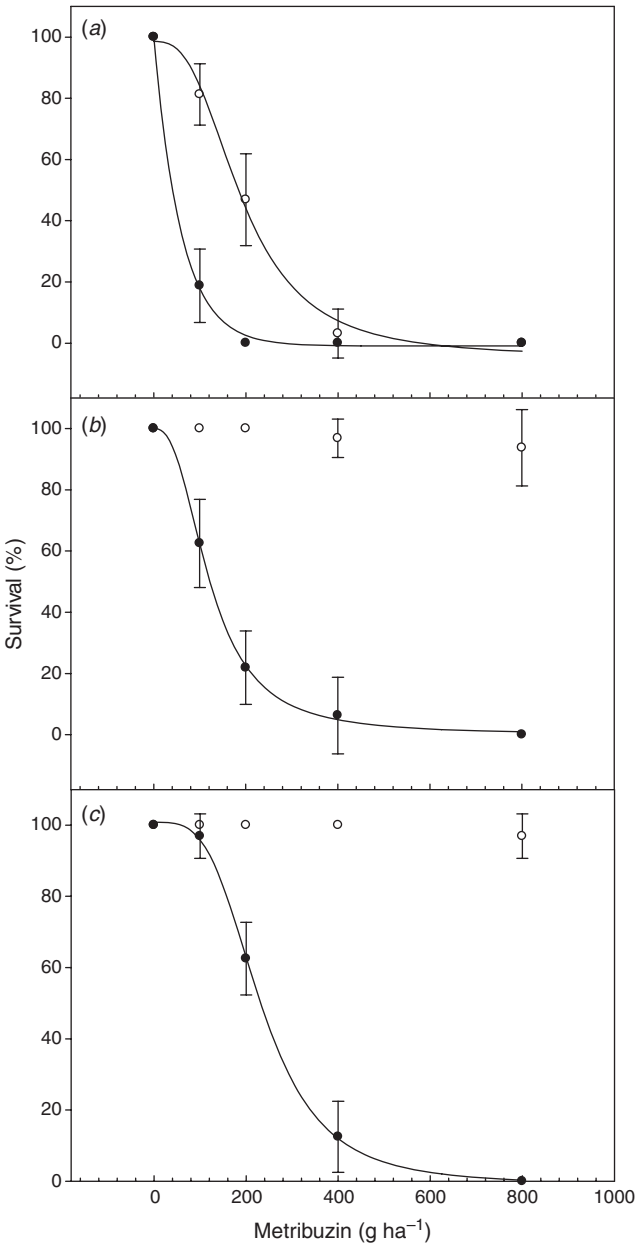

Fig. 2. Dose responses for survival (%) of (*a*) susceptible wild-type cv. Tanjil, (*b*) tolerant mutant Tanjil-AZ-33 and (*c*) tolerant mutant Tanjil-AZ-55 with a range of metribuzin doses plus (●) or minus (○) omethoate. Each data point represents the mean of four replicates and bars on the data points are standard errors.

metribuzin LD_{50} in the presence of tridiphane for Tanjil-AZ-55 decreased to 260 g ha^{-1}, lower than that for the S (435 g ha^{-1} in the absence of tridiphane). Therefore, metribuzin tolerance in T Tanjil-AZ-55 was clearly reversed by tridiphane. The different responses of the two T mutants to tridiphane indicate that different herbicide detoxifying enzymes/isoenzymes are likely involved in metribuzin tolerance in these two tolerant mutants.

Discussion

Metribuzin tolerance in lupin mutants is non-target site based

In plants, a range of herbicides, including triazines, triazinones, ureas and uracils, block the photosynthetic electron transport chain on the reducing side of PSII. Herbicides that target PSII compete with Q_B[1] for binding to the D1 protein of PSII and inhibit electron transport from Q_A to Q_B[1] by acting as non-reducible analogues of plastoquinone. The D1 protein is a subunit of the PSII core complex and is encoded by the chloroplast *psb*A gene. Mutations in positions 184, 219, 251, 255, 264 and 266 are known to confer resistance to metribuzin in weed species (Schwenger-Erger *et al*. 1993, 1999; Mengistu *et al*. 2000, 2005; Park and Mallory-Smith 2006). Triazine-tolerant canola has the *psb*A gene mutated in position 264 (Reith and Straus 1987). Because the *psb*A gene is encoded in the chloroplast genome, a characteristic of PSII target site resistance is its maternal inheritance. In the two metribuzin-tolerant lupin mutants studied here, we did not find any known mutations in the highly conserved *psb*A gene. Our inheritance study of the tolerant mutants reveals that metribuzin tolerance in the two lupin mutants (Tanjil-AZ-33 and Tanjil-AZ-55) is nuclear controlled (Si *et al*. 2011). Therefore, the metribuzin tolerance in the lupin mutants is not due to target site mutation in the chloroplast *psb*A gene. This result was further supported by leaf photosynthesis measurements. In comparing the metribuzin-tolerant T mutants with S wild type of lupin in the absence of the herbicide, there was no difference in leaf photosynthetic rates (Fig. 1). In addition, the initial inhibition after metribuzin treatment, but later recovery in photosynthetic rate of the T mutants suggests that the target site chloroplast is susceptible and the tolerance mechanism is non-target site based.

Cytochrome P450 likely involved in metribuzin tolerance

The ability of P450 inhibitors (omethoate, malathione and phorate) to reverse tolerance in two metribuzin tolerant lupin mutants suggests that the tolerance mechanism is likely to involve cytochrome P450 with enhanced rates of metribuzin metabolism (Table 1, Figs 2 and 3). Metribuzin tolerance in soybean and tomato is associated with higher rates of metribuzin metabolism (Frear *et al*. 1983, 1985; Davis *et al*. 1991). P450 inhibitors are a useful tool to indirectly establish the involvement of P450 in the metabolism of numerous herbicides (Siminszky 2006). The combination of the P450 inhibitor phorate with metribuzin was found to be lethal to soybean, whereas metribuzin or phorate alone had no effect (Waldrop and Banks 1983; Hammond 1986; Christianson 1991). Combination of phorate and clomazone also reduced metribuzin tolerance in cotton (Ferhatoglu *et al*. 2005). Synergistic interaction between malathion and the acetolactate synthase herbicide chlorsulfuron was observed in resistant annual ryegrass with enhanced rates of chlorsulfuron metabolism (Christopher *et al*. 1994). Our results with metribuzin-tolerant lupins suggest the involvement of P450 enzymes in metribuzin metabolism.

[1] Q_A and Q_B are the two sites for electron transfer during photosynthesis and they are key components of D1 protein in Photosystem II.

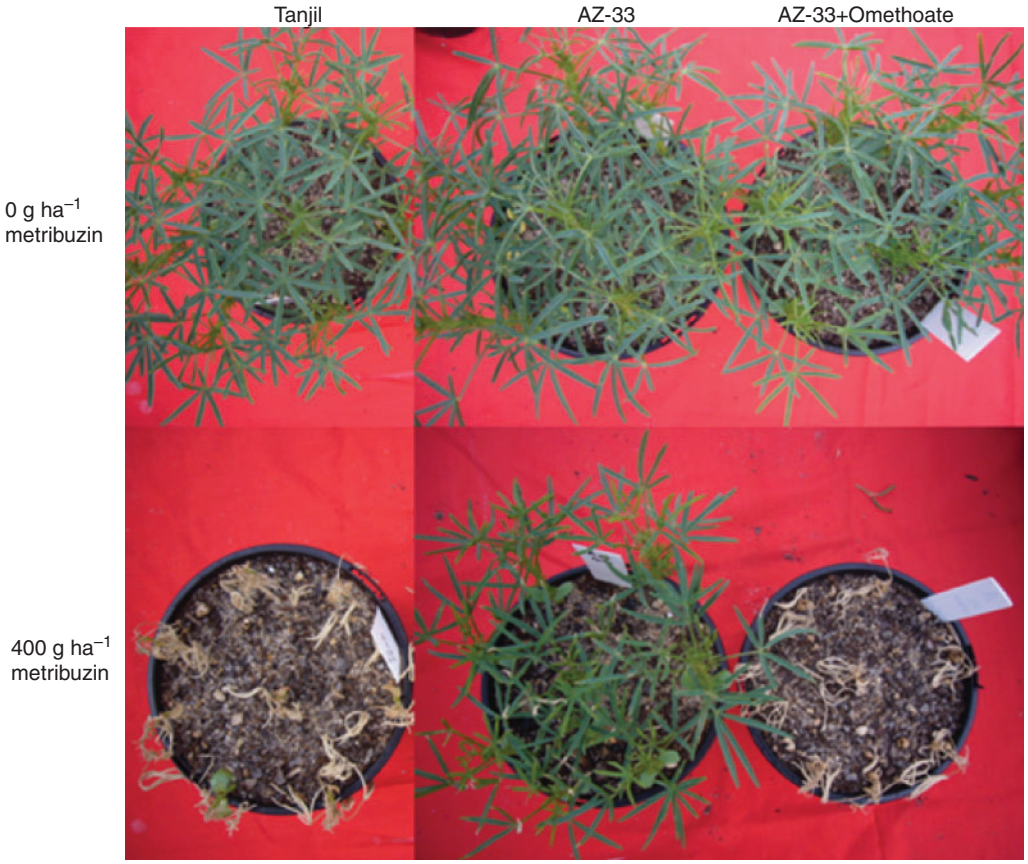

Fig. 3. Reversal of metribuzin tolerance in the tolerant mutant Tanjil-AZ-33 at 2 weeks after metribuzin treatment when P450 inhibitor omethoate was present. Left column: the susceptible Tanjil to 0, 400 g ha^{-1} metribuzin; central: the tolerant mutant Tanjil-AZ-33 to 0, 400 g ha^{-1} metribuzin; right: the tolerant mutant Tanjil-AZ-33 to 0, 400 g ha^{-1} metribuzin + 1500 g ha^{-1} omethoate.

Table 1. Metribuzin LD_{50} values (g ha^{-1}) (mean ± s.e.) of two tolerant mutants Tanjil-AZ-33 and Tanjil-AZ-55 and the susceptible wild-type cv. Tanjil of narrow-leafed lupin in the absence or presence of 1500 g ha^{-1} malathion, 1500 g ha^{-1} omethoate and 600 g ha^{-1} phorate

Genotypes	Metribuzin alone[A]	Metribuzin + malathion	Metribuzin + omethoate	Metribuzin + phorate
Tanjil-AZ-33	2748 ± 9.7	274.3 ± 0.2	122.1 ± 2.8	260.5 ± 35.9
Tanjil-AZ-55	2864 ± 7.1	571.7 ± 15.0	231.0 ± 3.6	320.6 ± 16.4
Tanjil	299.0 ± 5.5	46.4 ± 6.0	41.0 ± 0.0	220.6 ± 15.8

[A]Average of the three experiments.

Tridiphane is regarded as an inhibitor for GST-mediated herbicide metabolism of metribuzin and atrazine (Gaul *et al*. 1995). GST is the primary pathway responsible for atrazine tolerance in maize in which atrazine is glutathione-conjugated (Shimabukuro 1971). Equally, soybean tolerance to metribuzin can be associated with GST, due to the production of non-toxic homoglutathione conjugates (Frear *et al*. 1985; Brown and Neighbors 1987; Simoneaux and Gould 2008). Inhibition of the GST metabolic pathway for atrazine in maize and metribuzin in soybean has been demonstrated by application of the GST inhibitor tridiphane (Ezra *et al*. 1985; Gaul *et al*. 1995), although tridiphane also increases atrazine uptake by corn (Boydston and Slife 1986). Pretreatment of tridiphane followed by metribuzin reduces soybean tolerance and reduces metribuzin glutathione conjugation (Gaul *et al*. 1995). Our experiments showed metribuzin tolerance in one tolerant mutant Tanjil-AZ-55 is reversed by tridiphane (Fig. 4), indicating a possible involvement of GST-based metabolism in Tanjil-AZ-55. However, tridiphane also selectively inhibits cytochrome P450 isozymes (Moreland *et al*. 1989), and there is evidence that tridiphane inhibits P450 activities in wheat microsomes (Mougin *et al*. 1991). Therefore, it could also be possible that different P450 enzymes may be associated with tolerance in the two mutants. These indicative results signify

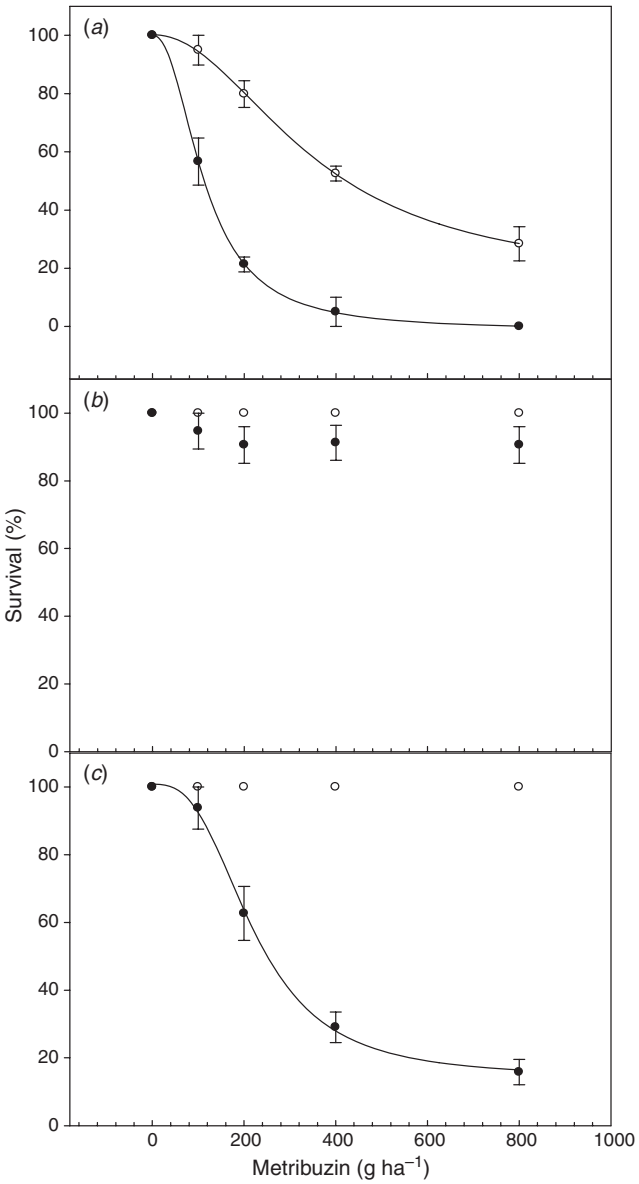

Fig. 4. Dose responses for survival (%) of (*a*) susceptible wild type cv. Tanjil, (*b*) tolerant mutant Tanjil-AZ-33 and (*c*) tolerant mutant Tanjil-AZ-55 with a range of metribuzin doses plus (●) or minus (○) tridipane. Each data point represents the mean of four replicates and bars on the data points are standard errors.

that further investigation of metribuzin metabolism in the two tolerant mutants is warranted.

Differences between the two mutants

Differences between the metribuzin-tolerant mutants Tanjil-AZ-33 and Tanjil-AZ-55 were observed in this study in terms of magnitude of tolerance reversal in response to P450 inhibitors omethoate and malathion and response to inhibitor tridiphane. Genetically, metribuzin tolerance in Tanjil-AZ-33 is controlled by the single nuclear gene *Mt3* and tolerance in Tanjil-AZ-55 by the *Mt5* gene (Si *et al*. 2011). These two genes are of non-allelic nature and of additive effects (Si *et al*. 2011). The consistent 2-fold difference in metribuzin LD_{50} values in the presence of malathion and omethoate between Tanjil-AZ-55 and Tanjil-AZ-33 suggest possible differences in tolerance mechanisms. The differences in their responses to the inhibitor tridiphane further support the interpretation. These indicative results from this study warrant further investigation of metribuzin metabolic pathway in the two tolerant mutants. It is likely for them to be complementary as plants containing the two tolerance genes are twice as tolerant as plants with only one tolerance gene.

In conclusion, the metribuzin tolerance mechanism in the two T lupin mutants is non-target site based, likely involving P450-mediated metribuzin metabolism. Lupin metribuzin tolerance is of different mechanism to the triazine-tolerant canola, which is target site based. The implication for plants with non-target site-based mechanism is that environmental factors may influence their tolerance levels, compared with that of target site-based mechanisms.

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