A novel amino acid substitution Ala-122-Tyr in ALS confers high-level and broad resistance across ALS-inhibiting herbicides

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Abstract

BACKGROUND: Wild radish, a problem weed worldwide, is a severe dicotyledonous weed in crops. In Australia, sustained reliance on ALS-inhibiting herbicides to control this species has led to the evolution of many resistant populations endowed by any of several ALS mutations. The molecular basis of ALS-inhibiting herbicide resistance in a novel resistant population was studied.

RESULTS: ALS gene sequencing revealed a previously unreported substitution of Tyr for Ala at amino acid position 122 in resistant individuals of a wild radish population (WARR30). A purified subpopulation individually homozygous for the Ala-122-Tyr mutation was generated and characterised in terms of its response to the different chemical classes of ALS-inhibiting herbicides. Whole-plant dose-response studies showed that the purified subpopulation was highly resistant to chlorsulfuron, metosulam and imazamox, with LD_{50} or GR_{50} R/S ratio of >1024, >512 and >137 respectively. The resistance to imazypyr was found to be relatively moderate (but still substantial), with LD_{50} and GR_{50} R/S ratios of >16 and >7.8 respectively. *In vitro* ALS activity assays showed that Ala-122-Tyr ALS was highly resistant to all tested ALS-inhibiting herbicides.

CONCLUSION: The molecular basis of ALS-inhibiting herbicide resistance in wild radish population WARR30 was identified to be due to an Ala-122-Tyr mutation in the ALS gene. This is the first report of an amino acid substitution at Ala-122 in the plant ALS that confers high-level and broad-spectrum resistance to ALS-inhibiting herbicides, a remarkable contrast to the known mutation Ala-122-Thr endowing resistance to imidazolinone herbicide.
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Keywords: acetolactate synthase (ALS); ALS-inhibiting herbicide; ALS resistance mutation; cross-resistance; wild radish (*Raphanus raphanistrum* L.)

1 INTRODUCTION

Acetolactate synthase (ALS) (EC 4.1.3.18), also known as ace-tohydroxy acid synthase (AHAS), is the first enzyme in the biosynthesis of the branched-chain amino acids (valine, leucine and isoleucine)[1] and is the target of many commercial herbicides. Herbicides from five structurally distinct chemical classes inhibit ALS activity: sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyl-benzoates (PBs) and sulfonyl-aminocarbonyl-triazolinones (SCTs).[2] Worldwide, biotypes of 112 weed species have evolved resistance to ALS-inhibiting herbicides (hereafter referred to as ALS herbicides).[3] In most cases where the underlying resistance mechanism has been investigated, resistance is due to point mutation(s) in the ALS gene reducing the sensitivity of the enzyme to herbicides.[2,4] At least 22 amino acid positions in the ALS genes of yeast, bacteria or plants have been identified where mutations confer ALS herbicide resistance (Gly-121, Ala-122, Met-124, Arg-142, Val-196, Pro-197, Arg-199, Ala-205, Phe-206, Lys-256, Met-351, His-352, Arg-373, Asp-375, Asp-376, Arg-377, Met-570, Val-571, Trp-574, Phe-578, Ser-653 and Gly-654).[2–8] In weed species with field-evolved ALS resistance, eight of these positions (those underlined

above) carry a total of 24 known resistance-endowing amino acid substitutions.[9]

Wild radish (*Raphanus raphanistrum* L.) infests large areas of the Australian cropping region[10] and is particularly problematic across the Western Australia (WA) grain belt. Across this region, the adoption of conservation cropping systems with minimal tillage has seen a high reliance on ALS herbicides, particularly of the SU class, for control of this genetically diverse weed. This has resulted in the rapid and widespread evolution of ALS resistance. Over the last decade, resistance to the SU herbicide

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chlorsulfuron has been documented as occurring in 54% of wild radish populations across a 10 million ha region.[11–13] Many of these populations displayed multiple resistance to herbicides such as 2,4-D amine, triazine or diflufenican with a different mode of action.[13,14]

The present authors' herbicide-resistance wild radish screening identified a population (WARR30) that is highly resistant to both SU and IMI herbicides. In this population, a new ALS-resistance-endowing amino acid substitution (Ala-122-Tyr) was identified. A subpopulation homozygous for this mutation was purified, and it was demonstrated that this mutation confers high-level and broad-spectrum resistance to ALS herbicides at both the whole-plant and enzyme level.

2 MATERIALS AND METHODS

2.1 Plant material

The ALS-herbicide-resistant (R) wild radish population (WARR30) originates from Yuna, Western Australia (28.34 °S, 115.01 °E). A well-characterised herbicide-susceptible (S) population (WARR7, referred to as S1)[14] and a second S population (WARR33, referred to as S2) collected at Green Hills, Western Australia (31.88 °S, 117.07 °E) served as controls.

2.2 Herbicide treatment and dose response

To release dormancy, seeds were germinated for 2 days on 0.6% agar-solidified water containing 1 µM karrikinolide.[15] Germinating seedlings were transplanted into commercial potting mix (ten plants per pot and four pots per herbicide treatment) and grown in a naturally lit glasshouse at the University of Western Australia, with regular watering and fertilisation. Seedlings at the 2–3-leaf stage were treated with commercial-formulation ALS herbicides (Table 1) plus adjuvant (0.2% v/v BS1000) using a cabinet sprayer with a spray volume of 118 L ha^{-1} at a pressure of 200 kPa. After treatment, the plants were returned to the glasshouse. Survival and dry weight of above-ground biomass were assessed 3 weeks after herbicide treatment.

2.3 ALS gene sequencing

DNA was extracted from bulked leaf material of untreated S plants from populations S1 and S2 (12 each, total 24) and from individual R plants (16) surviving imazamox treatment, according to Yu *et al.*[16] Primers to amplify and sequence wild radish ALS gene fragments

Table 2. Primers used for wild radish ALS gene sequencing

Primer	Sequence (5′–3′)
WR122F	TCTCCCGATACGCTCCCGACG
WR653R	TCAGTACTTAGTGCGACCATC
WR205R	GCAAGCTGCTGCTGAATATCC
WR376R	TTGCGAGTACTTTGATGGGG
WR574F	TTGTCATCATCAGGCCTTGGA

containing the five conserved regions (domains C, A, D, B and E)[17] were designed from alignments of plant ALS gene sequences from *Arabidopsis thaliana* (AY042 819), *R. raphanistrum* (AJ344986), *Brassica napus* (Z11 524) and *Lolium rigidum* (EF411171), using the software Oligo (v.6.31) (Table 2). PCR was conducted using the GoTaq Green Master Mix® kit (Promega) according to the manufacturer's instructions, with the following reaction profile: 94 °C for 4 min, then 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30–120 s, followed by a final extension step of 5 min at 72 °C. The primer pair WR122F/WR653R (Table 2) amplified a 1751 bp PCR product covering all five conserved domains and accounting for >90% of the full *Arabidopsis* ALS coding sequence and 100% of the conserved plant ALS sequences. The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega) and sequenced using additional primers (Table 2). DNA and deduced amino acid sequences from R and S plants were aligned and compared.

2.4 Generation of the purified resistant population

Seeds of the R and S populations were planted in plastic trays (50 seeds per tray) and grown as above. Seedlings at the 2–3-leaf stage were treated with the IMI herbicide imazamox at the field rate of 50 g ha^{-1}, and surviving plants were sampled for ALS gene sequencing. Nine resistant plants established as being homozygous for the Ala-122-Tyr mutation were isolated at the commencement of flowering, hand pollinated with each other (wild radish is an insect-pollinated species) and grown on for seed production. In this way, a purified population (referred to hereafter as WARR30-Tyr122) homozygous for the Ala-122-Tyr mutation was obtained. To confirm ALS resistance in the purified population, 50 seedlings from each of the S1, S2 and WARR30-Tyr122 populations at the 2–3-leaf stage were treated with either

Table 1. Herbicide application rates used in the dose–response study for susceptible and purified resistant wild radish populations

Herbicide	Field rate (g ha^{-1})	Application rate (g ha^{-1})	
		WARR7 (S1)	WARR30-Tyr122 (R)
SU			
Chlorsulfuron	20	0, 0.625, 1.25, 2.5, 5, 10, 20	0, 20, 40, 80, 160, 320, 640
Sulfometuron	15	0, 7.5, 15	0, 15, 60
TP			
Metosulam	6	0, 0.75, 1.5, 3, 6, 12, 24	0, 12, 24, 48, 96, 192, 384
Florasulam	10	0, 5, 10	0, 10, 40
IMI			
Imazamox	50	0, 6.25, 12.5, 25, 50, 100, 200	0, 50, 100, 200, 400, 800, 1600
Imazapyr	62	0, 62, 125, 250, 500, 1000	0, 125, 250, 500, 1000, 2000
Imazethapyr	72	0, 36, 72	0, 72, 288

20 g ha^{-1} chlorsulfuron or 50 g ha^{-1} imazamox. No S plants but all Tyr-122 plants survived both herbicides. Twelve individual plants from WARR30-Tyr122 were randomly selected for ALS sequencing, and all were found to be homozygous for the Ala-122-Tyr mutation.

2.5 ALS *in vitro* inhibition assay

Seedlings at the 3–4-leaf stage from the S1, S2 and WARR30-Tyr122 populations were used for *in vitro* assays of ALS enzyme activity. Leaf blades (not including the petiole) were harvested, snap frozen in liquid nitrogen and stored at −80 °C. The ALS assay was conducted according to Yu *et al.*[18], with modifications in the enzyme extraction procedure. Briefly, leaf tissue (4 g) was ground to a fine powder in liquid nitrogen with a mortar and pestle and further homogenised in 2.5 volumes of extraction buffer.[18] The homogenate was filtered through two layers of Miracloth and centrifuged at 20 000 × *g* for 15 min at 4 °C. The supernatant (10 mL) was brought to 50% $(NH_4)_2SO_4$ saturation and centrifuged at 20 000 × *g* for 20 min at 4 °C. The pellet was redissolved in reaction buffer[18] (6 mL), and the protein concentration in the extracts was determined using the Bradford method.[19] The Sephadex G25 column desalting step could be eliminated without effects on ALS activity and the Bradford assay under the present experimental conditions. ALS enzyme reactions were performed immediately and contained 320 µg of total protein, as pilot studies showed that this protein level catalysed a linear rate of acetoin formation over 60 min at 37 °C. For inhibition assays, technical-grade herbicides chlorsulfuron, sulfometuron, imazamox, imazapyr, metosulam and flumetsulam and the commercial formulation of imazethapyr (70% active ingredient) were used. The assay was repeated 2–3 times with independent extracts.

2.6 Statistics

For herbicide dose–response and ALS *in vitro* activity assays, data were expressed as the percentage of untreated controls and subjected to regression analysis using SigmaPlot 12.0. The LD$_{50}$ (herbicide rate causing 50% plant mortality), GR$_{50}$ (herbicide rate causing 50% growth reduction) and I$_{50}$ (herbicide concentration causing 50% inhibition of *in vitro* ALS activity) were estimated using two regression models, depending on which one fitted better, either the four-parameter log-logistic model

$$y = \frac{C + (D - C)}{1 + (x/I_{50})^b}$$

where *C* is the lower limit, *D* is the upper limit and *b* is the slope of the best-fitting curve through I$_{50}$ or LD$_{50}$, or the three-parameter exponential decay model

$$y = y_0 + ae^{-bx}$$

where Y_0 is the lower limit, $y_0 + a$ is the upper limit and *b* is the slope of the curve. The R/S ratios for LD$_{50}$, GR$_{50}$ and I$_{50}$ were calculated to indicate the level of resistance.

3 RESULTS

3.1 ALS gene sequencing revealed a novel Ala-122-Tyr mutation in R plants

A 1751 bp PCR fragment of the wild radish ALS gene covering the five highly conserved domains was amplified from 16
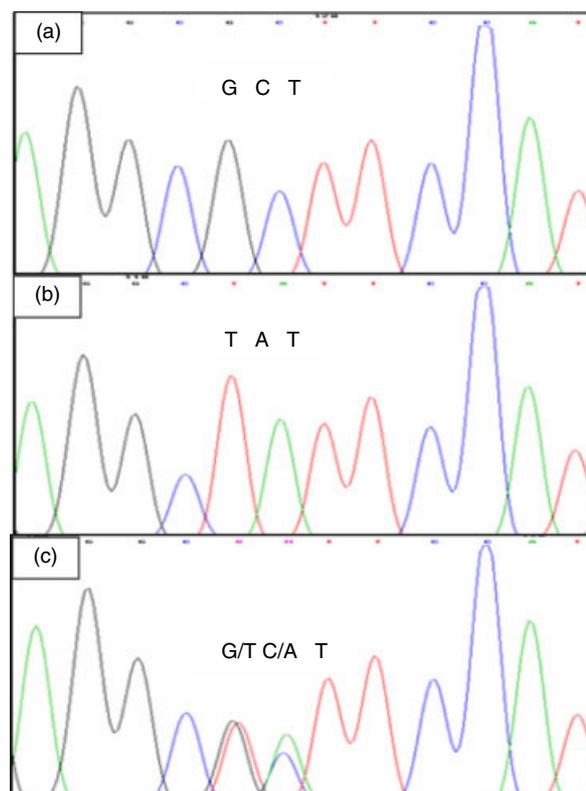

Figure 1. DNA sequencing result showing (a) the GCT codon for Ala-122 in homozygous susceptible plants, (b) the TAT codon for Tyr-122 in homozygous resistant plants and (c) the GCT/TAT codons for Ala/Tyr-122 in heterozygous resistant plants.

individuals of the R population (WARR30) that survived imazamox treatment, as well as from 12 plants (bulked) from each of two S populations. Comparison of the PCR fragment between R and S samples revealed 24 single nucleotide polymorphisms (SNPs) resulting in only two amino acid substitutions: a single nucleotide change of CCT to ACT, leading to a Pro-197-Thr substitution, and double nucleotide changes of GCT to TAT, causing an Ala-122-Tyr substitution (Figs 1a and b). Of the 16 R individuals sequenced, two individuals were homozygous (Fig. 1b) and eight individuals were heterozygous (Fig. 1c) for the Ala-122-Tyr mutation, one individual was homozygous for the Pro-197-Thr mutation and five individuals were heterozygous for both mutations (compound heterozygotes). The Pro-197-Thr mutation is a well-known mutation endowing ALS herbicide resistance and has been reported in many resistant weed species.[2] The Ala-122-Tyr mutation has not previously been reported in a plant species and very likely confers ALS herbicide resistance. Additional R plants were sequenced to identify individuals homozygous for the Ala-122-Tyr mutation, and, in total, nine such individuals were bulked to produce the purified R population (WARR30-Tyr122) specific for this mutation.

3.2 Whole-plant dose response revealing high-level and broad resistance of Tyr-122 plants to ALS herbicides

Plants homozygous for the Ala-122-Tyr mutation displayed high-level resistance to all SU, TP and IMI ALS herbicides examined, as outlined below.
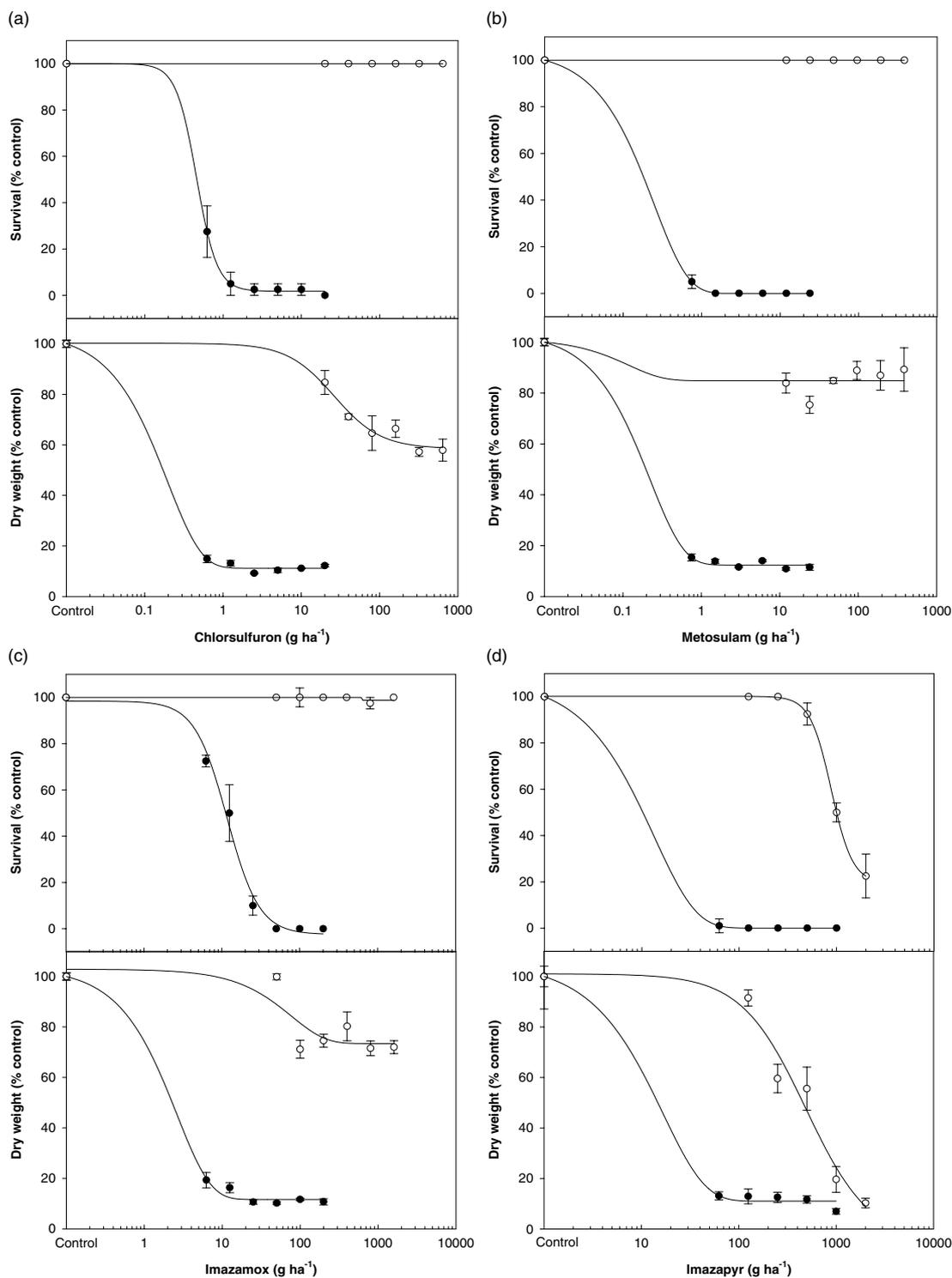
Figure 2. Effect of ALS herbicides (a, chlosulfuron; b, metosulam; c, imazamox; d, imazapyr) on survival (upper panel) and dry weight (lower panel) of purified R (WARR30-Tyr122, ○) and susceptible (S1, ●) wild radish populations. Plants were herbicide treated at the 2–3-leaf stage, and data were recorded 3 weeks after treatment. The 100% dry weight in controls in the absence of herbicide was 1.21 ± 0.009 and 1.28 ± 0.017 g plant^{-1} for S1 and R respectively. Data are means $\pm$ SE ($n = 4$).

3.2.1 The SU herbicide chlorsulfuron

At the upper limit of the normal chlorsulfuron field rate (20 g ha^{-1}), all Tyr-122 plants survived with only 15% growth reduction, and even at 32-fold the field rate (640 g ha^{-1}) no mortality was observed (Fig. 2a). In contrast, no S plants survived the field rate

and 72% of seedlings were controlled at the lowest rate used (0.625 g ha^{-1}), which is 32-fold lower than the field rate. Owing to the high-level resistance of the Tyr-122 plants and the extreme susceptibility of the S plants, the chlorsulfuron LD$_{50}$ or GR$_{50}$ values cannot be accurately determined, and therefore were estimated

Table 3. LD$_{50}$, GD$_{50}$ and I$_{50}$ values of the purified R population WARR30-Tyr122 (R) and the S populations (S1 and S2) for ALS-inhibiting herbicides, and the level of resistance indicated by the R/S ratios

Herbicide	LD$_{50}$ (g ha^{-1})			GR$_{50}$ (g ha^{-1})			I$_{50}$ (μM)		
	R	S1	R/S ratio	R	S1	R/S ratio	R	S1, S2 average	R/S ratio
SU									
Chlorsulfuron	>640	<0.625	>1024	>640	<0.625	>1024	>100	0.007 ± 0.002	>14 285
Sulfometuron							>100	0.023 ± 0.005	>4348
TP									
Metosulam	>384	<0.75	>512	>384	<0.75	>512	94	<0.001	>94 500
Flumetsulam							>100	1.658 ± 0.904	>60
IMI									
Imazamox	>1600	11.7	>137	>1600	<6.3	>256	>100	4.776 ± 0.617	>21
Imazapyr	1000	<63	>16	486	<63	>7.8	>100	6.838 ± 0.048	>14.6
Imazethapyr							>1000	1.353 ± 0.341	>741

by the highest (for R) or lowest (for S) rates used. Based on these calculations, the Ala-122-Tyr mutation confers >1024-fold resistance to chlorsulfuron (Table 3).

3.2.2 The TP herbicide metosulam

All Tyr-122 plants survived metosulam rates of up to 384 g ha^{-1}, 64-fold the field rate (6 g ha^{-1}), with only slight (10%) growth reduction (Fig. 2b). Conversely, no S plants survived the metosulam field rate, and even one-eighth of the field rate (0.75 g ha^{-1}) controlled 95% of the S plants (Fig. 2b). The estimated level of metosulam resistance endowed by the Ala-122-Tyr mutation was >512-fold (Table 3).

3.2.3 The IMI herbicides imazamox and imazapyr

Imazamox rates of up to 1600 g ha^{-1}, which is 32-fold the field rate (50 g ha^{-1}), did not control the Tyr-122 plants and inhibited growth by about 20% (Fig. 2c). In contrast, the field rate (62 g ha^{-1}) killed all S plants. The LD$_{50}$ and GR$_{50}$ R/S ratios indicated a >137–256-fold resistance to imazamox (Table 3). The level of resistance to imazapyr conferred by the Ala-Tyr-122 mutation was lower than that to imazamox (Fig. 2d) but still substantial (>7.8–16-fold). For example, the LD$_{50}$ for Tyr-122 plants was 1000 g ha^{-1}, which is 20-fold the field rate (50 g ha^{-1}) at which all S plants were killed (Table 3).

3.2.4 Additional ALS herbicides

The resistance of Tyr-122 plants to field rates (or higher) of additional SU (sulfometuron), TP (florasulam) and IMI (imazethapyr) herbicides (Table 1) was examined in comparison with the S plants. All Tyr-122 plants (50) survived each herbicide, while no S plants survived at any of the rates used (data not shown).

3.3 *In vitro* ALS activity and inhibition assays confirmed that Ala-122-Tyr mutation endows highly resistant ALS

In the absence of ALS herbicides, the extractable specific activity of Tyr-122 ALS (1.33 ± 0.03 μmol acetoin formed mg^{-1} protein h^{-1}) was found to be only slightly higher than that of the S populations (1.14 ± 0.06 and 1.24 ± 0.1 respectively for S1 and S2). However, in the presence of ALS herbicides, Tyr-122 ALS exhibited marked resistance to all representative ALS herbicides selected from three chemical classes.

ALS extracted from the two S populations was found to be similarly sensitive to all ALS herbicides selected, with herbicide I$_{50}$ values close to those reported for wild radish and other plant species.[18,20] Therefore, I$_{50}$ values for each herbicide from the two S populations were averaged for calculation of R/S ratios. In contrast, Tyr-122 ALS displayed dramatically reduced sensitivity to representatives of all three ALS herbicide classes, even at the highest herbicide concentrations used (Fig. 3), so that, as for the whole-plant LD$_{50}$ values, I$_{50}$ values for most of the herbicides tested could not be determined. Consistent with the whole-plant dose–response results, Tyr-122 ALS was highly resistant to the SU herbicides chlorsulfuron and sulfometuron (>14 285- and >4348-fold respectively) and to the TP herbicides metosulam and flumetsulam (>94 500- and >60-fold respectively). Tyr-122 ALS resistance to IMI herbicides varied from relatively moderately resistant to imazamox and imazapyr (>21- and >14.6-fold respectively) to highly resistant to imazethapyr (>741-fold). Therefore, it is evident that the Ala-122-Tyr mutation endows a highly resistant ALS, conferring broad resistance across ALS herbicides.

4 DISCUSSION

Previous studies have shown that ALS mutations at Pro-197 generally confer resistance to SU and TP herbicides, mutations at Ala-122, Ser-653 or Gly-654 endow resistance to the IMI group and mutations at Asp-376 or Trp-574 confer broad-spectrum resistance to many ALS herbicides (reviewed by Powles and Yu[2]). Resistance-endowing mutations at Pro-197, Trp-574 and Ser-653 have been found in biotypes of many resistant weed species,[9] whereas resistance mutations at the other positions are less common.[2] For example, the only known resistance-endowing mutation at Ala-122 (Ala-122-Thr) has been reported in just five weed species (*Xanthium strumarium*,[21] *Solanum ptycanthum*,[22] *Amaranthus retroflexus*,[17] *A. powellii*[17] and *A. hybridus*[23,24]) and confers high-level resistance to IMI but no resistance to SU and TP herbicides. The present study reports for the first time a double nucleotide mutation (GCT to TAT) in the plant ALS gene, resulting in Ala substitution by Tyr at position 122 and conferring high-level and broad-spectrum resistance across ALS herbicides.

Whole-plant dose–response studies and ALS *in vitro* enzyme activity assays using a purified population homozygous for this mutation have confirmed that the Ala-122-Tyr mutation bestows
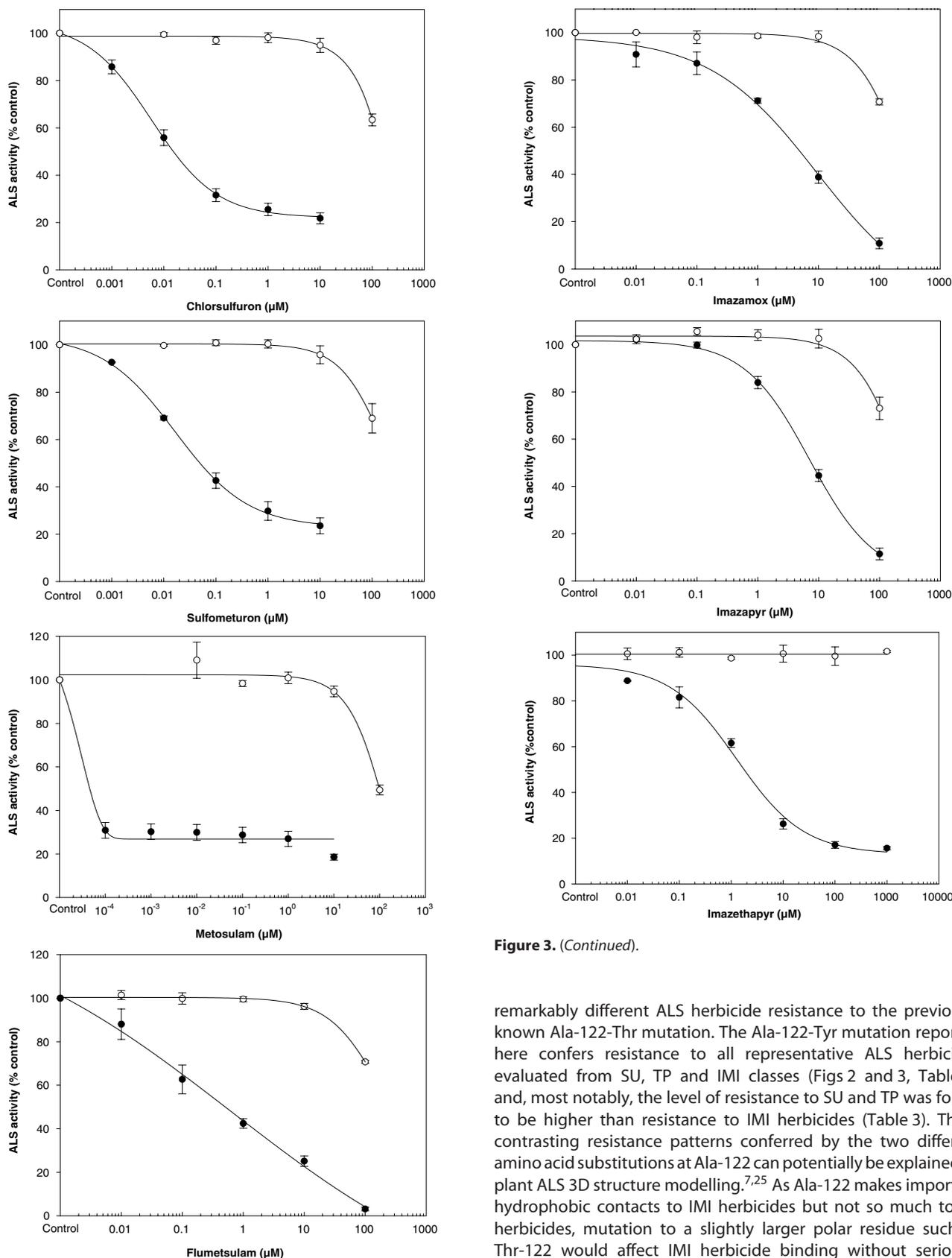
Figure 3. (*Continued*).

remarkably different ALS herbicide resistance to the previously known Ala-122-Thr mutation. The Ala-122-Tyr mutation reported here confers resistance to all representative ALS herbicides evaluated from SU, TP and IMI classes (Figs 2 and 3, Table 3), and, most notably, the level of resistance to SU and TP was found to be higher than resistance to IMI herbicides (Table 3). These contrasting resistance patterns conferred by the two different amino acid substitutions at Ala-122 can potentially be explained by plant ALS 3D structure modelling.[7,25] As Ala-122 makes important hydrophobic contacts to IMI herbicides but not so much to SU herbicides, mutation to a slightly larger polar residue such as Thr-122 would affect IMI herbicide binding without seriously compromising binding of SU; this is why the Ala-122-Thr mutation is commonly associated with IMI herbicide resistance. However, substitution with a very large aromatic residue such as Tyr-122 would impair both IMI and, particularly, SU binding, conferring

Figure 3. *In vitro* inhibition of ALS activity by ALS herbicides in the purified R (WARR30-Tyr122, ○) and susceptible (S1, ●) wild radish populations. ALS was extracted from 3–4-leaf stage plants. Data are means ± SE (*n* = 2 or 3).

resistance to both IMI and SU herbicides. In fact, a mutant yeast strain possessing the same Ala-122-Tyr mutation obtained via site-directed mutagenesis has been shown to be SU herbicide resistant.[26] This demonstrates that the cross-resistance pattern conferred by a given resistance mutation is dependent not only on the site of the amino acid substitution (such as at Pro-197 versus Ser-653) but also on the specific amino acid substitution.

Among the 24 resistance-endowing amino acid substitutions in plant ALS identified so far in field-evolved resistant weeds, only the Trp-574-Leu and the Asp-376-Glu mutation (and potentially the Arg-377-His[8]) have been shown to endow a broad-spectrum resistance across ALS herbicides (reviewed by Tranel and Wright[4] and by Powles and Yu[2]). Therefore, the Ala-122-Tyr substitution reported here is one of only a few broad-spectrum resistance-endowing mutations identified in plants. Moreover, the Ala-122-Tyr mutation confers high-level resistance without any major effect on growth (Fig. 2, Table 3). Research is currently under way in the authors' laboratory to evaluate properly the potential fitness costs associated with different ALS-resistance-endowing mutations in wild radish in the absence and presence of herbicide selection, using purified populations homozygous for each mutation.

Compared with other ALS resistance mutations (at Pro-197, Asp-376 and Trp-574) already identified in wild radish populations,[20,27,28] the Ala-122-Tyr mutation was less commonly identified in wild radish populations, likely owing to the fact that this mutation requires two nucleotide changes, while the others require only one nucleotide change, among other factors. Nevertheless, selection of several broad-spectrum resistance-endowing ALS gene mutations (122, 376 and 574) in wild radish populations (the present paper and Yu *et al*.[28]) reduces herbicide control options, having a significant negative impact on wild radish control, especially in IMI-tolerant Clearfield crops. As many wild radish populations have evolved multiple resistance to herbicides with other modes of action,[13,14] herbicide choices must be considered carefully within the context of integrated weed control practices that will acceptably lower selection pressures.

ACKNOWLEDGEMENTS

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