Research Article

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# ALS herbicide resistance mutations in *Raphanus raphanistrum*: evaluation of pleiotropic effects on vegetative growth and ALS activity

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## Abstract

**BACKGROUND: Gene mutations that endow herbicide resistance may cause pleiotropic effects on plant ecology and physiology. This paper reports on the effect of a number of known and novel target-site resistance mutations of the ALS gene (Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu or Trp-574-Leu) on vegetative growth traits of the weed *Raphanus raphanistrum*.**

**RESULTS: The results from a series of experiments have indicated that none of these ALS resistance mutations imposes negative pleiotropic effects on relative growth rate (RGR), photosynthesis and resource-competitive ability in *R. raphanistrum* plants. The absence of pleiotropic effects on plant growth occurs in spite of increased (Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu) and decreased (Trp-574-Leu) extractable ALS activity.**

**CONCLUSION: The absence of detrimental pleiotropic effects on plant growth associated with the ALS target-site resistance mutations reported here is a contributing factor in resistance alleles being at relatively high frequencies in ALS-herbicide-unselected *R. raphanistrum* populations.**


**Keywords:** adaptation; acetolactate synthase (ALS); ALS activity; mutations; resistance mechanism; resistance allele; target-site mutation

## 1 INTRODUCTION

The core of plant defence theory maintains that, depending on the environmental selective conditions, evolution favours plant functions and traits that maximise either growth or survival (i.e. resistance).[1–4] The evolution of herbicide resistance in plants provides an excellent model system to test the predicted growth–resistance trade-off.[5] Gene mutations endowing herbicide resistance involve a constitutively evolved plant defence mechanism that is expected to result in pleiotropic effects on plant fitness, which are manifested by the absence of herbicide selection.[5–7] An example of this is the reduced relative growth rate (RGR), net assimilation rate (NAR) and impaired ability to compete for resources of *Lolium rigidum* exhibiting cytochrome-P450-enhanced herbicide metabolism.[8,9]

In contrast to enhanced herbicide metabolism, in which herbicides are prevented from reaching their target (non-target-site resistance mechanism), herbicide resistance in plants can also be endowed by DNA mutations leading to single amino acid changes in herbicide target proteins that prevent effective herbicide binding (target-site resistance mechanism).[10] A single amino acid change in a herbicide target-site protein can cause detrimental pleiotropic effects on fitness traits at the whole-plant level.[11] A recent analysis, however, has shown that pleiotropic effects (i.e. resistance costs) associated with target-site herbicide resistance genes are difficult to predict and occur on a case-by-case basis.[5]

Acetolactate synthase (ALS) is a key plant enzyme responsible for the biosynthesis of branched-chain amino acids, and is also the target of many commercial ALS-inhibiting herbicides (hereafter referred to as ALS herbicides).[12] Evolved target-site ALS herbicide resistance is widespread in many weed species, but, in spite of the large number ($n = 22$) of known mutations endowing ALS herbicide resistance that have been reported hitherto, only a few limited attempts have been made to evaluate the expression and magnitude of resistance costs associated with ALS target-site herbicide resistance alleles.[13–16] A recent study in evolved ALS-herbicide-resistant *L. rigidum* populations has shown that various ALS resistance alleles in homozygous status had negligible effects on ALS kinetics or plant growth, and there was only a reduction in plant relative growth rate (RGR) if certain ALS kinetic parameters

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(e.g. substrate affinity and feedback inhibition) and specific activity were altered.[17]

*Raphanus raphanistrum* (wild radish) is a widespread, economically important dicot weed of Australian and global agriculture.[18] A large-scale random survey across 10 million ha of Australian crop land revealed very widespread and high-level evolved resistance to ALS herbicides in *R. raphanistrum*.[19] Many evolved ALS-herbicide-resistant *R. raphanistrum* populations exhibit diverse ALS gene mutations endowing target-site resistance.[10,20–22] Recently, the present authors identified in *R. raphanistrum* populations a previously unknown Ala-122-Tyr mutation that confers high-level and broad resistance to ALS herbicides,[21] as well as a number of known ALS gene resistance mutations (e.g. Pro-197-Ser, Asp-376-Glu, Trp-574-Leu).[22] In this study, *R. raphanistrum* populations with all plants individually homozygous for four specific ALS resistance mutations (Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu or Trp-574-Leu) were used. An investigation was conducted to establish whether these ALS resistance mutations leading to amino acid changes in the ALS translated into pleiotropic effects on plant vegetative growth or ALS activity.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

In order to minimise genetic background differences between genotypes, the assessment of pleiotropic effects of herbicide resistance mutations on fitness traits requires genetically well-characterised plants.[23] In this study, the effect of genetic background differences within each ALS *R. raphanistrum* resistant and susceptible population was largely eliminated after identification and selection of individuals homozygous (RR) for the ALS-resistant and wild-type mutations. Here, each *R. raphanistrum* genotype included all plants individually homozygous for the unique resistance mutation Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu or Trp-574-Leu. Furthermore, the inclusion of two ALS-susceptible genotypes was planned to minimise the effect of genetic background differences between ALS-resistant and ALS-susceptible genotypes.[5,23,24] This methodological approach enables the independent comparison of each ALS-resistant population versus both ALS-susceptible populations.

The effect of these ALS herbicide resistance mutations on plant growth was examined by determining RGR and its components NAR and LAR, as well as resource-competitive responses to wheat competition. The effect of the ALS resistance mutations on ALS activity was also evaluated.

Four ALS-herbicide-resistant (R) and two ALS-herbicide-susceptible (S) *R. raphanistrum* populations were collected from the northern cropping region of Western Australia (Table 1). The chosen wild-type populations represent two susceptible genetic backgrounds with a range of phenotypic variability that served as a comparison for the R populations.[23] A number of herbicides were used for phenotypic identification of R and S individuals from within each population.[22] Phenotypically identified R and S plants were sampled for genomic DNA extraction, followed by ALS gene sequencing and derived cleaved amplified polymorphic sequence [(d)CAPS] marker analysis. Briefly, primers were designed on the basis of plant ALS gene sequences of *Arabidopsis thaliana* (AY042819), *R. raphanistrum* (AJ344986) and *Brassica napus* (Z11524) to amplify the regions containing all potential resistance-endowing ALS gene mutation sites.[22] PCR products were purified from agarose gel and directly sequenced. ALS DNA and deduced amino acid sequences from R and S plants were aligned and compared. The total amplified fragment length covers >90% of the *Arabidopsis* full ALS coding sequence (excluding the transit peptide sequence) and 100% of the conserved coding sequence across plant species. These molecular tools enabled the identification of plants homozygous for the wild-type (susceptible) and resistance ALS mutations Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu or Trp-574-Leu.[21,22]

At least five plants identified as susceptible or homozygous for each ALS resistance mutation were isolated and cross-pollinated manually, and seed was obtained. In this way, purified subpopulations were obtained, grown under the same environmental conditions and each possessing all homozygous individuals with only a specific ALS resistance (Tyr-122, Ser-197, Glu-376 or Leu-574) or susceptible allele (referred to as wild-type $S_1$ and $S_2$).[21,22] Twelve progeny plants were randomly selected from within each subpopulation for ALS sequencing/marker analysis, and all were found to be homozygous for a specific ALS-resistance-endowing allele. These purified ALS-herbicide-resistant subpopulations were tested and found to be susceptible to herbicides of other modes of action (data not shown).

### 2.2 Seed germination and growth

Seeds of the purified R and S subpopulations were weighed so as to ensure no difference in size, then germinated on water-solidified agar (0.6% w/v) at 25/15 °C in the dark for 48 h. To overcome seed dormancy, 1 $\mu$M of karrikinolide was incorporated into the agar medium. Germinating seedlings of similar size were transplanted into plastic pots (see below) containing a mix of 25% moss peat, 25% river sand and 50% mulched pine bark.

### 2.3 Evaluation of growth traits

Experiments were designed to estimate RGR and its components NAR and LAR associated with ALS R and S subpopulations endowed with different ALS gene mutations. Growth assessments were performed with both isolated individuals (physiological resistance cost) and plants growing in a competitive resource-limited environment imposed by a range of wheat (*Triticum aestivum*) crop densities (ecological resistance cost). Estimations of RGR and its components involved a classic[25] and combined[26] growth analysis. A target–neighbourhood experimental design[27] enabled the analysis of resource-competitive responses of ALS R and S individuals under crop competition.[9,28] All growth traits were evaluated within 40 days of vegetative growth in an outdoor experimental garden during the normal growing season for *R. raphanistrum* in the Southern Hemisphere (April–September).

#### *2.3.1 Classic growth analysis approach*

Uniform-size seedlings were transplanted into large pots (one plant per pot, 15 cm diameter × 15 cm height) containing the same soil substrate as above. Pots were regularly fertilised, irrigated, periodically rearranged to randomise environmental differences and spatially distributed to avoid light competition. Individual above-ground dry biomass and leaf area were assessed by harvesting plants at 15 and 29 days after transplanting (DAT). Leaf area was determined with a digital leaf area meter (LI-3100, LI-COR; John Morris Scientific Pty Ltd) at each harvest time. Harvested plants (7–8-leaf stage) were then oven dried for 72 h at 65 °C. Each experimental treatment consisted of 25 replicate plants.

Photosynthesis rates (μmol $CO_2$ $m^{-2}$ $s^{-1}$) were estimated at 17 and 26 DAT (LI-COR 6400; LI-COR® Biosciences, Lincoln, NE).

**Table 1.** Geographical locations of field collected ALS-herbicide-resistant (R) and ALS-herbicide-susceptible (S) *Raphanus raphanistrum* populations and purified subpopulations homozygous for various ALS resistance mutations/alleles

| Original population | Geographical location of collected populations | Purified subpopulations homozygous for specific ALS resistance mutation | ALS resistance allele |
|---|---|---|---|
| WARR7 ($S_1$) | Yuna (28° 20.4′ S, 115° 0.6′ E) | Wild type | $S_1$ |
| WARR33 ($S_2$) | Green Hills (31° 52.8′ S, 117° 4.2′ E) | Wild type | $S_2$ |
| WARR30 (R) | Yuna (28° 20.4′ S, 115° 0.6′ E) | Ala-122-Tyr | Tyr-122 (R) |
| WARR32 (R) | Three Springs (29° 34.2′ S, 115° 28.2′ E) | Pro-197-Ser | Ser-197 (R) |
| WARR12 (R) | Nabawa (28° 30′ S, 115° 0.6′ E) | Asp-376-Glu | Glu-376 (R) |
| WARR31(R) | Tardun ( 28° 42.6′ S, 115° 4.2′ E) | Trp-574-Leu | Leu-574 (R) |

Photosynthesis was measured at photon flux densities of 1500 µmol $m^{-2}$ $s^{-1}$, with 400 ppm $CO_2$ at 20 °C. Each time, eight plants from each R and S subpopulation were randomly selected for measurement.

*2.3.1.1 Statistical analysis.* RGR, NAR and LAR were estimated for each R and S subpopulation over time. The unbiased formula proposed by Hoffmann and Poorter[29] was used to determine RGR. The variance ($V$) of RGR was estimated according to Venus and Causton.[30,31] One-way analysis of variance (ANOVA) with Dunnett's post-test ($\alpha = 5\%$) was performed to assess pairwise differences in growth (RGR, NAR, LAR) estimates between each ALS R subpopulation and both ALS S subpopulations (Graphpad Prism v.6.0; GraphPad Software, San Diego, CA).

*2.3.2 Combined growth analysis approach*

This methodology enables the comparison of time trends in classically derived RGR, NAR and LAR after data fitting to a polynomial model.[26] Seedlings from ALS R and S subpopulations were transplanted into pots (five plants per pot, 17 cm diameter × 17 cm height) containing the same substrate as described above. Plants were harvested at 9, 15, 20, 25, 30, 35 and 40 DAT (7–8-leaf stage), and there were four replicates per harvest. Individual plant leaf area and above-ground biomass were determined as described above. The experimental conditions were also as described above.

*2.3.2.1 Statistical analysis.* The mean values of RGR, NAR and LAR estimated for each harvest interval were fitted to a splined cubic polynomial model:[25,32]

$$y = y_0 + ax + bx^2 + cx^3$$

where $y$ represents the RGR, NAR or LAR of the plant, $x$ is time, $y_0$ is the $y$ value when $x = 0$ and $a$, $b$ and $c$ are the rates of increase in RGR, NAR and LAR at different harvest times. Positive and negative values of $a$, $b$ and $c$ indicate, respectively, increase and decrease in RGR, NAR and LAR.

### 2.4 Evaluation of resource-competitive responses

Using the neighbourhood competition experimental design,[28] target ALS R and S *R. raphanistrum* individuals were subjected to asymmetric competition from increasing neighbour wheat (*Triticum aestivum*) densities from 0 to 600 plants $m^{-2}$. *Raphanus raphanistrum* seeds were germinated as described above and transplanted into pots when wheat plants were at the 2–3-leaf stage. Experimental units were arranged in a completely randomised design using six replicates for the control treatment (no competition) and five replicates for all other target–neighbour combinations. Above-ground biomass of target (*R. raphanistrum*) and neighbour (wheat) plants was harvested 38 days after transplanting of the former. The biomass was oven dried for 72 h at 65 °C and then weighed. The leaf area of ALS R and S target plants was evaluated as above. Variations in photosynthetic active radiation (PAR) imposed by increasing wheat densities to target plants were estimated over time at the top of the canopy of target plants in each target–wheat combination (SKP 200; Skye instruments Ltd).

*2.4.1 Statistical analysis*

To standardise for differences in productivity, data for above-ground biomass and leaf area of target plants in the presence of neighbours were expressed as a percentage of dry matter production or leaf area in the absence of competition.[9,33] Per-unit-size and per-individual competitive responses were analysed using a hyperbolic non-linear model to describe the response of target plants to increasing biomass of neighbour wheat plants:[9,27,34]

$$y = a/(1 + bx)$$

where $y$ represents the biomass of the target plant at neighbour biomass $x$, $a$ is the biomass of the target plant in the absence of competitors (neighbours) ($x = 0$) and $b$ is the slope of the regression. Whereas steep slopes denote weak competitive responses,[35,36] the coefficient of determination ($R^2$) indicates the importance of resource competition relative to other factors affecting target-plant performance.[34] Estimates of parameter $b$ and corresponding standard errors were obtained after data for each ALS R (Tyr-122, Ser-197, Glu-376 or Leu-574) and S (wild-type $S_1$ and $S_2$) subpopulation had been fitted to the model by least-squares regression analysis using SigmaPlot software (v.12.0; Systat Software). The relative per-unit-size competitive responses of ALS R and S subpopulations were established after comparison of regression slopes ($b$ parameter) by one-way analysis of variance (ANOVA) (Graphpad Prism v.6.0; GraphPad Software, San Diego, CA).

### 2.5 ALS *in vitro* activity

Seedlings at the 3–4-leaf stage from the two susceptible and four resistant genotypes were used for *in vitro* assays of ALS enzyme activity. Leaf blades (4 g, not including the petiole) were harvested from each genotype, snap-frozen in liquid nitrogen and stored at −80 °C. The ALS assay was conducted according to Han *et al.*[21] The assay was repeated 3 times with independent extractions.

  

**Table 2.** Mean estimates of relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and leaf $CO_2$ assimilation rate of ALS-susceptible ($S_1$, $S_2$) and ALS-resistant (Tyr-122, Ser-197, Glu-376, Leu-574) *Raphanus raphanistrum* subpopulations. Different lower-case letters indicate significant differences in growth traits and photosynthesis after pairwise comparisons of each ALS-resistant with both ALS-susceptible subpopulations according to Dunnett's post-test ($\alpha = 5\%$). Values in parentheses denote SE of the mean

| | $S_1$ | $S_2$ | Tyr-122 | Ser-197 | Glu-376 | Leu-574 |
|---|---|---|---|---|---|---|
| **Growth traits** | | | | | | |
| RGR (mg $mg^{-1}$ $day^{-1}$) | 0.181 (0.004) abc | 0.174 (0.004) bc | 0.170 (0.004) c | 0.189 (0.003) ab | 0.193 (0.004) a | 0.179 (0.004) abc |
| NAR (mg $cm^{-2}$ $day^{-1}$) | 0.721 (0.019) b | 0.699 (0.018) b | 0.650 (0.018) b | 0.829 (0.017) a | 0.833 (0.018) a | 0.847 (0.026) a |
| LAR ($cm^2$ $mg^{-1}$) | 0.260 (0.007) a | 0.256 (0.005) ab | 0.267 (0.005) a | 0.231 (0.004) cd | 0.236 (0.005) bc | 0.213 (0.006) d |
| **Photosynthesis** (μmol $CO_2$ $m^{-2}$ $s^{-1}$) | | | | | | |
| 17 DAT* | 33.7 (1.3) b | 36.3 (0.6) ab | 33.5 (0.5) b | 36.5 (1.1) ab | 37.6 (0.8) a | 34.6 (0.8) ab |
| 26 DAT | 27.7 (1.0) a | 28.0 (0.7) a | 28.6 (0.9) a | 29.7 (1.9) a | 29.2 (0.8) a | 29.2 (1.0) a |

* DAT: days after transplanting (of seedlings).

# 3 RESULTS

## 3.1 Vegetative growth analysis

Growth analysis over 2 weeks (15–29 DAT), indicated that the two wild-type ALS-herbicide-susceptible *R. raphanistrum* subpopulations exhibited comparable RGR values (Table 2). None of the subpopulations homozygous for the various ALS resistance alleles showed consistent RGR differences to both S wild-type subpopulations (Table 2). Interestingly, plants homozygous for the Ser-197, Glu-376 or Leu-574 ALS resistance alleles showed consistently greater NAR values than individuals from both ALS S subpopulations (Table 2). As individuals carrying these ALS resistance alleles displayed similar RGR values to those of S plants, it was expected that the higher NAR would have been compensated by lower LAR, a result that highlights a different plant leaf architecture associated with these ALS resistance alleles (Table 2). It was also evident that the higher NAR associated with the particular ALS resistance alleles (Ser-197, Glu-376 or Leu-574) was not driven by higher photosynthesis rates (Table 2).

A second growth analysis involved a longer time period (4 weeks) that commenced 9 days post-transplanting (9–40 DAT). During this growth period, three growth phases were observed, evident in the overall negative and positive signs of the estimated polynomial parameters. All ALS R and S subpopulations displayed a similar RGR during the 9–20 DAT time period, followed by a linear RGR decrease phase (20–30 DAT), again in which no apparent RGR differences were observed (30–40 DAT) (Fig. 1). In spite of transient differences at particular growth phases, similar NAR and LAR time trends were also observed for ALS R and S populations (Fig. 1).

## 3.2 Analysis of resource-competitive responses

As expected, growth responses of ALS R and S target plants were significantly driven by the density and biomass increase of competing neighbouring wheat plants ($P < 0.0001$, all $R^2 \geq 0.90$) (Fig. 2, Table 3). After ca 40 days of competition, the number of leaves attained by ALS R and S target plants decreased in a similar manner from 8–9 leaves (control), then 6–7 (40 plants $m^{-2}$), 5–6 (100 plants $m^{-2}$), 4–5 (200 plants $m^{-2}$), 3–4 (480 plants $m^{-2}$) to 2–3 leaves (600 plants $m^{-2}$) under increasing wheat competition. Increasing competition from wheat plants greatly reduced PAR interception by target plants with increasing wheat plant densities (Fig. 3).

The two ALS S wild-type subpopulations showed contrasting abilities to grow under the effect of increasing wheat competition. Target individuals from the $S_1$ subpopulation exhibited a steeper slope (parameter *b*) and thus displayed a weaker competitive response than the $S_2$ subpopulation (Table 3, Fig. 2). This variation in the competitive response from the two ALS S wild-type subpopulations reflects genetic background variability and served as a comparison for the ALS R subpopulations. Analysis of biomass changes of ALS R target plants (Tyr-122, Ser-197, Glu-376) under increasing wheat competition revealed competitive responses falling within the range of the competitive responses of the two ALS S wild-type target plants (Figs 2a to c). Biomass changes of target plants with the Leu-574 resistance allele mirrored the response associated with the ALS $S_2$ wild-type subpopulation (Fig. 2d). Estimates of regression slopes for ALS R target-plant biomass showed no significant differences from estimates for ALS S wild-type target plants, indicating that ALS R and S target plants performed in a similar fashion to competition from neighbouring wheat crop plants. Leaf area changes exhibited by ALS R target plants under wheat competition followed a similar pattern to the changes observed in above-ground biomass (data not shown).

## 3.3 *In vitro* specific ALS activity

The extractable specific ALS activity of the two ALS S wild-type subpopulations was similar (Fig. 4). However, the ALS activity in plants homozygous for the resistance alleles Tyr-122, Ser-197 or Glu-376 was 1.5–1.9-fold greater than that of the ALS S subpopulations (Fig. 4). In contrast, in plants homozygous for the Leu-574 resistance allele, the ALS activity was about half that determined for the ALS S subpopulations.

# 4 DISCUSSION

## 4.1 No evidence of pleiotropic effects on plant growth but on ALS activity

The present study has sought to test for pleiotropic effects on plant growth and ALS activity associated with specific ALS gene herbicide resistance endowing mutations that have evolved in field wild populations of the weed *R. raphanistrum*. The studies were conducted with individuals each homozygous for one of the specific ALS gene mutations Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu or Trp-574-Leu. These ALS gene mutations individually endow resistance to ALS herbicides.

Changes in RGR, NAR and LAR usually lead to changes in plant establishment and resource use strategies and overall plant fitness.[37] While the plant growth analyses revealed transient differences for RGR, NAR and LAR traits among the ALS R versus S

  

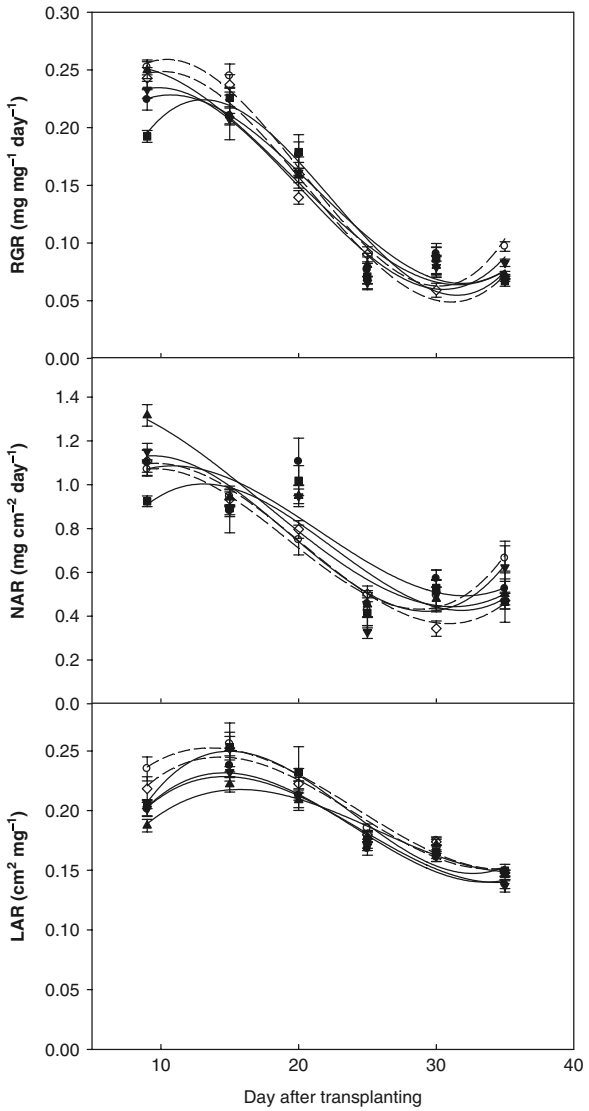


**Figure 1.** Changes in mean estimates of relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) over time for ALS-susceptible (broken line) (○, $S_1$; ◇, $S_2$), and ALS-resistant (solid line) (■, Tyr-122; ▼, Ser-197; ●, Glu-376; ▲, Leu-574) *R. raphanistrum* subpopulations. Derived RGR, NAR and LAR data were fitted to the splined cubic polynomial model $y = y_0 + ax + bx^2 + cx^3$.

subpopulations during the vegetative growth phase, there was no evidence of consistent impaired growth traits associated with any of these specific ALS resistance gene mutations (Table 2, Fig. 1). Equally, when experiments were conducted under high wheat competition, there were no significant differences in growth traits between R and S plants. When compared with two ALS wild-type herbicide-susceptible subpopulations, leaf area and above-ground biomass of R plants with these four resistance mutations were similarly inhibited by increasing competition from wheat plants (i.e. *b* parameter) (Table 3, Fig. 2). This result indicates similar competitive responses in S plants versus R plants with any of these four specific resistance mutations. Results also highlight the importance of using more than one susceptible genetic background in studies assessing resistance costs, as different and erroneous conclusions can be made when no other protocols are used to minimise genetic background differences among R and S genotypes.[23,24]

**Table 3.** Resource-competitive responses of ALS-resistant (Tyr-122, Ser-197, Glu-376, Leu-574) and ALS-susceptible ($S_1$, $S_2$) target *R. raphanistrum* subpopulations. Values represent the mean estimate of the slope (parameter *b*), derived from the hyperbolic regression $y = a/(1 + bx)$. Different lower-case letters indicate significant differences in regression parameter *b* after pairwise comparisons of each ALS-resistant with both ALS-susceptible subpopulations according to Dunnett's post-test ($\alpha = 5\%$). Values in parentheses denote SE of the mean

| Target subpopulation | *b* parameter | $R^2$ |
|---|---|---|
| $S_1$ | 0.028 (0.003) a | 0.97 |
| $S_2$ | 0.016 (0.002) b | 0.97 |
| Tyr-122 | 0.022 (0.004) ab | 0.93 |
| Ser-197 | 0.022 (0.003) ab | 0.96 |
| Glu-376 | 0.021 (0.004) ab | 0.90 |
| Leu-574 | 0.015 (0.002) b | 0.91 |

Current understanding suggests that resistance-conferring mutations in herbicide target enzymes not only limit herbicide binding but may also compromise normal plant function or metabolism by altering protein activity, regulation and kinetics.[5,10] A shortage, excess and/or imbalance of the herbicide target protein products can have pleiotropic effects on cell metabolism and correlate with diminished plant growth. Correlation of whole-plant pleiotropic effects with the degree of alteration of the ALS functionality has proved to be difficult. In the cases where pleiotropic effects have been detected in both weed and laboratory-derived ALS-gene-based herbicide-resistant plants, both increased ALS activity and decreased ALS activity have been reported.[5] In the present study, where pleiotropic effects have been shown to be negligible, both higher ALS activity (Pro-197-Ser, Ala-122-Tyr and Asp-376-Glu) and lower activity (Trp-574-Leu) were observed. The lower ALS activity associated with the Trp-574-Leu resistance mutation contrasts with the results of Yu *et al.*,[17] with a higher activity of the mutation in *L. rigidum*. In both cases, no evident pleiotropic effects on plant growth were estimated. The discrepancies between ALS activity and pleiotropic effects suggest that (1) the optimal plant range of tissue ALS activity level is relatively wide and/or (2) there exist unquantified *in vitro* (extractable) and *in vivo* differences in ALS activities.

### 4.2 Role of genetic background (Trp-574-Leu) and double nucleotide change (Ala-122-Tyr)

Of the 25 identified ALS-herbicide-resistance-endowing mutations of the ALS gene,[21,38] very few ALS gene resistance mutations have been assessed for associated pleiotropic effects in weed species.[5] A recent investigation by the present authors with homozygous resistant *L. rigidum* carrying the ALS Trp-574-Leu and various resistance mutations at ALS Pro-197 (including the Pro-197-Ser) has shown no associated detrimental effects on plant growth of these mutations.[17] However, in resistant populations of *Amaranthus powellii*, the Trp-574-Leu mutation has been shown significantly to alter leaf morphology and reduce vegetative and reproductive growth.[15] The discrepancy across the results may be related to the genetic background effects (i.e. plant species, resistance populations/biotypes and ALS gene sequences).[39]

The Ala-122-Tyr is an ALS gene resistance mutation that endows high-level and broad-spectrum resistance to ALS herbicides, yet this mutation must be rare because, in spite of many other mutations being reported[40] thus far, this mutation has only been

  

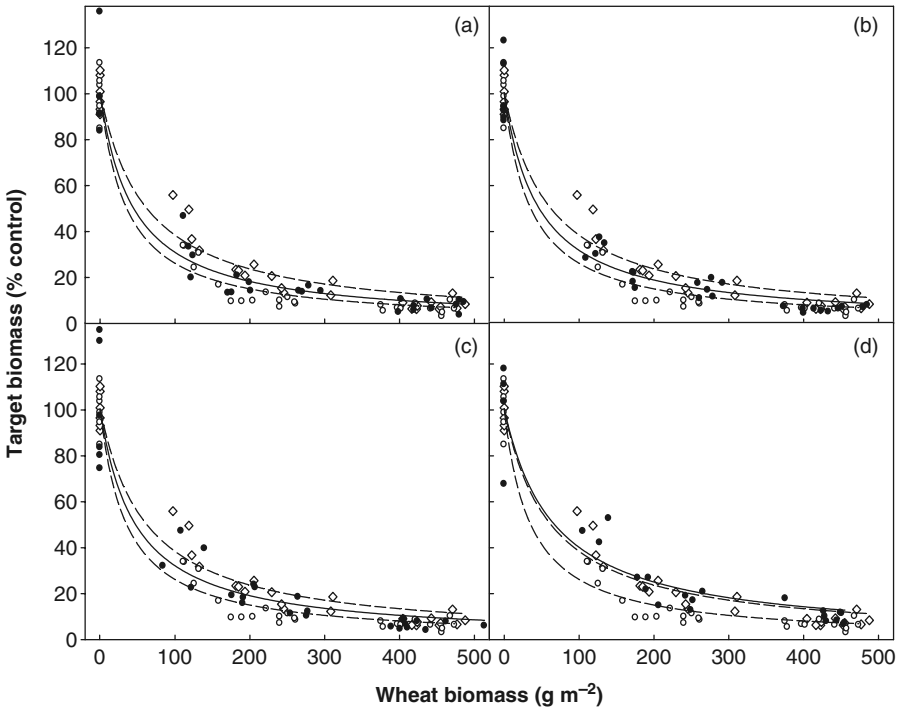


**Figure 2.** Above-ground biomass response of ALS-resistant (solid line) (•) (a, Tyr-122; b, Ser-197; c, Glu-376; d, Leu-574) and ALS-susceptible (broken line) (○, $S_1$; ◇, $S_2$) and target *R. raphanistrum* plants to increasing above-ground vegetative biomass of wheat neighbour plants. Broken and solid lines are predictive values derived from non-linear regression analysis [$y = a/(1 + bx)$] (parameters shown in Table 3).

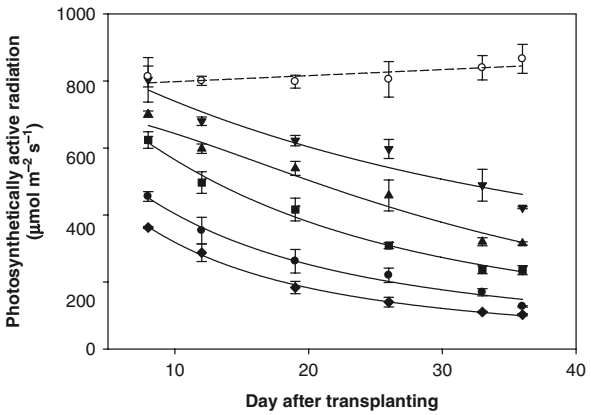


**Figure 3.** Photosynthetically active radiation (µmol m$^{-2}$ s$^{-1}$) intercepted by target ALS-herbicide-resistant and ALS-susceptible plants as a function of time (7–36 DAT) and increasing wheat crop densities (○, control; ▼, 40; ▲, 100; ■, 200; ●, 480; ◆, 600 plants m$^{-2}$).

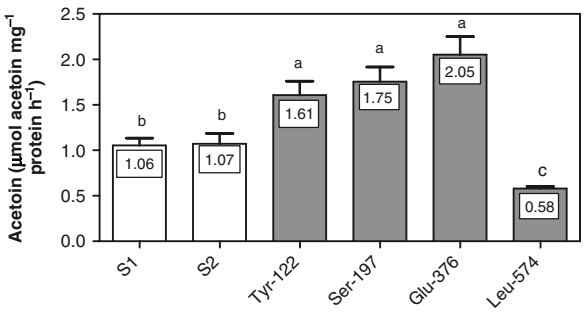


**Figure 4.** Extractable ALS activity measured from partially purified enzyme extracts of the ALS-resistant and ALS-susceptible *Raphanus raphanistrum* subpopulations. Values inside each column denote mean estimates of ALS specific activity. Columns ($n = 3$) with different lower-case letters indicate significant differences in ALS activity after pairwise comparisons of each ALS-resistant with both ALS-susceptible subpopulations according to Dunnett's post-test ($\alpha = 5\%$).

identified once.[21] While this novel resistance mutation may be rare, its rarity, as demonstrated here, is not the result of any associated impaired plant growth (Tables 2 and 3, Figs 1 and 2). Rather, it is most likely due to the probability of two nucleotide changes (either sequential or simultaneous) required for this mutation to occur, which contrasts with other ALS resistance mutations that require only one nucleotide change.[21] The low occurrence probability of mutations involving a double-nucleotide change in agricultural weeds is evident, as only one previous report has documented a double-nucleotide change (CCT→ATT) in the ALS gene, leading to Pro-197-Ile amino acid substitution.[41] No studies have been conducted to assess resistance costs of this other rare ALS herbicide resistance mutation.

### 4.3 ALS target-site herbicide resistance evolution

The results of the present study show that, either in the absence or in the presence of resource competition, the ALS gene resistance endowing mutations Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu and Trp-574-Leu do not impose negative pleiotropic effects on *R. raphanistrum* vegetative growth in isolated and competing plants.

Rapid and widespread evolution of ALS resistance in *R. raphanistrum* and other plant species selected for only a few generations with ALS herbicides is known.[19,40] The population frequency of ALS resistance alleles depends on their selective advantage or disadvantage (resistance cost) in, respectively, the presence and absence of ALS herbicide selection. The absence of detrimental effects on plant growth associated with the four ALS resistance mutations reported here would be a contributing

factor in resistance alleles being at relatively high frequencies in ALS-herbicide-unselected plant populations.[42] Provided that the observed lack of pleiotropic effects on plant growth and competitive ability are also evident in reproductive fitness traits, the ALS resistance mutations studied here are expected to persist at high frequencies upon removal of ALS herbicide selection and/or to be fixed in *R. raphanistrum* populations regardless of ALS herbicide selection. In spite of the absence of associated pleiotropic effects, the ALS Tyr-122 resistance allele is still expected to be a rare allele, given the relatively lower occurrence probability of the associated double-nucleotide mutation in the ALS gene.

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## REFERENCES

1 Bergelson J and Purrington CB, Surveying patterns in the cost of resistance in plants. *Am Nat* **148**:536–558 (1996).
2 Coustau C, Chevillon C and ffrench-Constant R, Resistance to xenobiotics and parasites: can we count the cost? *Trends Ecol Evol* **15**:378–383 (2000).
3 Herms DA and Mattson WJ, The dilemma of plants – to grow or defend. *Q Rev Biol* **67**:283–335 (1992).
4 Coley PD, Bryant JP and Chapin FS, Resource availability and plant antiherbivore defense. *Science* **230**:895–899 (1985).
5 Vila-Aiub MM, Neve P and Powles SB, Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytol* **184**:751–767 (2009).
6 Bergelson J, Purrington CB, Palm CJ and Lopez-Gutierrez JC, Costs of resistance: a test using transgenic *Arabidopsis thaliana*. *Proc R Soc Lond Ser B Biol Sci* **263**:1659–1663 (1996).
7 Purrington CB, Costs of resistance. *Curr Opin Plant Biol* **3**:305–308 (2000).
8 Vila-Aiub MM, Neve P and Powles SB, Resistance cost of a cytochrome P450 herbicide metabolism mechanism but not an ACCase target site mutation in a multiple resistant *Lolium rigidum* population. *New Phytol* **167**:787–796 (2005).
9 Vila-Aiub MM, Neve P and Powles SB, Evidence for an ecological cost of enhanced herbicide metabolism in *Lolium rigidum*. *J Ecol* **97**:772–780 (2009).
10 Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61**:317–347 (2010).
11 Holt JS and Thill DC, Growth and productivity of resistant plants, in *Herbicide Resistance in Plants Biology and Biochemistry*, ed. by Powles SB and Holtum JAM. Lewis Publishers, Boca Raton, FL, pp. 299–316 (1994).
12 Saari LL, Cotterman JC and Thill DC, Resistance to acetolactate synthase inhibiting herbicides, in *Herbicide Resistance in Plants, Biology and Biochemistry*, ed. by Powles SB and Holtum JAM. Lewis Publishers, Boca Raton, FL, pp. 141–170 (1994).
13 Dyer WE, Chee PW and Fay PK, Rapid germination of sulfonylurea-resistant *Kochia scoparia* l accessions is associated with elevated seed levels of branched-chain amino-acids. *Weed Sci* **41**:18–22 (1993).
14 Ashigh J and Tardif FJ, An amino acid substitution at position 205 of acetohydroxyacid synthase reduces fitness under optimal light in resistant populations of *Solanum ptychanthum*. *Weed Res* **49**:479–489 (2009).
15 Tardif FJ, Rajcan I and Costea M, A mutation in the herbicide target site acetohydroxyacid synthase produces morphological and structural alterations and reduces fitness in *Amaranthus powellii*. *New Phytol* **169**:251–264 (2006).
16 Alcocer-Ruthling M, Thill DC and Mallorysmith C, Monitoring the occurrence of sulfonylurea-resistant prickly lettuce (*Lactuca serriola*). *Weed Technol* **6**:437–440 (1992).
17 Yu Q, Han H, Vila-Aiub MM and Powles SB, AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth. *J Exp Bot* **61**:3925–3934 (2010).
18 Tamarin RH, *Principles of Genetics*. WCB/McGraw-Hill, New York, NY (1999).
19 Walsh MJ, Owen MJ and Powles SB, Frequency and distribution of herbicide resistance in *Raphanus raphanistrum* populations randomly collected across the Western Australian wheatbelt. *Weed Res* 542–550 (2007).
20 Yu Q, Zhang XQ, Hashem A, Walsh MJ and Powles SB, ALS gene proline (197) mutations confer ALS herbicide resistance in eight separated wild radish (*Raphanus raphanistrum*) populations. *Weed Sci* **51**:831–838 (2003).
21 Han H, Yu Q, Purba E, Li M, Walsh M and Powles SB, A novel amino acid substitution Ala-122-Tyr in ALS confers high-level and broad resistance across ALS-inhibiting herbicides. *Pest Manag Sci in press* (2012).
22 Yu Q, Heping H, Li M, Purba E, Walsh M and Powles SB, Resistance evaluation for herbicide resistance-endowing ALS gene mutations using *Raphanus raphanistrum* populations homozygous for specific ALS mutations. *Weed Res* **52**:178–186 (2012).
23 Vila-Aiub MM, Neve P and Roux F, A unified approach to the estimation and interpretation of resistance costs in plants. *Heredity* **107**:386–394 (2011).
24 Cousens RD, Gill GS and Speijers EJ, Comment: number of sample populations required to determine the effects of herbicide resistance on plant growth and fitness. *Weed Res* **37**:1–4 (1997).
25 Hunt R, *Plant Growth Curves. The Functional Approach to Plant Growth Analysis*. Edward Arnold, London, UK (1982).
26 Poorter H, Plant-growth analysis – towards a synthesis of the classical and the functional-approach. *Physiologia Plantarum* **75**:237–244 (1989).
27 Weiner J, A neighborhood model of annual-plant interference. *Ecology* **63**:1237–1241 (1982).
28 Goldberg DE, Components of resource competition in plant communities, in *Perspectives in Plant Competition*, ed. by Grace JB and Tilman D. Academic Press, San Diego, CA (1990).
29 Hoffmann WA and Poorter H, Avoiding bias in calculations of relative growth rate. *Ann Bot* **90**:37–42 (2002).
30 Venus JC and Causton DR, Plant-growth analysis – re-examination of the methods of calculation of relative growth and net assimilation rates without using fitted functions. *Ann Bot* **43**:633–638 (1979).
31 Causton DR and Venus JC, *The Biometry of Plant Growth*. Edward Arnold, London, UK (1981).
32 Hunt R and Evans GC, Classical data on the growth of maize: curve fitting with statistical analysis. *New Phytol* **86**:155–180 (1980).
33 Goldberg DE and Scheiner SM, ANOVA and ANCOVA. Field competition experiments, in *Design and Analysis of Ecological Experiments*, ed. by Scheiner SM and Gurevitch J. Oxford University Press, New York, NY, pp. 77–98 (2001).
34 Goldberg DE and Fleetwood L, Competitive effect and response in four annual plants. *J Ecol* **75**:1131–1143 (1987).
35 Goldberg DE and Werner PA, Equivalence of competitors in plant communities: a null hypothesis and a field experimental approach. *Am J Bot* **70**:1098–1104 (1983).
36 Goldberg DE, Neighborhood competition in an old-field plant community. *Ecology* **68**:1211–1223 (1987).
37 Grime JP, Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *Am Nat* **111**:1169–1194 (1977).
38 Tranel PJ, Wright TR and Heap IM, *ALS Mutations from Herbicide-resistant Weeds*. [Online]. Available: http://www.weedscience.org/mutations/MutDisplay.aspx Accessed [01 February 2012].
39 Paris M, Roux F, Berard A and Reboud X, The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in *Arabidopsis thaliana*. *Heredity* **101**:499–506 (2008).
40 Tranel PJ and Wright TR, Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci* **50**:700–712 (2002).
41 Boutsalis P, Karotam J and Powles SB, Molecular basis of resistance to acetolactate synthase-inhibiting herbicides in *Sisymbrium orientale* and *Brassica tournefortii*. *Pestic Sci* **55**:507–516 (1999).
42 Preston C and Powles SB, Evolution of herbicide resistance in weeds: initial frequency of target site-based resistance to acetolactate synthase-inhibiting herbicides in *Lolium rigidum*. *Heredity* **88**:8–13 (2002).

  