

ALS gene proline (197) mutations confer ALS herbicide resistance in eight separated wild radish (*Raphanus raphanistrum*) populations

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The biochemical and molecular basis of resistance to acetolactate synthase (ALS)–inhibiting herbicides was investigated in eight resistant (R) and three susceptible (S) wild radish populations. In vitro enzyme assays revealed an ALS herbicide-resistant ALS enzyme in all R populations. ALS enzyme extracted from the shoots of all eight R populations was highly resistant to the ALS-inhibiting sulfonylurea herbicide chlorsulfuron (20- to 160-fold) and the triazolopyrimidine herbicide metosulam (10- to 46-fold) and moderately resistant to metsulfuron (three to eightfold). There was little or no cross-resistance to the imidazolinone herbicides imazapyr and imazethapyr. The ALS gene fragment covering potential mutation sites in these populations was amplified, sequenced, and compared. All eight R populations had point mutations in the codon for the proline residue in Domain A. However, the point mutations varied and encoded four different amino acid substitutions: histidine, threonine, alanine, and serine. No nucleotide difference in the DNA sequence of Domains C and D resulting in amino acid substitutions was observed between the R and S populations examined. In addition, a three- to fivefold higher ALS-specific activity was consistently observed in all R populations compared with S populations, whereas Northern blot analysis detected a similar level of ALS mRNA, suggesting a possible translational–posttranslational regulation of the enzyme. It is concluded that selection pressure from chlorsulfuron on eight separate wild radish populations has resulted in target site mutation at the same proline residue in the ALS gene. Higher ALS activity also may play a role in the resistance level.

Nomenclature: Chlorsulfuron; flumetsulam; imazapyr; imazethapyr; metosulam; metsulfuron; wild radish, *Raphanus raphanistrum* L. RAPRA.

Key words: Acetolactate synthase, target site.

Acetolactate synthase (ALS) is the first enzyme common to the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine. Five chemical classes of commercial herbicides inhibit ALS: sulfonylureas (SU), imidazolinones (IM), triazolopyrimidines (TP), pyrimidinyl thiobenzoates (Saari et al. 1994 and references therein), and sulfonylamino-carbonyl-triazolinones (Santel et al. 1999). ALS-inhibiting herbicides are widely used because of their low dose rate, sound environmental properties, low mammalian toxicity, wide crop selectivity, and high efficacy. However, globally there are biotypes of 83 weed species that have evolved resistance to these herbicides so far (Heap 2002). In Australia, resistance to ALS-inhibiting herbicides is widespread in rigid ryegrass (*Lolium rigidum* Gaud.) and wild radish (Hashem et al. 2001; Llewellyn and Powles 2001; Walsh et al. 2001) and has been reported in several dicot weeds, e.g., Indian hedge mustard (*Sisymbrium orientale* Torn.) (Boutsalis and Powles 1995a; Boutsalis et al. 1999), common sowthistle [*Sonchus oleraceus* (L.)] (Boutsalis and Powles 1995b), turnip weed [*Rapistrum rugosum* (L.) All.], climbing buckwheat [*Fallopia convulvulus* (L.) A. Loef], and American turnip weed (*Sisymbrium thellungii* O. Schultz) (Adkins et al. 1997).

Two major mechanisms have been identified that endow resistance to ALS-inhibiting herbicides. In many cases, evolved resistance is due to a reduction in target site sensi-

tivity conferred by one of several mutations within the ALS gene (reviewed by Devine and Eberlein 1997; Saari et al. 1994; Tranel and Wright 2003), whereas ALS overexpression has only been indicated in resistant corn inbred lines and resistant Indian hedge mustard (Boutsalis et al. 1999; Forlani et al. 1991). Nontarget site resistance due to enhanced rates of ALS herbicide metabolism (reviewed by Preston and Mallory-Smith 2001) has so far been reported in blackgrass (*Alopecurus myosuroides* Huds.) (Menendez et al. 1997), rigid ryegrass (Christopher et al. 1991, 1992; Cotterman and Saari 1992) late watergrass [*Echinochloa phyllopogon* (Stapf.) Koss.] (Fisher et al. 2000), and wild mustard (*Sinapis arvensis* L.) (Veldhuis et al. 2000). Multiple resistance, due to both mechanisms, an insensitive ALS, and enhanced rate of ALS herbicide metabolism has been reported (Christopher et al. 1992).

Wild radish is a widespread and damaging weed of Australian agriculture, second only to annual ryegrass, especially in Western Australia (WA). ALS-inhibiting herbicides, especially chlorsulfuron, have been widely and frequently used to control wild radish, and, as a consequence, resistance is now widespread in WA. Many wild radish populations exhibit resistance to chlorsulfuron and other ALS-inhibiting herbicides (Hashem et al. 2001), and a random survey revealed that 21% of the wild radish populations across a large region of the WA wheat belt were chlorsulfuron resistant

TABLE 1. Geographical origins of susceptible (S) and resistant (R) populations used in the study.

Populations	Resistant status	Shire/Town	Latitude (°)	Longitude (°)
960019	S	Yuna	28.33	115.00
WARR7	S			
990040	R			
WARR6	R			
960042	S	Mullewa	28.53	115.51
990041	R			
WARR3	R			
960071	R	Geraldton	28.78	114.61
960107	R			
96FS11	R	Walkaway	28.94	114.80
990025	R	Mingenew	29.19	115.44

(Walsh et al. 2001). Resistance poses practical control problems and raises important and intriguing research questions. The objective of this study is to identify the biochemical and molecular basis of resistance to ALS herbicides in eight distinct wild radish populations to establish whether there is diversity in their resistance mechanisms.

Materials and Methods

Plant Materials

This study used eight chlorsulfuron-resistant populations collected from distinct, separate fields within a region of the WA wheat belt (Table 1): 990025, 960107, 960071, 990040, 96FS11, and 990041 (Hashem et al. 2001) and WARR3 and WARR6 (Walsh et al. 2001). Three susceptible populations (960042, 960019, and WARR7) were collected from sites with no herbicide-use history. The 11 fields are scattered across an area of approximately 500 km².

Germination

Wild radish seeds were soaked in commercial bleach (6% sodium hypochlorite) for 20 to 30 min followed by a 30-min tap water rinse, then placed on 0.6% agar solidified water with or without chlorsulfuron (commercial formulation, concentration ranging from 0.01 to 100 µM). Germination conditions were a 12-h light period at 25 C at a photon flux density of 50 µmol m⁻² s⁻¹ and a 12-h dark period at 15 C. Each treatment contained three replicates. After 7 d, germination and root and stem elongation were compared between R and S populations. Seedlings in which root growth was unaffected (R) were transplanted into pots containing potting mix, and untreated seedlings from S populations were transplanted. The pots were maintained in a glasshouse (temperature controlled at approximately 22 C, natural sunlight) with regular watering and fertilization. Shoot tissue of R and S populations at the three- to four-leaf stage was harvested, snap-frozen in liquid nitrogen, and stored at -80 C until use.

ALS In Vitro Assay

Technical grade SU, TP, and IM herbicides used in the in vitro ALS assays were provided by commercial manufacturers. A modified method from Ray (1984) was adopted

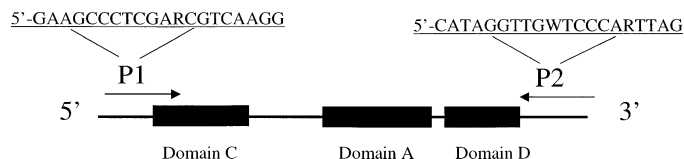


FIGURE 1. Primers P1 and P2 designed to amplify a 501-bp fragment from the acetolactate synthase gene of *Raphanus raphanistrum*-resistant and -susceptible populations, including Domains A, C, and D.

for wild radish ALS assay. The enzyme was extracted in a 2× assay buffer containing 50 mM *N*(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), pH 7.5, 200 mM sodium pyruvate, 20 mM MgCl₂, 2 mM thiamine pyrophosphate, and 20 µM flavin adenine dinucleotide. Insoluble polyvinylpyrrolidone (PVP) was added at the ratio of leaf tissue:insoluble PVP = 6:1. The homogenate was filtered through one layer of miracloth, centrifuged at 27,000 × *g* for 15 min, and immediately used for enzyme assay. Crude extract (100 µl) and ALS herbicide solution (100 µl) were incubated at 37 C for 60 min. The reaction was stopped by adding 40 µl of 6 N H₂SO₄ and incubated at 60 C for 15 min. Afterward, 190 µl of creatine solution (0.55%) and 190 µl of α-naphthol solution (5.5% in 5 N NaOH) were added, and the mixture was incubated at 60 C for 15 min. Enzyme activity was determined colorimetrically (530 nm) by measuring acetoin production. Protein concentration of the crude extract was measured by the Bradford method (Bradford 1976). The *I*₅₀ (herbicide concentration causing 50% inhibition of enzyme activity) was calculated using SigmaPlot 2000¹ software capable of non-linear regression analysis (Sigmoidal, logistic, four parameters). For each population, three independent protein extractions were prepared as replicates from plants from three pots. The enzyme experiment was repeated at least twice.

Molecular Basis of Resistance

Genomic DNA Extraction

Individual plants from R and S populations were harvested, and DNA was extracted from 100 mg shoot tissue of each plant using the cetyltrimethylammonium bromide method (Doyle and Doyle 1990).

Oligonucleotide Primers

Two primers were designed for amplifying and sequencing the highly conserved region 1 of the wild radish ALS gene based on the ALS gene sequences of Arabidopsis [*Arabidopsis thaliana* (L.)], canola (*Brassica napus* L.) (Z11524, Z11525, and Z11526), and redroot pigweed (*Amaranthus retroflexus* L.) (AF363369) from the GenBank database (Figure 1). The upstream primer (5'-GAAGCCCTCGARCGTCAAGG) is homologous to Arabidopsis ALS gene (AY042819) region 352 to 371, and the downstream primer (5'-CATAGGTTGWTCCCATTTAG) is homologous to region 833 to 842 in the gene.

DNA Amplification and Sequencing

Platinum[®] *Taq* DNA polymerase High Fidelity system² was used to amplify ALS gene fragments from wild radish genomic DNA according to the manufacturer's instructions.

A single DNA fragment of 501 bp covering ALS gene Domains A, C, and D was amplified, and the polymerase chain reaction (PCR) product was purified from agarose gel with Qiagen Gel extraction kit.³ The purified PCR products were directly sequenced with the AB-Big Dye Terminators system using a commercial sequencing service.⁴ Three plants from each population were analyzed, and for each plant, three sequencing reactions were performed on each purified PCR fragment and sequenced from both ends. The sequences from R populations were aligned and compared with those of S populations.

Northern Blot

Shoot tissue harvested for ALS in vitro assay was used for total RNA extraction by the guanidine method (Venugopalan and Kapoor 1997). Denatured total RNA (50 µg) of each sample was separated on a 1.2% agarose-formaldehyde gel, and the RNA was transferred to a Hybond+ nylon membrane. The membrane was probed with a ³²P-labeled ALS gene fragment (501 bp, PCR produced as above) in formamide hybridization solution (50% formamide, 5× Denhardt's, 0.5% sodium dodecyl sulfate [SDS]) at 42 C. The hybridized blots were then washed at 42 C for 5 min (2× standard saline citrate [SSC], 0.1% SDS) followed by 15 min washing at 55 C (0.2 × SSC, 0.1% SDS). The hybridized ALS messenger RNA (mRNA) transcripts from the total RNA were autoradiographed onto a Kodak X film. Northern blot analysis was conducted in four R and three S populations, each with three independent total RNA extractions. The experiment was repeated at least twice.

Results and Discussion

Biochemical Basis of Resistance

Germination Screening to Identify R Individuals

Germination and early seedling growth on agar-solidified water containing chlorsulfuron was used to further confirm R individuals. Chlorsulfuron (0.01 to 100 µM) had no effect on germination and initial shoot elongation of R or S populations. However, above 1 µM chlorsulfuron, root growth of S controls was seriously inhibited (70 to 80%), although not significantly affected in R populations at concentrations ≤ 10 µM (Figure 2). Furthermore, when transplanted to pots and foliar sprayed with 20 g chlorsulfuron ha⁻¹ (two- to three-leaf stage), all seedlings identified as R in the germination screening survived foliar treatment (data not shown). Therefore, germination on 2 to 5 µM chlorsulfuron was used to discriminate R and S individuals for subsequent biochemical and molecular analyses.

Comparison of ALS Sensitivity for the R and S Populations

To determine ALS sensitivity, crude tissue extracts from R and S populations were compared for ALS herbicide inhibition of pyruvate conversion to acetoin. Herbicide concentration required to inhibit ALS activity by 50% (I_{50} values) for all selected populations was calculated based on a nonlinear regression analysis of the respective inhibition curves (Figure 3), and the I_{50} ratio between R and S was determined (Table 2). ALS isolated from all R populations was found to be highly resistant to the SU herbicide chlor-

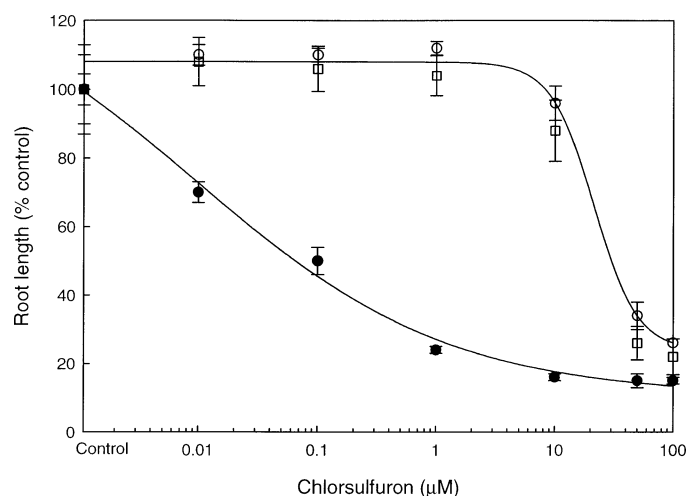


FIGURE 2. Effect of chlorsulfuron incorporated into 0.6% agar-solidified water medium on root elongation (6 d) of resistant populations (○, WARR3; □, WARR6) and a susceptible (●) wild radish population (WARR7). Root length was expressed as a percentage of root length in the absence of chlorsulfuron. Data are means ± SE of two experiments, each conducted in triplicates. Untreated controls had root length of 5.96 (± 0.08) – 6.93 (± 0.93) cm for R populations and 5.62 (± 0.09) cm for the S population.

sulfuron. In some populations the R/S I_{50} ratio was over 100-fold higher, whereas other populations were only 60- to 20-fold higher than the S populations (Table 2). ALS from the R populations also displayed moderate level (three- to eightfold) resistance to the SU herbicide metsulfuron. ALS from the R populations was highly cross-resistant to the TP herbicide metosulam, the I_{50} ratio ranging from 10 to 46, whereas cross-resistance, if any, to the TP herbicide flumetsulam was low, as evidenced by the I_{50} ratio of 0.3 to 2.2. There was no cross-resistance to the IM herbicides imazapyr or imazethapyr except for a marginal level of resistance in populations 990025, 990041, and WARR3. Collectively, these data strongly established that, despite originating from fields separated by considerable distance, all eight R populations are resistant to ALS herbicides due to an insensitive ALS enzyme, and all have a similar cross-resistance pattern across ALS herbicides. However, quantitative differences in the level of resistance are evident among these populations. Taken together, these results suggested that different amino acid substitutions might have occurred in the same ALS gene domain.

Intriguingly, we have consistently observed that all eight R populations displayed a (three- to fivefold) higher basal ALS activity compared with the three S populations (Table 2). The results were further confirmed by applying different assay methods, using fresh or frozen tissue, crude or partially purified enzyme preparations (data not shown).

Molecular Basis of Resistance

Identification of the ALS Gene Mutations

To determine the molecular basis for the differential sensitivity of ALS enzyme detected in crude extracts (Table 2), highly conserved ALS gene regions covering potential mutation sites in these populations were amplified, sequenced, and compared between R and S populations. Amplification of the wild radish genomic DNA with the primers (Figure

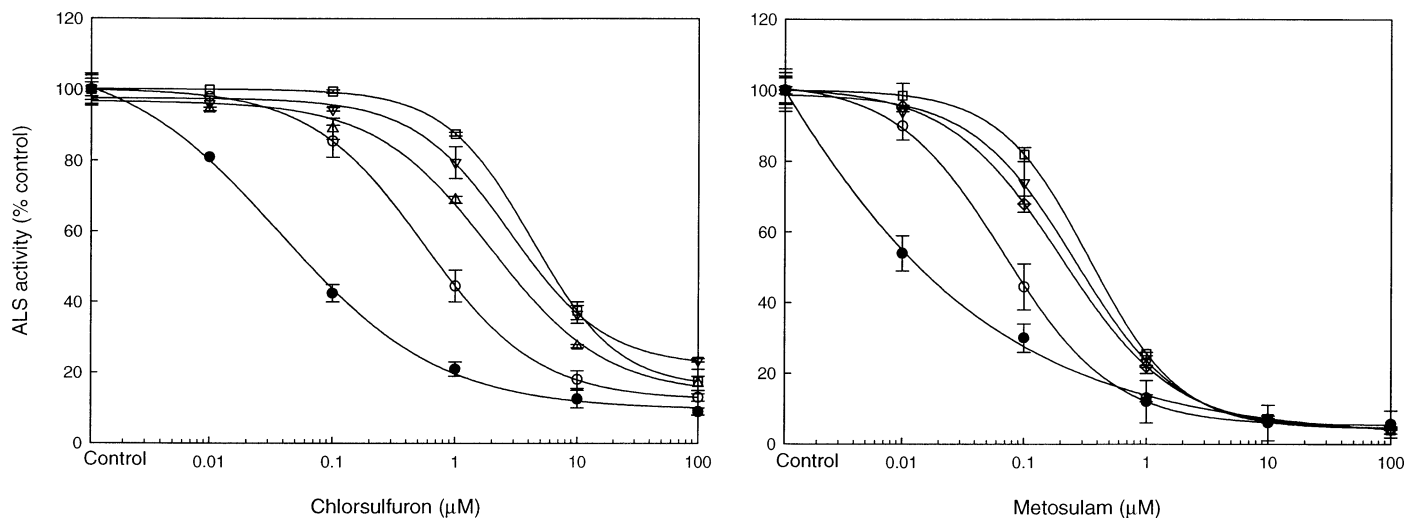


FIGURE 3. In vitro inhibition of ALS activity by chlorsulfuron and metosulam for resistant ($\circ$, 990025; $\diamond$, 990040; $\square$, 990041; $\triangle$, 960071; ∇ , 96FS11) and susceptible ($\bullet$, 960019) wild radish populations. ALS was extracted from R and S plants at the three- to four-leaf stage. ALS activity was expressed as a percentage of activity in the absence of herbicide. Hundred percent ALS activity was $1,407 (\pm 130) - 2,442 (\pm 182)$ nmol acetoin mg^{-1} protein h^{-1} for R populations and $545 (\pm 111)$ for the S population. Data are means $\pm$ SE of at least two experiments each conducted in triplicate.

1) produced a single DNA fragment with the expected size of 501 bp. The DNA fragment was sequenced with the same primers from both ends and was confirmed to cover ALS gene Domains A, C, and D, relative to the *A. thaliana* ALS sequence. Amplified DNA and derived amino acid sequences in these domains from all 11 populations were aligned and compared. It was found that there were 24 base polymorphisms; four of which resulted in amino acid changes. The unique amino acid substitution site was at proline-197 (numbering to *Arabidopsis* ALS gene sequence) in Domain A. Among the eight resistant populations, at least four different single point mutations were found, all at Pro-197, resulting in four amino acid substitutions (Table 3). The first nucleotide C for Pro-197 (CCT) in S biotypes was replaced by T, G, or A, resulting in the amino acid Pro being substituted, respectively, by Ser, Ala, or Thr in R populations. In one population (990025) the second nucleotide C for Pro-197 (CCT) in S biotypes was replaced by A, resulting in the amino acid being substituted by His. It is emphasized that R and S populations differed only in one nucleotide in Domain A, and no mutations in Domains C and D were observed.

Domains B and E were not investigated based on the fact that mutations in Domain B generally confers broad resistance to four chemical classes of ALS inhibitors (Bernasconi et al. 1995; Hattori et al. 1995; Woodworth et al. 1996), whereas Domain E mutations confer resistance mostly to IM herbicides (Devine and Eberlein 1997). The cross-resistance pattern revealed by our in vitro ALS inhibition assay (Table 2) did not show an appreciable level of resistance to IM herbicides, most likely indicating a Domain A mutation.

In addition, in two populations (960107, 990040) we have noticed the presence of two peaks at a single position in the DNA sequence chromatogram (data not shown) representing a mutated and a native base (RS), similar to the heterozygosity recently reported in ALS-resistant giant ragweed (*Ambrosia trifida*) (Patzoldt and Tranel 2002) and wild radish populations (Tan and Medd 2002). In particular, in two other R populations (WARR6, WARR3), we found two different mutated bases at the same position (R1R2) (Table

3). This phenomenon is obviously attributable to the out-crossing nature of wild radish.

It is clear that the amino acid substitutions at Pro-197 in Domain A of the ALS gene from wild radish are responsible for resistance to ALS herbicides in all eight R populations studied. On the basis of in vitro ALS assay data, the specific amino acid modifications in the ALS gene encoded a resistant ALS. All the mutations occurring at Pro-197 in Domain A result in a similar cross-resistance pattern in the eight R populations examined. Of the four resistant ALS alleles, the allele containing Ser at position 197 is most common in the R populations analyzed.

Target-site change is often identified to be predominately responsible for evolved ALS herbicide resistance in weed species (Saari et al. 1994; Tranel and Wright 2003), and single amino acid substitution within the ALS gene Domains A, C, and D, and Domains B and E has been reported to confer resistance to ALS-inhibiting herbicides (Boutsalis et al. 1999; Devine and Eberlein 1997). The most common mutation site within the ALS gene is proline (197), especially in populations selected by sulfonylurea herbicides. However, there is a considerable diversity in the mutations within the ALS gene that endow resistance. Amino acid substitutions for proline (197) by Ser, His, Leu, Gln, Ala, or Thr have been observed and result in resistance to SU and TP herbicides (Saari and Mauvais 1996; Saari et al. 1994). A Pro to Thr change was identified in resistant *Kochia* [*Kochia scoparia* L. (Schrad)] (Guttieri et al. 1992). Replacement of Pro with Ala, Ser, or Gln is involved in resistance to SU herbicides in seven of 14 resistant biotypes of *Lindernia* spp. (Uchino and Watanabe 2002). Pro substitution by Ala or Thr was reported in three of four resistant wild radish populations (Tan and Medd 2002). The relationship between amino acid substitution and cross-resistance pattern has been established for a few biotypes. A Pro to Ser mutation in *A. thaliana* and sugarbeet (*Beta vulgaris*) was demonstrated to result in resistance to SU and TP but not IM herbicides (Haughn and Somerville 1986; Mourad and King 1992; Wright et al. 1998). An African mustard (*Brassica tournefortii* Gouan.) biotype with a Pro to Ala substitution

TABLE 2. ALS I_{50} values (herbicide concentration causing 50% inhibition of ALS activity) for a range of ALS-inhibiting herbicides on ALS isolated from susceptible (S) and resistant (R) wild radish populations. I_{50} ratio was calculated by dividing I_{50} value of each R population with the average I_{50} value from three S populations. Data are means $\pm$ SE of at least two experiments each conducted in triplicate.

Populations	ALS activity (nmol acetoin mg ⁻¹ protein h ⁻¹)	<i>I</i> ₅₀ (μM) and R/S ratios												
		R/S ratio	Chlorsulfuron (SU) ^a	R/S ratio	Metsulfuron (SU) ^a	R/S ratio	Metosulam (TP) ^a	R/S ratio	Flumetsulam (TP) ^a	R/S ratio	Imazapyr (IM) ^a	R/S ratio	Imazethapyr (IM) ^a	R/S ratio
Susceptible populations														
960019	545 ± 72		0.06 ± 0.03		0.07 ± 0.02		0.01 ± 0.00		6.89 ± 0.75		14.57 ± 0.04		7.89 ± 0.95	
960042	386 ± 111		0.04 ± 0.00		0.024 ± 0.00		0.01 ± 0.00		3.5 ± 0.2		16.4 ± 0.92		6.84 ± 1.42	
WARR7	407 ± 74		0.024 ± 0.01		0.05 ± 0.01		0.01 ± 0.00		8.70 ± 1.8		19.91 ± 1.89		7.77 ± 1.46	
Resistant populations														
9990025	1,407 ± 130	3.2	0.82 ± 0.15	21	0.27 ± 0.12	5.4	0.10 ± 0.01	10	1.83 ± 0.38	0.3	33.64 ± 3.93	2.0	11.64 ± 1.5	1.6
9600071	1,864 ± 33	4.2	2.21 ± 0.15	55	0.19 ± 0.01	3.8	0.11 ± 0.01	11	8.43 ± 0.7	1.3	20.06 ± 0.53	1.2	6.15 ± 1.21	0.8
9600107	1,625 ± 221	3.6	1.90 ± 0.86	48	0.24 ± 0.08	4.8	0.32 ± 0.11	32	11.88 ± 0.52	1.9	13.94 ± 0.07	0.8	3.64 ± 1.22	0.5
9990040	1,747 ± 98	3.9	2.43 ± 1.04	61	0.22 ± 0.03	4.4	0.21 ± 0.09	21	7.19 ± 0.93	1.1	17.10 ± 0.2	1.0	8.37 ± 1.26	1.1
9990041	2,442 ± 182	5.5	6.48 ± 0.35	162	0.18 ± 0.02	3.6	0.46 ± 0.01	46	10.01 ± 0.36	1.6	30.23 ± 8.9	1.8	7.96 ± 3.52	1.1
96FS11	1,559 ± 240	3.6	5.09 ± 1.23	127	0.20 ± 0.08	4.0	0.34 ± 0.12	34	10.56 ± 2.88	1.7	17.44 ± 1.46	1.0	4.93 ± 0.19	0.7
WARR3	1,563 ± 164	3.5	4.39 ± 0.72	110	0.23 ± 0.05	4.6	0.37 ± 0.04	37	14.27 ± 2.46	2.2	32.30 ± 5.11	1.9	7.75 ± 0.2	1.0
WARR6	1,672 ± 169	3.7	4.48 ± 0.52	112	0.39 ± 0.05	7.8	0.17 ± 0.03	18	10.88 ± 0.33	1.7	14.90 ± 3.10	0.9	4.83 ± 0.97	0.6

^a Abbreviations: SU, sulfonylureas; TP, triazopyrimidines; IM, imidazolinones.

also was reported as highly resistant to SU and TP but not to IM herbicides (Boutsalis et al. 1999). Multiple mutations including Pro to Thr, Ser, or Ala have been identified in kochia-resistant biotypes and confer a high level of resistance to SU and TP and slight resistance to IM herbicides (Guttieri et al. 1995; Saari et al. 1994 and references therein). Our biochemical and molecular data, showing that four different mutations across R populations all resulted in a high level of resistance to SU herbicide chlorsulfuron and TP herbicide metosulam but little or no resistance to IM herbicides, strongly support these findings.

Although amino acid substitutions occurring at specific sites of the ALS gene have been documented for particular cross-resistance patterns (reviewed by Devine and Eberlein 1997), different amino acid substitutions at the same mutation site may confer different levels of resistance to one or a group of specific herbicides. In this study, the cross-resistance pattern was basically similar across eight R populations, but quantitative levels of resistance to chlorsulfuron were evident. For instance, a relatively low level of resistance to chlorsulfuron was evident in one population (990025) with Pro to His substitution (Tables 2 and 3). Apparently, His substitution is associated with a lower level of resistance to chlorsulfuron than other amino acid substitutions. However, a Pro to His change was reported in prickly lettuce (*Lactuca serriola*), which endowed a high level of resistance to SU, low level to TP, but moderately high level to IM (Guttieri et al. 1992). Correlating specific amino acid substitution and levels of resistance to chlorsulfuron is difficult from the results of the present study, and this should be examined more carefully (e.g., individual-plant or mutation-type-based enzyme assay, types of ALS preparations and homozygosity or heterozygosity status identification).

It is noticeable from our results and those of others that mutations conferring resistance to SU and TP herbicides are largely confined to Domain A at the proline site, with diverse substitutions. It was demonstrated that mutations at Domain A reduced the sensitivity of ALS to feedback inhibition by branched-chain amino acids, physiologically altering amino acid pools in leaves and seeds of resistant plants (Eberlein et al. 1999; Rathinasabapathi et al. 1990). Different amino acid substitutions may have differential consequences on ALS structure and function. In Domain A, a proline residue may have a special function in the configuration of the enzyme molecule strongly influencing herbicide binding. One may speculate that the high frequency of proline-site mutation is due to the possibility that changes at this proline site are not linked with major fitness penalty.

Northern Blot Analysis of the ALS mRNA

To examine the possibility that higher basal ALS activity in all R populations detected in the in vitro ALS assay (Table 2) is due to overexpression of the ALS gene, the level of ALS gene transcription was analyzed in four R and three S populations. As shown in Figure 4, no appreciable difference in abundance of steady-state ALS mRNA was observed between populations R and representative S based on equal total RNA loading, despite a more than three- to fourfold difference observed in enzyme activity (Table 2). This suggested that there was no correlation between the amount of ALS mRNA and the level of ALS-specific activity, assuming in vitro enzyme assay reflects the in vivo enzyme levels. Al-

TABLE 3. DNA and derived amino acid sequences in Domain A of ALS gene from susceptible (S) and resistant (R) populations of wild radish. The bold nucleotide bases encode Pro-197 in S populations and the mutations in R populations. K, G/T; R, A/G; Y, C/T.

Susceptible populations														
960019	GCT	ATY	ACA	GGA	CAG	GTG	CCT	CGT	CGG	ATG	ATT	GGT	ACC	
	Ala	Ile	Thr	Gly	Gln	Val	Pro	Arg	Arg	Met	Ile	Gly	Thr	
960042	GCT	ATT	ACA	GGA	CAG	GTC	CCT	CGT	CGG	ATG	ATC	GGT	ACC	
							Pro							
WARR7	GCT	ATT	ACA	GGA	CAG	GTC	CCT	CGT	CGG	ATG	ATC	GGT	ACC	
							Pro							
Resistant populations														
990025	GCT	ATT	ACA	GGA	CAG	GTC	CAT	CGT	CGG	ATG	ATC	GGT	ACC	
							His							
960071	GCT	ATT	ACA	GGA	CAG	GTC	ACT	CGT	CGG	ATG	ATC	GGT	ACC	
							Thr							
960107	GCT	ATC	ACA	GGA	CAG	GTC	YCT	CGT	CGG	ATG	ATT	GGT	ACC	
							Ser/Pro							
990040	GCT	ATT	ACA	GGA	CAG	GTC	YCT	CGT	CGG	ATG	ATC	GGT	ACC	
							Ser/Pro							
990041	GCT	ATT	ACA	GGA	CAA	GTS	TCT	CGT	CGG	ATG	ATT	GGT	ACC	
							Ser							
96FS11	GCT	ATC	ACA	GGA	CAG	GTC	TCT	CGT	YGG	ATG	ATC	GGT	ACC	
							Ser							
WARR3	GCT	ATT	ACA	GGA	CAG	GTC	RCT	CGT	CGG	ATG	ATC	GGT	ACC	
							Thr/Ala							
WARR6	GCT	ATT	ACA	GGA	CAG	GTC	KCT	CGT	CGG	ATG	ATC	GGT	ACC	
							Ala/Ser							
Amino acid position	191	192	193	194	195	196	197	198	199	200	201	202	203	

though ALS-resistant plant mutants due to ALS overexpression have been isolated only in the laboratory (Saari et al. 1994), higher ALS activity was demonstrated to be responsible for chlorsulfuron resistance in inbred corn lines (Forlani et al. 1991). Boutsalis et al. (1999) also indicated that overexpression of ALS in Indian hedge mustard populations may contribute to ALS resistance. However, although a significant difference in ALS-specific activity was found between S and R chicory (*Cichorium intybus* L.), a similar level of ALS mRNA was detected by Northern blot analysis (De-waele et al. 1996). Conversely, expression of wild-type ALS

gene from *A. thaliana* in transgenic tobacco (*Nicotiana tabacum* L.) resulted in an over 25-fold increase in ALS mRNA but only a twofold increase in ALS-specific activity, indicating a lack of correlation between the amount of physiologically active enzyme and mRNA (Odell et al. 1990). This information is important for elucidating ALS overexpression as a resistance mechanism and for understanding the role and the limitations that ALS overexpression might have in an agronomic setting.

Although resistance in all eight populations clearly involves mutation of ALS, we have not investigated whether

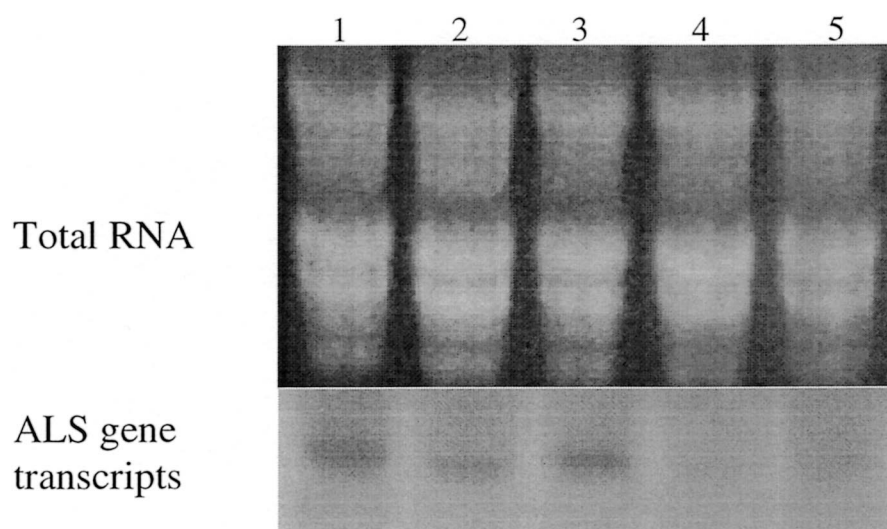


FIGURE 4. Northern blot analysis of expression of ALS mRNA in susceptible (S) and resistant (R) populations of wild radish. Total RNA (50 µg), isolated from shoot tissue of R and S populations, was loaded into each lane (lane 1, 960019; 2, 990025; 3, 990040; 4, 990041; 5, WARR6) and resolved on formaldehyde agarose gel, transferred to a nylon membrane probed with a ³²P-labeled 501-bp fragment. The blot shown was representative among the several blots from one experiment, and similar results were obtained in several independent experiments.

nontarget site mechanisms (e.g., enhanced rates of metabolism) also contribute to the level of resistance in any of these populations.

To conclude, we have established that eight R populations of wild radish, collected from separate locations within the WA wheat belt and primarily selected by sulfonylurea herbicides, have all evolved target-site-based resistance. Diverse resistant alleles in these R populations encode insensitive ALS and confer high level of resistance to SU herbicide chlorsulfuron and TP herbicide metosulam. A higher basal ALS activity, which was regulated at least not at the transcriptional stage, also may contribute to the resistance level.

Sources of Materials

¹ SigmaPlot 2000 for Windows version 6.10. SPSS Inc., 233 South Wacker Drive, Chicago, IL 60606.

² Platinum[®] Taq DNA polymerase High Fidelity system, Invitrogen[®] Life Technologies, 9800 Medical Center Drive, P.O. Box 6482, Rockville, MD 20850.

³ Qiagen Gel extraction kit, QIAGEN GmbH, Max-Volmer-Straß 4, Hilden 40724, Germany.

⁴ AB-Big Dye Terminators system, Royal Perth Hospital, Wellington Street, Perth, WA 6000, Australia.

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