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Effects of a novel combination of two mutated acetolactate synthase (ALS) isozymes on resistance to ALS-inhibiting herbicides in flixweed (*Descurainia sophia*)

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Research Article

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Abstract

Flixweed [*Descurainia sophia* (L.) Webb ex Prantl] is a notorious broadleaf weed that is widely distributed in winter wheat–growing areas of China and has evolved resistance to tribenuron-methyl mainly due to target-site resistance (TSR) mutations in acetolactate synthase (ALS). In the current research, two *ALS* genes were identified in tribenuron-methyl–susceptible (TS) or tribenuron-methyl–resistant (TR) *D. sophia*. Resistance mutations of Asp-376-Glu and Pro-197-Ala were identified on ALS1 and ALS2 isozymes in TR *D. sophia*, respectively. The TR *D. sophia* evolved 10,836.3-fold resistance to tribenuron-methyl and displayed cross-resistance to multiple ALS-inhibiting herbicides with different chemical structures. Dose response experiments and ALS activity assay indicated that two mutated ALS isozymes contributed differentially in resistance to tribenuron-methyl, flucetosulfuron, and pyribenzoxim. In addition, the relative expression level of the *ALS1* gene was 2.2- and 1.6-fold higher than *ALS2* genes in TR *D. sophia* at 1 and 7 d after tribenuron-methyl treatment, respectively. In contrast, the relative expression level of *ALS1* and *ALS2* in TS *D. sophia* is similar. This is the first research that explored different roles of ALS isozymes in resistance to ALS-inhibiting herbicides, which might provide a new perspective for the weed resistance management.

Introduction

Acetolactate synthase (ALS) (EC 2.2.1.6), also recognized as acetohydroxyacid synthase (AHAS; EC 4.1.3.18), catalyzes the formation of 2-acetolactate or 2-aceto-2-hydroxybutyrate in the biosynthesis of branched-chain amino acids (Duggleby et al. 2008). It is well known that ALS is the target of ALS-inhibiting herbicides, which have been used worldwide since the early 1980s. Based on their different chemical structures, ALS-inhibiting herbicides are divided into the following classes: sulfonylurea (SU), pyrimidinyl-thiobenzoate (PTB), sulfonylamino-carbonyl-triazolinone (SCT), triazolopyrimidine (TP), and imidazolinone (IMI). To date, more than 160 weed species have evolved resistance to ALS-inhibiting herbicides worldwide due to continuous and intensive application (Heap 2020). Resistance mutations in ALS isozymes that reduce the binding ability of ALS with herbicides are the most important and universal target-site resistance (TSR) mechanisms (Powles and Yu 2010). To date, resistance mutations have been reported at the Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, or Gly-654 loci of the *ALS* gene (corresponding to the sequence of *ALS* in *Arabidopsis thaliana*) (Heap 2020; Yu and Powles 2014).

Multiple ALS isozymes have been reported in many plants (Table 1). Existing evidence suggests that different ALS isozymes might play different roles in the growth and development of plants. For example, the *ALS1* and *ALS3* genes are constitutively expressed in all somatic and reproductive tissues of oilseed rape (*Brassica napus* L.), while *ALS2* transcripts have only been detected in flowers and young siliques and are regulated in an organ-specific manner (Ouellet et al. 1992). Two *ALS* genes (*SurA* and *SurB*) have been detected in tobacco (*Nicotiana tabacum* L.), while the *SurB* gene is consistently expressed at higher levels than the *SurA* gene (Keeler et al. 1993). Similarly, two or more ALS isozymes were also found in various ALS-inhibiting herbicide-resistant weeds, and up to six ALS isozymes were found in quinoa (*Chenopodium quinoa* Willd.) (Mestanza et al. 2015) and monochoria [*Monochoria vaginalis* (Burm. f.) C. Presl ex Kunth] (Imaizumi et al. 2008) (Table 1). In resistant weeds, resistance-endowing mutations harbor only one of the ALS isozymes in a single plant, and the other isozyme is usually a wild-type copy. Although there are some reports for double resistance mutations in an individual plant, whether the mutations occur in the same or different target isozymes is still uncertain. However, in our previous studies, two ALS isozymes carrying resistance mutations were found in an individual tribenuron-methyl–resistant (TR) flixweed

Table 1. The information and characteristics of multiple acetolactate synthase (ALS) isozymes in plants, including weeds.

Plant species	ALS isozymes	Characteristic difference on *ALS* gene	Reference
Annual bluegrass (*Poa annua* L.)	Two unnamed ALS	The resistance mutations occurred only in one *ALS* gene.	McElroy et al. 2013
Bog bulrush [*Schoenoplectiella mucronata* (L.) J. Jung & H.K. Choi]	ALS1, ALS2, and ALS3	The *ALS1* gene was more frequently detected in leaves than the *ALS2* and *ALS3* genes, and the resistance mutations were identified only in the *ALS1* gene.	Scarabel et al. 2010
Cotton (*Gossypium hirsutum* L.)	A19, A5, and another four unnamed ALS	The *A19* and *A5* genes constitutively expressed and encoded the main housekeeping *ALS* gene, and the expression of the other four genes was tissue specific.	Grula et al. 1995
False flax (*Camelina microcarpa* Andrz. ex DC.)	ALS1 and ALS2	Resistance mutations were identified only in the *ALS1* gene.	Hanson et al. 2004
Flixweed [*Descurainia sophia* (L.) Webb ex Prantl]	ALS1, ALS2, ALS3, and ALS4	The *ALS1* and *ALS2* genes were constitutively expressed in all populations, while the *ALS3* and *ALS4* genes were expressed only in some populations.	Xu et al. 2020
Hairy beggarticks (*Bidens pilosa* L.)	ALS1, ALS2, and ALS3	The resistance mutation of Trp-574-Leu was identified in only one of three *ALS* genes.	Lamego et al. 2009
Mayweed chamomile (*Anthemis cotula* L.)	ALS1 and ALS2	Different resistance mutations were identified only in the *ALS1* gene.	Intanon et al. 2011
Monochoria [*Monochoria vaginalis* (Burm f.) C. Presl ex Kunth]	ALS1, ALS2, ALS3, ALS4, ALS5, and ALS6	The resistance mutations occurred only in the *ALS1* or *ALS3* gene.	Imaizumi et al. 2008
Oilseed rape (*Brassica napus* L.)	ALS1, ALS2, ALS3, ALS4, and ALS5	The *ALS1* and *ALS3* genes were constitutively expressed in somatic and reproductive tissues, while the *ALS2* gene was expressed only in reproductive tissues. The *ALS4* and *ALS5* genes were pseudogenes.	Ouellet et al. 1992
Quinoa (*Chenopodium quinoa* Willd.)	CqAHAS1, CqAHAS2, CqAHAS3, CqAHAS4, CqAHAS5, and CqAHAS6	The *CqAHAS1* and *CqAHAS2* genes were the functional genes; the other four genes were silent or expressed in a specific tissue.	Mestanza et al. 2015
Rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.]	ALS1 and ALS2	The expression level of the *ALS2* gene was significantly higher than that of the *ALS1* gene in rapidly growing organs, such as roots, culms, leaf sheaths, and lamina joints.	Iwakami et al. 2012
Russian thistle (*Salsola tragus* L.)	Two unnamed ALS	The resistance mutation occurred only in one *ALS* gene.	Warwick et al. 2010
Shortawn foxtail (*Alopecurus aequalis* Sobol.)	ALS1, ALS2, ALS3, and ALS4	The *ALS1* and *ALS2* genes were constitutively expressed in all populations, while the *ALS3* and *ALS4* genes were pseudogenes and were expressed only in some populations.	Iwakami et al. 2017
Sunflower (*Helianthus annuus* L.)	AHAS1, AHAS2, and AHAS3	Resistance mutations occurred only in the *AHAS1* gene, and inducible expression of three *AHAS* genes was tissue specific and gene dependent.	Breccia et al. 2013; Kolkman et al. 2004
Tobacco (*Nicotiana tabacum* L.)	SurA and SurB	The *SurB* gene was consistently expressed at higher levels than the *SurA* gene in all tobacco organs examined.	Keeler et al. 1993
Wheat (*Triticum aestivum* L.)	ahas-D1, ahas-B1, and ahas-A1	The resistance mutation of Ser-653-Asn occurred only in the *ahas-D1* or *ahas-B1* gene, and the mutation on the *ahas-D1* gene coded for more-resistant ALS activity.	Pozniak et al. 2004

[*Descurainia sophia* (L.) Webb ex Prantl] (Deng et al. 2017). In the current study, a novel combination of two mutated ALS isozymes is identified in a single TR *D. sophia* plant.

Descurainia sophia is a troublesome weed infesting winter wheat (*Triticum aestivum* L.) and has evolved resistance to tribenuron-methyl since its introduction into China in 1988. Amino acid substitutions in one or multiple ALS isozymes are responsible for *D. sophia*'s resistance to tribenuron-methyl. In addition, enhanced metabolism in TR *D. sophia* mediated by cytochrome P450s heightens *D. sophia* resistance (Deng et al. 2014, 2015, 2017; Xu et al. 2020; Yang et al. 2016, 2018a, 2018b). In the current study, a new resistance mutation combination of mutated ALS1 (Asp-376-Glu) and ALS2 (Pro-197-Ala) is identified in individual *D. sophia*. Do weeds accumulate multiple mutated ALS enzymes in a single plant as a mechanism for combating higher herbicide selection pressure in fields? Are resistance mutations occurring on ALS isozymes randomly, or are some ALS isozymes are more preferentially mutated? Do different ALS isozymes play different roles in resistance evolution? Studies of these problems will help to reveal the resistance mechanisms to ALS-inhibiting herbicides. The current research aims to evaluate the contributions of different mutated ALS1 and ALS2 in resistance to different ALS-inhibiting herbicides in *D. sophia* through (1) determining the herbicide concentration that inhibits 50% of the activity of ALS1 and ALS2 (I_{50}) and (2) comparing the expression levels of *ALS1* and *ALS2* genes in *D. sophia*.

Materials and Methods

Plant Materials

In 2016, seeds of the TR *D. sophia* population (SD1637) were collected from winter wheat fields in Liaocheng City, China (36.71°N, 115.84°E), where tribenuron-methyl had been used repeatedly for more than 20 yr. The tribenuron-methyl–susceptible (TS) *D. sophia* population (BJ1602) was harvested from the roadside in Beijing (40.21°N, 116.33°E), where tribenuron-methyl is unlikely to be used. To confirm the frequency of resistance mutations, the *ALS* genes for 50 plants in each population were sequenced, and all plants investigated had the same mutation combination.

Seeds of *D. sophia* were immersed in 20% H_2O_2 solution for 30 min, followed by a 24-h soak in 0.03% gibberellin solution, and germinated in petri dishes at room temperature for 4 d after rinsing with water. Sixteen germinated seeds were planted in one pot containing moist soil, which was kept in artificial climate chambers at

Table 2. Information on primers for *ALS* gene cloning (F_1/R_1), reference gene *18S rRNA* (F_2/R_2) and *ALS* expression determination (F_3/R_3, F_4/R_4) in tribenuron-methyl–susceptible (TS) and tribenuron-methyl–resistant (TR) *Descurainia sophia*.

Amplification gene	Primers[a]	Sequence(5′→3′)	Amplification size
			bp
ALS1 and *ALS2*	F_1	CGCTCCTCTCCTGAAGCTCACCA	2004 (*ALS1*)/1998 (*ALS2*)
	R_1	CAAACAAACAGCAGTAGCGTCTGAAG	
18S rRNA	F_2	TAGTTGGTGGAGCGATTTGTCTG	114
	R_2	CTAAGCGGCATAGTCCCTCTAAG	
ALS1	F_3	CTCCACCACTTCTTCTCCT	236
	R_3	CGAAAACACCTCCTTGTT	
ALS2	F_4	CCACTTCTTCTCCAGCGA	257
	R_4	ACCTGAGGATCGAGCGTA	

[a]F, forward primer; R, reverse primer.

25/20 C (light/dark) for a 16-h photoperiod with a photosynthetic photon flux density of approximately 270 µmol m^{-2} s^{-1}.

Determination of Resistance Mutations in ALS Genes

A DNA extraction kit (Plant Genomic DNA Kit®, DP305, Tiangen China, No. 86, Shuang Ying West Road, Changping District, Beijing, China) was used to extract genomic DNA from fresh leaves of TS and TR *D. sophia*. An F_1/R_1 primer pair was designed to amplify the *ALS* genes to full length (Table 2). The polymerase chain reaction (PCR) mixtures and program were the same as those described by Deng et al. (2017). The PCR products were purified from agar gel with a purification kit (TIANgel Midi Purification Kit®, DP209, Tiangen China). The purified PCR products were ligated to the pLB-Simple vector and then transformed into TOP10 competent cells. Six clones of each DNA sample were selected for sequencing. Resistance mutations were identified by comparing the *ALS* sequence with that of susceptible *D. sophia* (accession no. JQ868736). The *ALS* genes for 50 plants in each population were sequenced to determine the proportion of plants carrying the resistance mutations.

Whole-Plant Dose Response Experiments for ALS-inhibiting Herbicides

Whole-plant dose response experiments were used to determine the resistance or cross-resistance levels of TR and TS *D. sophia* populations to different ALS-inhibiting herbicides, including tribenuron-methyl (SU), flucetosulfuron (SU), pyribenzoxim (PTB), flucarbazone-sodium (SCT), flumetsulam (TP), and imazethapyr (IMI). *Descurainia sophia* plants at the 4-leaf stage were used for whole-plant dose response experiments. The herbicides were diluted to a series of concentrations with 0.2% Tween-80 solution. The spray doses used for ALS-inhibiting herbicides are listed in Table 3. Notably, due to the great difference in the susceptibility to tribenuron-methyl, the rates varied between TS and TR *D. sophia* populations. Herbicides were applied by using an automatic cabinet sprayer (ASP-1098 automatic sprayer, Zhejiang University Xinnong Pesticide Model Technology Development, Hangzhou, China) at a spray volume of 600 L ha^{-1}. Control individuals were treated with water containing 0.2% Tween-80. The aboveground fresh *D. sophia* seedlings were weighed at 21 d after treatment. The experiments were conducted twice with three replicates in one dose.

In Vitro ALS Extraction and Activity Assay

Approximately 40 d after planting, 4 g of leaf material was used for ALS extraction and activity assays in vitro according to the methods of Deng et al. (2014). ALS activity in vitro was determined by colorimetry (520 nm) with a microplate photometer (Thermo Fisher, Waltham, MA, USA) by measuring acetoin production. The final concentrations in the reaction mixtures for the different herbicides are listed in Table 3. The experiments were repeated twice using independent enzyme extracts with three replicates for each herbicide concentration.

Relative Expression of ALS1 and ALS2 in TR and TS Descurainia sophia

Each *D. sophia* plant at 40 d after transplanting was treated evenly with 5 mg L^{-1} technical tribenuron-methyl (15 µl per plant) dissolved in a mixture of acetone and water (4:6 v/v) by using a micro-applicator (Hamilton PB 600 dispenser, Hamilton, Lancaster, PA, USA). The TS population exhibited no observable adverse response to the applied dose. Three plants were selected for RNA extraction by an RNA extraction kit (RNAprep pure Plant Kit®, DP432, Tiangen China) before tribenuron-methyl treatment (BT) or at 1, 3, 5, and 7 d after tribenuron-methyl treatment (DAT). Plants treated with only an equal volume of 40% acetone were used as controls.

18S rRNA was selected as a reference gene that was confirmed to be stably expressed in *D. sophia* (Yang et al. 2018b). Two primer pairs (F_3/R_3 and F_4/R_4) were designed for quantitative real-time polymerase chain reaction (qPCR) according to the differences between *ALS1* and *ALS2* gene sequences (Table 2). First-strand complementary DNA (cDNA) was synthetized according to the instructions of the FastQuant RT Kit (TIANScript II RT Kit®, KR107, Tiangen China). The expression level for *ALS1* or *ALS2* was determined by performing qPCR. The reaction mixtures with a volume of 20 µl consisted of 0.4 µl 50× ROX reference dye, 0.6 µl primers, 1 µl diluted cDNA, 7.4 µl ribonuclease-free distillation-distillation H_2O (RNase-free ddH_2O), and 10 µl 2× SuperReal PreMix Plus (SuperReal PreMix Plus®, FP205, Tiangen China). There were four replicates per cDNA. qPCR was carried out with programs of 15-min incubation at 95 C, 40 cycles at 95 C for 10 s, 60 C for 20 s, and 72 C for 32 s. There were four replicates per cDNA and three cDNA samples at each time point in the TS or TR populations.

Statistical Analysis

Whole-Plant Dose Response Experiments and In Vitro ALS Activity Assay

The GR_{50} (herbicide dose causing 50% plant growth reduction) and I_{50} were calculated using GraphPad Software (v. 5.0, San Diego, CA, USA) with Equation 1. This equation is based on a

Table 3. Herbicide rates of acetolactate synthase (ALS)-inhibiting herbicides in whole-plant dose response experiments and in vitro ALS activity assay.

Herbicide	Rates in whole-plant dose response experiments[f]		Rates for in vitro ALS activity assay
	TS	TR	TS and TR
	g ai ha^{-1}		μM
Tribenuron-methyl[a]	0.0006, 0.0023, 0.0092, 0.036, 0.15, 0.59, 2.34, 9.38	0.036, 0.15, 0.59, 2.34, 9.38, 37.5, 75, 150	1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 0.1, 1, 10, 100, 200, 400, 800
Flucetosulfuron[a]	0.0005, 0.0012, 0.0047, 0.019, 0.075, 0.3, 1.2	0.075, 0.3, 1.2, 4.8, 19.2, 76.8, 307.2	1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 0.1, 1, 10, 100, 500, 1,000
Pyribenzoxim[b]	0.019, 0.075, 0.3, 1.2, 4.8, 19.2, 76.8	0.075, 0.3, 1.2, 4.8, 19.2, 76.8, 307.2	1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 0.1, 1, 10, 50, 100, 500, 1,000
Flucarbazone-sodium[c]	0.0002, 0.0009, 0.0036, 0.015, 0.059, 0.024, 0.098	0.94, 3.75, 15, 60, 240, 480, 960	1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 0.1, 1, 10, 100, 500, 1,000
Flumetsulam[d]	0.0017, 0.019, 0.075, 0.3, 1.2, 4.8, 19.2	0.075, 0.3, 1.2, 4.8, 19.2, 76.8, 307.2	1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 0.1, 1, 10, 100, 500, 1,000
Imazethapyr[e]	0.0012, 0.0047, 0.019, 0.075, 0.3, 1.2, 4.8	0.019, 0.075, 0.3, 1.2, 4.8, 19.2, 76.8	1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 0.1, 1, 10, 100, 500, 1,000

[a,b,c,d,e]indicate the herbicide subfamily of sulfonylurea (SU), pyrimidinyl-thiobenzoate (PTB), sulfonylamino-carbonyl-triazolinone (SCT), triazolopyrimidine (TP), and imidazolinone (IMI), respectively.
[f]TS, tribenuron-methyl–susceptible; TR, tribenuron-methyl–resistant.

double-sigmoid model, which is constructed as the sum of two different logics. The double-sigmoid model suggests the coexistence of two inhibition targets with different susceptibilities.

$$y = Frac \times \frac{100}{1 + 10^{(a-x)}} + (1 - Frac) \times \frac{100}{1 + 10^{(b-x)}} \quad [1]$$

In this equation, y is the percentage of fresh weight or ALS activity (% control); x is the log of the herbicide dose; and a and b are the logarithms of the GR_{50} (or I_{50}) of two single-sigmoid curves ($b > a$). The *Frac* value is considered to be a putative proportion of the resistance contribution of more-susceptible ALS (Lipovetsky 2010; Tsuneki et al. 2004; Yamato et al. 2013).

Relative Expression of ALS1 and ALS2

The relative expression ratio (as $2^{-\Delta\Delta C_T}$) was calculated by the cycle threshold (C_T) method (Schmittgen and Livak 2008), where $\Delta C_T = C_T$ target gene $- C_T$ internal control gene. Data for the relative expression level of *ALS* genes were tested for normality using the Kolmogorov-Smirnov test with SPSS software (v. 16.0, IBM, Armonk, NY, USA), and homogeneity of variance was confirmed by Levene's test with SPSS. The data obtained from the relative expression of genes met the assumptions of an independent-samples *t*-test and ANOVA by testing. The relative expression of two *ALS* genes between TS or TR *D. sophia* was analyzed by the independent-samples *t*-test ($P < 0.05$). Dunnett's test at the 5% level of significance in ANOVA was carried out to compare the relative expression of the *ALS1* or *ALS2* gene at different times in TS or TR *D. sophia*.

Results and Discussion

Determination of Resistance Mutations in ALS Genes

Two *ALS* genes with full lengths of 2,004 bp (*ALS1*) and 1,998 bp (*ALS2*) were cloned from TS (BJ1602) and TR (SD1637) *D. sophia*. A new combination of resistance mutations (ALS1 with Asp-376-Glu, ALS2 with Pro-197-Ala) was identified from a single TR *D. sophia* plant in the current study. The *ALS1* and *ALS2* genes in TS and TR *D. sophia* show high homology. A single different nucleotide caused the resistance mutation at the site of Pro-197 (in ALS2) or Asp-376 (in ALS1). Fifty TS or TR *D. sophia* plants were sequenced, and the *ALS1* or *ALS2* of each plant was the same in each *D. sophia* population, which indicated that the genetic backgrounds of the different plants were very similar or the same.

Two resistance mutations on ALS in a single plant were also reported in kochia [*Bassia scoparia* (L.) A.J. Scott; syn. *Kochia scoparia* (Linn.) Schrad] (Warwick et al. 2008), rigid ryegrass (*Lolium rigidum* Gaudin) (Kaundun et al. 2012), and Palmer amaranth (*Amaranthus palmeri* S. Watson) (Singh et al. 2019), but it is not clear whether these mutations occur in the same ALS isozyme or in different ALS isozymes. To the best of our knowledge, a total of eight different amino acid mutations have been identified at Pro-197 (substituted by Ser, Thr, Leu, His, Ala, or Arg), Asp-376 (by Glu), and Trp-574 (by Leu) in ALS isozymes in TR *D. sophia* (Xu et al. 2020). Initially, resistance mutations were only identified in one ALS isozyme, and the most common resistance mutation was identified at the site of Pro-197 (Xu et al. 2020). However, resistance mutations at Asp-376 or Trp-574 in ALS, which can cause higher levels of resistance to tribenuron-methyl and broader cross-resistance to ALS-inhibiting herbicides than resistance mutations at Pro-197, were identified only in a few TR *D. sophia* (Deng et al. 2017; Xu et al. 2015; Yang et al. 2018a). Deng et al. (2017) first reported that two mutated ALS isozymes (Trp-574-Leu in ALS1, Pro-197-Thr in ALS2) in a single TR *D. sophia* plant exhibit a higher resistance level (789.3-fold) to tribenuron-methyl than TR *D. sophia* populations carrying a single mutated isozyme (211.8- and 366.3-fold) and exhibit cross-resistance to more ALS-inhibiting herbicides. In the current study, a novel combination of two mutated ALS isozymes (Asp-376-Glu in ALS1 and Pro-197-Ala in ALS2) was identified in a single TR *D. sophia* plant. Hence, we speculated that the accumulation of two or multiple mutated ALS isozymes in a single plant results in a higher level of resistance.

Whole-Plant Dose Response Experiments for ALS-inhibiting Herbicides

Whole-plant dose response experiments established the different sensitivities of TS (BJ1602) and TR (SD1637) *D. sophia* populations to different ALS-inhibiting herbicides (Table 4; Figure 1). The TS *D. sophia* were killed completely by tribenuron-methyl at a dose of 0.59 g ai ha^{-1}, while all TR *D. sophia* survived at the highest dose of 150 g ai ha^{-1} (Figure 1A). The resistance index

Table 4. The GR_{50} and I_{50} values of acetolactate synthase (ALS)-inhibiting herbicides for tribenuron-methyl–susceptible (TS) and tribenuron-methyl–resistant (TR) *Descurainia sophia*.[a]

	TS		TR					
Herbicide	GR_{50}	I_{50}	GR_{50}	*Frac*[b]	RI	I_{50}	*Frac*	RI
	g ai ha^{-1}	μM	g ai ha^{-1}			μM		
Tribenuron-methyl	0.03	0.17	325.09	0.09	10,836.33	508.39	0.08	2,990.53
Flucetosulfuron	0.03	3.07	14.51	0.19	483.67	295.12	0.16	96.13
Pyribenzoxim	1.29	0.13	14.00	0.18	10.85	107.52	0.16	827.08
Flucarbazone-sodium	0.01	0.03	28.39	NA	2,839.00	1.97	NA	65.66
Flumetsulam	0.59	0.45	20.15	NA	34.15	18.12	NA	40.27
Imazethapyr	0.05	5.75	3.49	NA	69.80	29.62	NA	5.15

[a]Abbreviations: GR_{50}, herbicide rates causing 50% plant growth reduction; I_{50}, herbicide rates inhibiting 50% ALS activity; RI, resistance index, GR_{50} value or I_{50} value of TR populations divided by that of TS population; NA, not applicable.
[b]*Frac* is considered to be a putative proportion of resistance contribution of more susceptible ALS.

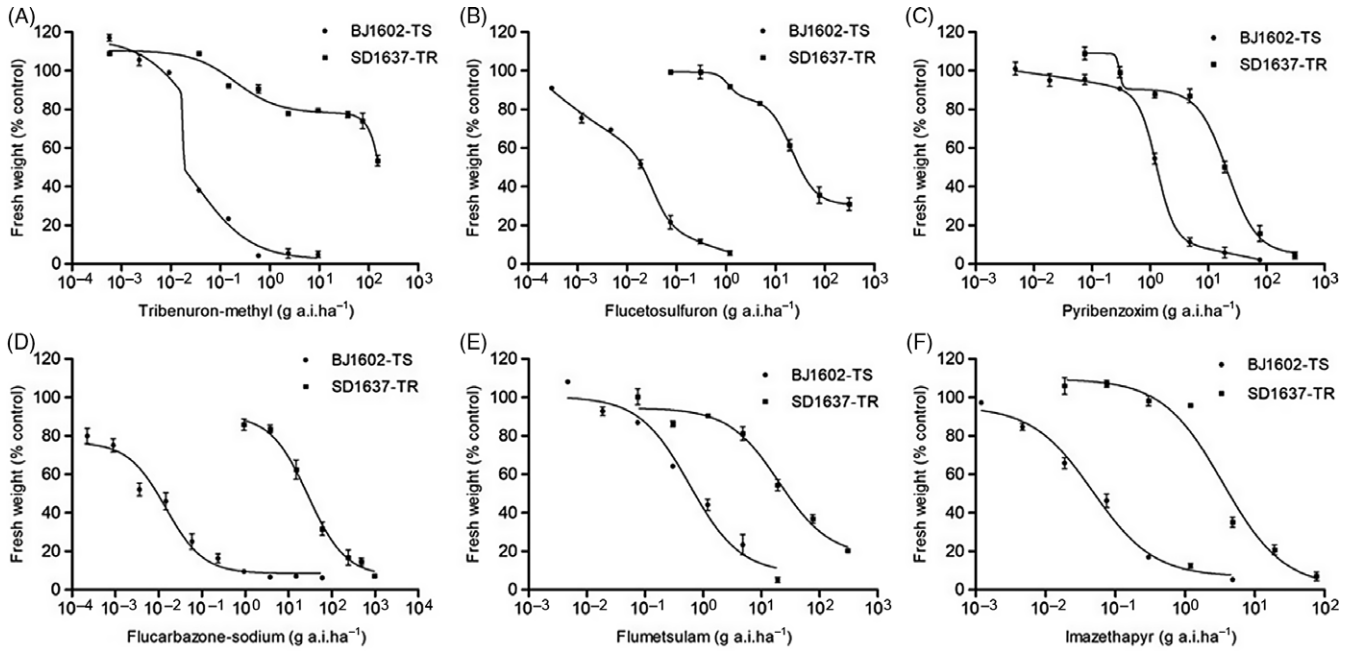

Figure 1. Dose response curves of tribenuron-methyl–susceptible (TS, BJ1602-TS) and tribenuron-methyl–resistant (TR, SD1637-TR) *Descurainia sophia* treated with acetolactate synthase (ALS)-inhibiting herbicides: (A) tribenuron-methyl, (B) flucetosulfuron, (C) pyribenzoxim, (D) flucarbazone-sodium, (E) flumetsulam, and (F) imazethapyr. The experiments had three replicates per herbicide dose and were repeated twice. Vertical bars represent the standard error of two experiments.

(RI) of TR *D. sophia* (SD1637) was 10,836.33, which is higher than that of the TR populations carrying any other known resistance mutations (Deng et al. 2017; Yang et al. 2018a). For example, the populations with a single resistance mutation of Pro-197-His, Pro-197-Leu, Pro-197-Thr, Pro-197-Ser, Trp-574-Leu, or Asp-376-Glu in the ALS1 isozyme exhibited 205.8-, 250.0-, 708.4-, 263.7-, 485.3-, or 2,844.7-fold resistance to tribenuron-methyl, respectively (Yang et al. 2018a), and the TR *D. sophia* population with two mutated ALS isozymes (Trp-574-Leu in ALS1, Pro-197-Thr in ALS2) evolved a 789.3-fold resistance to tribenuron-methyl (Deng et al. 2017). Therefore, the presence of two mutated ALS isozymes in individual plants helped weeds survive higher doses of ALS-inhibiting herbicide compared with weeds carrying a single mutated ALS isozyme. In addition, the accumulation of multiple *ALS* mutations in individual plants was also observed in *A. palmeri* (Singh et al. 2019). In recent years, double mutations have been reported in a variety of resistant weeds, and these double mutations lead to weeds evolving higher resistance levels than weeds with a single resistance mutation. However, it is not clear whether double resistance mutations occur on the same or different target isozymes. For example, double mutations (Ala-251-Val and Phe-273-Val) in *psbA* resulted in *Amaranthus* spp. evolving a higher resistance to linuron and diuron compared with each single substitution (Davis et al. 2020). Goosegrass [*Eleusine indica* (L.) Gaertn.] with double mutations (Thr-102-Ile and Pro-106-Ser in 5-enolpyruvylshikimate-3-phosphate synthase [EPSPS]) developed a more than 32-fold higher resistance to glyphosate than *E. indica* carrying a single mutation (Pro-106-Ser) (Yu et al. 2015). Similar to *E. indica*, a different EPSPS double mutation (Thr-102-Ile and Pro106-Thr) conferring a high level of glyphosate resistance was reported in tetraploid beggarticks (*Bidens pilosa* L.) (Takano et al. 2020). Notably, a triple mutation (Thr-102-Ile, Ala-103-Val, and Pro-106-Ser) in *EPSPS* was reported, providing a 314-fold resistance to glyphosate compared with the wild-type strain in smooth pigweed (*Amaranthus hybridus* L.) (Perotti et al. 2018).

The results obtained indicate that TR *D. sophia* exhibits obvious cross-resistance to representative herbicides of ALS-inhibiting

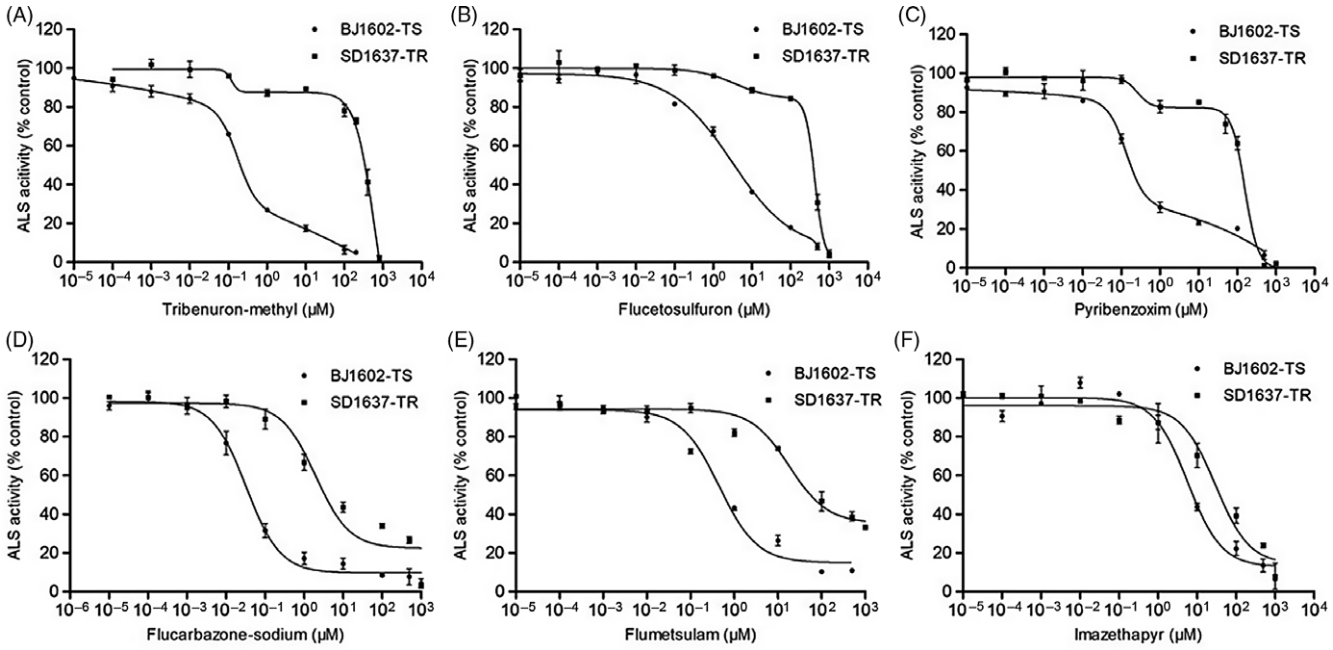

Figure 2. The acetolactate synthase (ALS) activity in vitro of tribenuron-methyl–susceptible (TS, BJ1602-TS) and tribenuron-methyl–resistant (TR, SD1637-TR) populations inhibited by ALS-inhibiting herbicides: (A) tribenuron-methyl, (B) flucetosulfuron, (C) pyribenzoxim, (D) flucarbazone-sodium, (E) flumetsulam, and (F) imazethapyr. The experiments had three replicates per herbicide concentration and were repeated twice. Vertical bars represent the standard error of two experiments.

herbicides with different chemical structures (Table 4). The cross-resistance levels to flucarbazone-sodium (SCT) and flucetosulfuron (SU) are much higher than to imazethapyr (IMI), flumetsulam (TP), and pyribenzoxim (PTB). Although the ALS isozymes are the common targets of these herbicides, their binding ability with ALS isozymes may be different due to different chemical structures. Therefore, TR *D. sophia* displays different cross-resistance levels to different ALS-inhibiting herbicides. The dose response of TR *D. sophia* to different ALS-inhibiting herbicides is different (Figure 1). The whole-plant dose response to tribenuron-methyl, flucetosulfuron, and pyribenzoxim can be fit to double-sigmoid models, demonstrating that two ALS isozymes in TR *D. sophia* have different sensitivities to these herbicides (Figure 1A–C). However, the curves fit for flucarbazone-sodium, flumetsulam, and imazethapyr are more consistent with single-sigmoid models, indicating that the ALS isozymes in TR *D. sophia* have similar sensitivities to these herbicides (Figure 1D–F). *Frac* is usually considered to be the proportion of resistance contributed by a more susceptible ALS isozyme to ALS-inhibiting herbicides (Yamato et al. 2013). The *Frac* value of TR *D. sophia* to tribenuron-methyl, flucetosulfuron, and pyribenzoxim indicates that the resistance contribution of the two ALS isozymes to these herbicides is obviously different. The resistance contributions to flucarbazone-sodium, imazethapyr, and flumetsulam are similar for the two ALS isozymes (Table 4).

In Vitro ALS Activity Assay

The results for I_{50} demonstrate that the herbicide concentrations inhibiting ALS activity in TR *D. sophia* increase greatly compared with those in TS *D. sophia* (Table 4). The curve-fitting models for the whole-plant dose response to and ALS activity inhibition by the same herbicide are nearly consistent for the TR population (Figures 1 and 2). The inhibition of ALS activity by tribenuron-methyl, flucetosulfuron, and pyribenzoxim can be fit by double-sigmoid models, indicating that two ALS isozymes in TR *D. sophia* contributed differently to the resistance to tribenuron-methyl, flucetosulfuron, and pyribenzoxim (Figure 2A–C). The ALS activity inhibition by flucarbazone-sodium, flumetsulam, and imazethapyr can be fit to single-sigmoid models, demonstrating that the two ALS isozymes exhibit similar contributions to the resistance to flucarbazone-sodium, flumetsulam, and imazethapyr (Figure 2D–F). One double-sigmoid curve can be regarded as the combination of two independent single-sigmoid curves. Compared with the single-sigmoid curves, the double-sigmoid curves for tribenuron-methyl, flucetosulfuron, and pyribenzoxim in TR *D. sophia* have a plateau between 1.0 to 10 μM, 10 to 100 μM, and 1.0 to 100 μM, respectively (Figure 2A–C), indicating that the more susceptible of the two ALS isozymes was saturated first. The activity of another ALS isozyme is gradually saturated with increasing herbicide concentration. In addition, the R^2 obtained by weighted least-squares for the GR_{50} and I_{50} values for six ALS-inhibiting herbicides are determined to be 0.908, which indicates that the reduced ALS activity in response to herbicides is highly related to herbicide resistance in TR *D. sophia*. In particular, the I_{50}-based RI value of flucarbazone-sodium is much lower than the GR_{50}-based RI value. Given that the two ALS isozymes contribute equally to resistance to flucarbazone-sodium, the higher RI value (GR_{50}-based) may also be due to non–target site resistance (NTSR) mechanisms. To date, NTSR to flucarbazone-sodium has been reported only in wild oat (*Avena fatua* L.) (Burns et al. 2018). Further research is needed to determine whether the NTSR mechanisms confer flucarbazone-sodium resistance in TR *D. sophia*.

Reduced ALS affinity with ALS-inhibiting herbicides, which is caused by resistance mutations in ALS isozymes, is the major TSR mechanism for *D. sophia* and other weed species (Deng et al. 2014, 2017; Heap 2020; Xu et al. 2015; Yang et al. 2018a). However, most

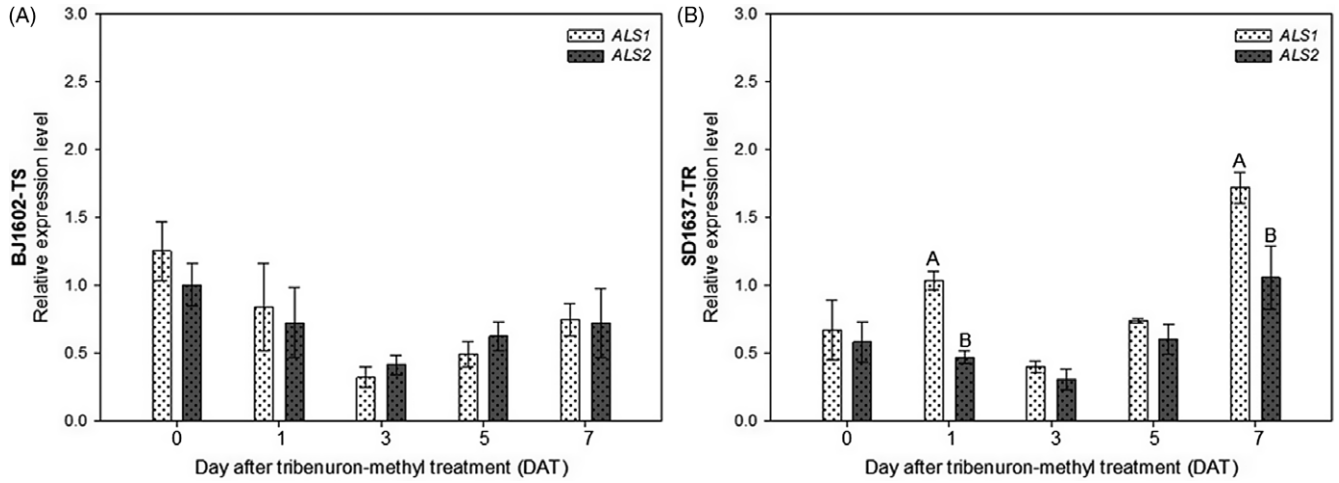

Figure 3. The relative expression of *ALS1* and *ALS2* genes in (A) tribenuron-methyl–susceptible (TS) or (B) tribenuron-methyl–resistant (TR) *Descurainia sophia* plants before tribenuron-methyl treatment (BT) or at 1, 3, 5, and 7 d after tribenuron-methyl treatment (DAT). Each data is the mean ± SE of four replicates. Means with different capital letters indicate the relative expression level of *ALS1* was significantly higher than that of *ALS2* at the same time point on level of $P < 0.05$. Means without letters show the relative expression level of two *ALS* genes at the same time point exhibited no significant difference.

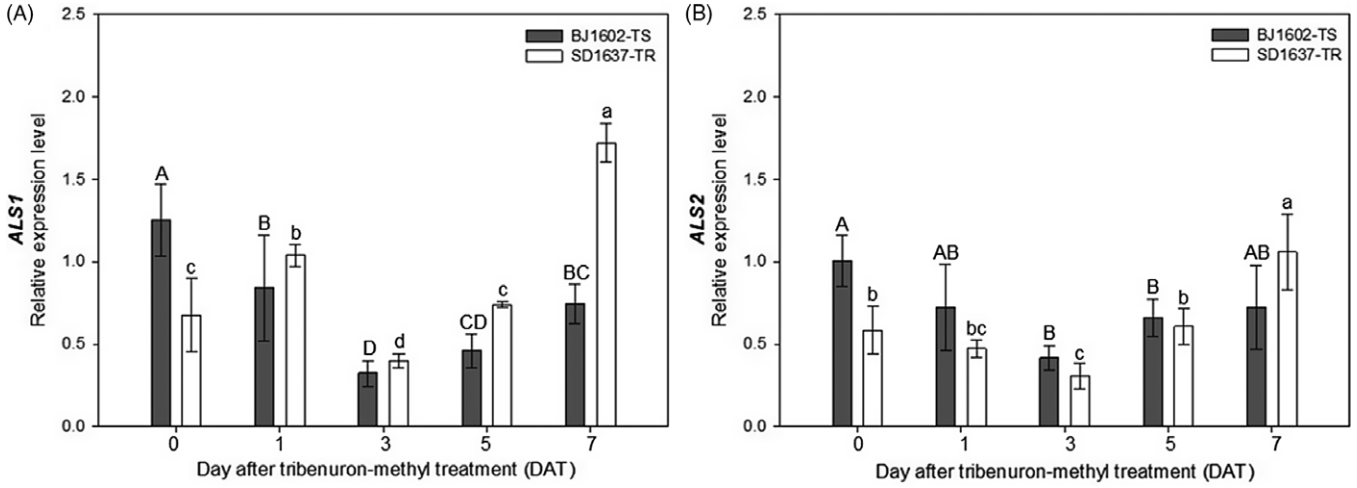

Figure 4. The relative expression levels of *ALS1* (A) (or *ALS2*, B) gene before tribenuron-methyl treatment (BT) or at 1, 3, 5, and 7 d after tribenuron-methyl treatment (DAT) in tribenuron-methyl–susceptible (TS) or (B) tribenuron-methyl–resistant (TR) *Descurainia sophia*. Each data is the mean ± SE of four replicates. Means with different capital (or lowercase) letters mean the relative expression level of *ALS* genes in TS (or TR) plants exhibited significant differences ($P < 0.05$) at BT or at 1, 3, 5, and 7 DAT, respectively.

studies on the TSR mechanism for ALS have mainly focused on the sites and amino acids of resistance mutations in ALS isozymes. With the presence of multiple mutations in a single gene or multiple mutated target isozymes in an individual plant, the resistance patterns and mechanisms will become more complex. For example, the EPSPS crystal structure from *Escherichia coli* revealed that the mutation Thr-97-Ile (Thr-102 in plants) in the presence of Ser-101 (Ser-106 in plants) causes a shift of residue Gly-96 toward the glyphosate binding site, which impairs the efficient binding of glyphosate (Funke et al. 2009). Compared with EPSPS with a double mutation, EPSPS with three mutations may form a new conformational structure with a smaller active site, leading to a higher exclusion of glyphosate (Perotti et al. 2018). The results here also indicate that the resistance contribution of two mutated ALS isozymes to tribenuron-methyl, flucetosulfuron, and pyribenzoxim is different, but there is no obvious difference in resistance to flucarbazone-sodium, flumetsulam, and imazethapyr (Table 4).

Relative Expression of ALS1 and ALS2 in TR and TS Descurainia sophia

The results in Figure 3 compare the relative expression levels of *ALS1* and *ALS2* in TS or TR *D. sophia* before treatment or at 1, 3, 5, and 7 DAT. At 1 and 7 DAT, the relative expression level of *ALS1* in TR *D. sophia* is significantly higher than that in *ALS2* by 2.2- and 1.6-fold, respectively. No obvious differences are observed before and at 3 and 5 DAT (Figure 3B). In contrast, the relative expression levels of *ALS1* and *ALS2* in TS *D. sophia* are not obviously different before or after tribenuron-methyl treatment (Figure 3A).

The results in Figure 4 compare the relative expression levels of *ALS1* (or *ALS2*) before or at 1, 3, 5, and 7 DAT in the same population. Compared with BT, the relative expression level of *ALS1* in TR *D. sophia* is significantly increased 1.5- and 2.6-fold at 1 and 7 DAT (Figure 4A). *ALS2* is not induced or slightly inhibited before 5 DAT and is increased 1.8-fold at 7 DAT (Figure 4B). In TS

D. sophia, the relative expression level of *ALS1* (or *ALS2*) at 1, 3, 5, and 7 DAT was approximately 0.7- (0.7-), 0.3- (0.4-), 0.4- (0.6-) and 0.6- (0.7-) fold that of BT, respectively (Figure 4).

The expression level of *ALS1* is significantly higher than that of *ALS2* in TR *D. sophia*, and *ALS1* is more easily induced by tribenuron-methyl than *ALS2*. Therefore, *ALS1* might be a preferred mutated target for weeds to gain resistance. The inducible expression of *ALS* has also been reported in other weeds. For example, the transcript levels for the *ALS* genes in *B. scoparia* were 7-fold higher 24 h after chlorsulfuron treatment (Varanasi et al. 2017). The transcript levels for *AHAS3* in the roots of sunflower (*Helianthus annuus* L.) were induced 4- to 5-fold by imazapyr (Breccia et al. 2013). *ALS* expression was upregulated 15.5- to 21.0-fold after mesosulfuron-methyl treatment in shortawn foxtail (*Alopecurus aequalis* Sobol.) (Zhao et al. 2018). Higher gene expression levels may lead to the accumulation of ALS isozymes, which can thus enhance the tolerance or resistance of weeds to herbicides by compensating for reduced ALS activity. However, enhanced transcription does not necessarily give rise to increased translation level, and posttranslational regulation may affect actual protein levels. Therefore, the contribution of increased *ALS* gene transcription to herbicide resistance needs more supporting data.

In summary, the novel resistance mutation combination (Asp-376-Glu in ALS1 and Pro-197-Ala in ALS2) leads to TR *D. sophia* evolving higher and broader resistance or cross-resistance to ALS-inhibiting herbicides than *D. sophia* with a single mutated ALS isozyme. The resistance contribution of two mutated ALS isozymes is different, but the contribution ratio of each ALS isozyme cannot be determined. The presence of two or multiple mutated ALS isozymes is probably the result of continuous herbicide selection on the basis of a single resistance mutation, which helps weeds survive higher doses of herbicides. However, it has to be pointed out that this study is based on a TS/TR *D. sophia* population. These results and speculations need to be verified by studying more *D. sophia* populations and even other weed species.

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