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First report of molecular basis of resistance to imazethapyr in common lambsquarters (*Chenopodium album*)

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Research Article

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Abstract

Common lambsquarters (*Chenopodium album* L.) is one of the most troublesome weeds in soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.) fields in northeast China. In 2017, a *C. album* population that survived imazethapyr at the recommended field rate was collected from a soybean field in Heilongjiang Province in China. Experiments were conducted to determine the basis of resistance to imazethapyr and investigate the herbicide-resistance pattern in *C. album*. Dose–response tests showed that the resistant population (R) displayed high resistance to imazethapyr (20-fold) compared with the susceptible population (S). An in vitro acetolactate synthase (ALS) activity assay indicated that the ALS of the R population was resistant to imazethapyr compared with the ALS of the S population. Sequence analysis of the *ALS* gene revealed that the GCA was replaced by ACA at amino acid position 122, which resulted in an alanine to threonine substitution (Ala-122-Thr) in the R population. The R population displayed cross-resistance to thifensulfuron-methyl and flumetsulam but susceptibility to bispyribac-sodium, flucarbazone, glyphosate, mesotrione, and fomesafen. These results confirmed that the basis of imazethapyr resistance in *C. album* was conferred by the Ala-122-Thr substitution in the ALS enzyme. This is the first report of the target-site basis of ALS-inhibiting herbicide resistance in *C. album*.

Introduction

Acetolactate synthase (ALS; EC 2.2.1.6) is an important enzyme for biosynthesis of the essential amino acids valine, leucine, and isoleucine (Durner et al. 1990; Umbarger 1978). It is present only in plants and microorganisms and is also the target enzyme for five classes of more than 50 commercial herbicides, including sulfonylureas (SU) (Chaleff and Mauvais 1984), imidazolinones (IMI) (Shaner et al. 1984), triazolopyrimidines (TP) (Gerwick et al. 1990), pyrimidinylthio-benzoates (PTB) (Stidham 1991), and sulfonylamino-carbonyltriazolinones (SCT) (Santel et al. 1999). These herbicides inhibit ALS activity, blocking the formation of branched-chain amino acids, and ultimately lead to plant death. ALS-inhibiting herbicides are very popular and have been used in various crops worldwide because of their potency at low use rates and their low mammalian toxicity (Mazur and Falco 1989).However, long-term and repeated use of these herbicides has accelerated the evolution of resistant weeds (Huang et al. 2019; Yu and Powles 2014).To date, 160 weed species have been reported to be resistant to one or more ALS-inhibiting herbicides (Heap 2019).

Two major mechanisms have been identified that endow resistance to ALS inhibitors in weeds. One is conferred by amino acid substitution in the ALS enzyme, which reduces sensitivity to ALS herbicides. Another is increased herbicide metabolism (Devine and Amit 2000; Liu et al. 2013). However, target-site substitution in ALS is the predominant mechanism conferring herbicide resistance. Generally, target site–based resistance to ALS inhibitors is associated with one of several amino acid substitutions in ALS. To date, eight sites (Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654) in ALS have been primarily reported to confer resistance to ALS-inhibiting herbicides (Tranel et al. 2019). It is noted that Pro-197 is the most common, followed by Trp-574 and Ser-653. Generally, a mutation at Pro-197 confers high-level resistance to SU herbicides but no resistance or low-level resistance to IMI herbicides. Ala-122 and Ser-653 mutations usually confer resistance to IMI herbicides. However, mutations at Asp-376 and Trp-574 confer broad cross-resistance to all five classes of ALS-inhibiting herbicides (Tranel et al. 2019).Moreover, different amino acid substitutions in the same ALS position conferred different cross-resistance patterns (Li et al. 2017). For example, barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] with the Ala-122-Asn/Thr substitution showed high

resistance to TP herbicides, whereas plants with the Ala-122-Val substitution displayed susceptibility to TP herbicides (Riar et al. 2013). This phenomenon was also observed for Pro-197-His/Thr substitution in corn poppy (*Papaver rhoeas* L.) (Kaloumenos et al. 2009).

Common lambsquarters (*Chenopodium album* L.) is a annual dicot weed and one of the five most widely distributed plants (Holm et al. 1977). It can produce large numbers of seeds (more than 10,000 seeds per plant) (Holm et al. 1977) and is considered to be a strong competitor with crops, causing severe crop yield losses (Thiel and Varrelmann 2014).Herbicides have been used for *C. album* control for many years. Unfortunately, overreliance on herbicides has resulted in *C. album* evolving resistance to two groups of herbicides, those targeting ALS and photosystem II (Heap 2019). However, the molecular basis of resistance to ALS-inhibiting herbicides in *C. album* remains unknown.

In autumn 2017, it was found that a *C. album* population survived the recommended field rate of imazethapyr, which is a popular ALS-inhibiting herbicide for control of broadleaf weeds in soybean [*Glycine max* (L.) Merr.] fields. Our specific objective was to investigate the basis of imazethapyr resistance and determine the herbicide resistance status of the resistant *C. album* population.

Materials and Methods

Plant Materials and Growth Conditions

The study was conducted on two *C. album* populations, one with suspected resistance (R) and one known to be susceptible (S). Seeds of the R population were collected from a soybean field where unsatisfactory control had been observed after repeated imazethapyr use in Heilongjiang Province of China (49.73°N, 125.88°E). The S seeds were obtained from a nearby fallow area without a history of herbicide use.

Chenopodium album seeds were dipped in a solution of gibberellin (100 mg L^{-1}) for 12 h to increase germination rate. Thirty seeds were sown in plastic pots (10-cm diameter) that contained mixed soil (50% peat, 25% pine bark, and 25% sand). Seedlings were thinned to 6 plants per pot at the 2-leaf stage, placed in a greenhouse (30/25 C and 16/8 h of day/night), and watered as required.

Whole-Plant Dose–Response Tests

A series of imazethapyr (50 g L^{-1}; Newpath®, BASF, Shanghai, China) doses (0, 22.5, 45, 90, 180, 360, and 720 g ai ha^{-1}) with a recommended field rate of 90 g ai ha^{-1} were applied to plants of the R and S populations (3- to 4-leaf stage) using a cabinet spray chamber (3WPSH-500D, National Engineering Research Center for Information Technology in Agriculture, Beijing, China). Dry weights of aboveground biomass from both populations were assessed at 21 d after treatment (DAT). All the treatments were replicated three times, and experiments were repeated twice to verify the results.

In Vitro ALS Inhibition Assay

Both R and S *C. album* seedlings at the 3- to 4- leaf stage were harvested and used for an ALS enzyme activity assay. The commercial formulation of imazethapyr was used at 0, 0.0078, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, and 1 µM. The ALS enzyme extraction and testing were performed according to the method of Yu et al. (2004). The ALS inhibition assay was repeated twice, with three replications per treatment.

Table 1. Primers used in this study.

Primer	Sequence	Tm	Amplification mutation site of the *ALS*
ALS-1f	5′- TTTGCCCCTGATGAACCC-3′	58	Ala-122, Pro-197, Ala-205, Asp-376, Arg-377
ALS-1r	5′- ACAAAAAAGAGCCAACGAG-3′		
ALS-2f	5′- AGCAGATTGTGAGGTTGATGAG-3′	57	Trp-574, Ser-653, Gly-654
ALS-2r	5′- TAACAAAAAAGAGCCAACGAG-3′		

ALS Gene Cloning and Sequencing

Seedlings of R and S populations at the 3- to 4-leaf leaf stage were sprayed with imazethapyr at the recommended field rate (90 g ai ha^{-1}), 10 surviving plants of the R population were individually harvested, and genomic DNA was extracted using a DNA extraction kit (TianGen Biotechnology, China).

To investigate whether the *ALS* gene mutation exists in the R population, two primer pairs (Table 1) were designed according to the *ALS* sequence of quinoa (*Chenopodium quinoa* Willd.) (GenBank no. LOC110717632) to clone the *ALS* gene of *C. album*. PCR was performed in a 30-µl reaction mixture containing 1 µl of genomic DNA, 1 µl each of both forward and reverse primers, 15 µl of Taq 2×master mix (Biomed Biotech, Beijing, China), and 13 µl of nuclease-free water. Each PCR was performed with the following temperature profile: 94 C for 5 min; 30 cycles of 94 C for 30 s; 30 s at 58 C and 57 C for the two primer pairs, respectively; 72 C for 1 min; and a final extension at 72 C for 5 min. PCR products from 10 individuals of each population were excised from agarose gel and sequenced by Biomed Biotech. The nucleotide and amino acid sequences of interest were aligned and compared using Vector NTI v. 9.0.

Single-Dose Herbicide-Resistance Assay

To study the R population's herbicide resistance status with respect to other ALS-inhibiting herbicides and herbicides with different sites of action, including 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), hydroxyphenylpyruvate dioxidase (HPPD), and protoporphyrinogen oxidase (PPO), seedlings of both R and S populations at the 3- to 4-leaf stage were treated with herbicides at the recommended field doses (Table 2). Herbicide treatment methods were performed as described for the dose–response tests. Plant mortality was recorded at 21 DAT. Plants were considered to be survivors if there was new shoot formation. A plant survival rate ≥80% was considered to represent resistance, and ≤20% was considered to represent susceptibility. For each herbicide treatment, 10 pots (6 plants per pot) each from the R and S population were tested. The experiment was conducted with a randomized complete block design and repeated twice.

Statistical Analysis

For dose–response and in vitro ALS activity assays (expressed as a percentage of the control), we used nonlinear regression analysis to determine the GR_{50} (herbicide dose causing 50% plant growth) and I_{50} (herbicide concentration that inhibited ALS activity by 50%) with SigmaPlot software (v. 12.0, SigmaPlot Software, Chicago, IL, USA). The data were fit to a log-logistic model (Seefeldt et al. 1995):

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Table 2. Information for herbicides used in the single-dose herbicide-resistance assay.

Target[a]	Herbicide[b]	Manufacturer	Website	Dose
				—g ai ha^{-1}—
ALS	Thifensulfuron-methyl (25% WP)	Fengle Agrochemical, Anhui, China	http://www.flnh.com.cn	30
ALS	Flumetsulam (80% WDG)	Dow AgroSciences, Midland, MI, USA	http://www.dow.com	57
ALS	Bispyribac-sodium (10% WP)	Hetian Chemical, Shenyang, China	http://www.agronet.com.cn	10
ALS	Flucarbazone (70% WDG)	Arysta LifeScience, Cary, NC, USA	http://www.arysta.com.co	30
EPSPS	Glyphosate (30% AS)	Monsanto, St Louis, MO, USA	https://monsanto.com	900
HPPD	Mesotrione (15% SC)	Jiner Agrochemical, Qingdao, China	http://www.qingdaojiner.com/index.html	135
PPO	Fomesafen (250 g L^{-1}AS)	Qiaochang Modern Agriculture, Binzhou, China	http://www.qiaochang.com	275

[a]ALS, acetolactate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; HPPD, hydroxyphenylpyruvate dioxidase; PPO, protoporphyrinogen oxidase.
[b]AS, aqueous solution; SC, suspension concentrate; WDG, water-dispersible granule; WP, wettable powder.

$$Y = C + [(D - C)/[1 + (x/\mathrm{ED}_{50})^{b}] \quad [1]$$

In this equation, Y is plant dry weight, C is the lower response limit, D is the upper response limit, b is the slope of the curve, and ED_{50} represents GR_{50} or I_{50}. The resistance index (RI) was used to calculate the resistance level of the R population compared with the S population. Tukey's test (P = 0.05) was used to calculate the significance of the parameters.

Results and Discussion

Whole-Plant Dose Response

The dose–response tests were conducted to determine the resistance levels of the R and S populations to imazethapyr. The results showed that the S plants were all killed when treated with imazethapyr at the recommended field dose (90 g ai ha^{-1}), while the R plants were 20% controlled. The dry weight of the R population was reduced by 50% with the 387.41 g ai ha^{-1} rate of imazethapyr, while it was reduced by half with the 19.04 g ai ha^{-1} imazethapyr rate in the S population (Table 3). Based on the GR_{50} value, the R population was 20-fold more resistant to imazethapyr compared with the S population (Table 3; Figure 1). However, this RI was lower than RI values observed in eastern black nightshade (*Solanum ptychanthum* Dunal) (Milliman et al. 2003) and wild radish (*Raphanus raphanistrum* L.) with the Ala-122 substitution (Han et al. 2012). Moreover, the RI for IMIs was much lower than those reported for Ser-653-Thr substitution and Trp-574-Leu substitutions in Powell amaranth (*Amaranthus powellii* S. Watson) (Ferguson et al. 2001), but similar to results for the Asp-376-Glu substitution in redroot pigweed (*Amaranthus retroflexus* L.) (Huang et al. 2016).

Herbicide-resistant weeds have seriously threatened the world's food security and caused great losses for crop producers (Lonhienne et al. 2018). Several weed species resistant to ALS-inhibiting herbicides have been documented in soybean fields, including waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] (Trucco et al. 2009), hairy beggarticks (*Bidens pilosa* L.) (Baio et al. 2013), and *A. retroflexus* (Huang et al. 2016). Therefore, investigating the molecular basis of the R *C. album* population is critical for developing successful control programs to slow the herbicide resistance evolution.

In Vitro ALS Inhibition Assay

Results for the in vitro ALS inhibition assay showed that the total ALS activity for the S population (10.24 ± 0.68 nmol acetoin mg^{-1} protein min^{-1}) was similar to that of the R population (11.39 ± 0.59 nmol acetoin mg^{-1} protein min^{-1}) (Table 4). ALS of the S population displayed sensitivity to imazethapyr (I_{50} = 0.015); in contrast, ALS of the R plants exhibited high resistance to imazethapyr (I_{50} = 0.27). As shown in Table 4, the RI was 18 (Table 4; Figure 2). This result was consistent with the dose–response tests. The large difference in I_{50} values between R and S populations supports the presence of a target site–based resistance mechanism. Therefore, we suppose that there may be an amino acid substitution in the ALS that reduced sensitivity to ALS inhibitors and confers resistance to imazethapyr in the R population.

The continuous use of herbicides with the same site of action has greatly increased the selection pressure of weed

Table 3. GR_{50} values of R and S populations treated with imazethapyr.[a]

Population	GR_{50} (SE)	RI[b]
	—g ai ha^{-1}—	
S	19.04 (2.15)	—
R	387.41 (26.34)	20.35

[a]GR_{50}, herbicide dose causing 50% plant growth compared with the untreated control; R, resistant population; S, susceptible population.
[b]RI (resistance index) was used to calculate the resistance level of the R population compared with the S population.

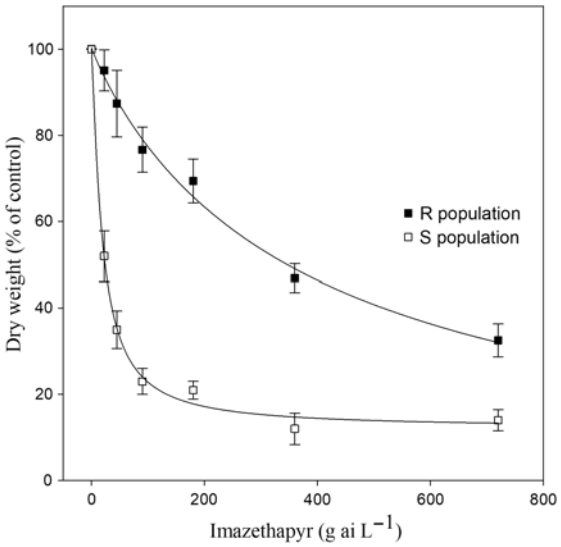

Figure 1. Dose–response curve for *Chenopodium album* dry weights of the resistant (R) and susceptible (S) populations. Dry weight was expressed as a percentage of the untreated control. Each point was calculated using three replications, and experiments were repeated twice to verify the results. Vertical bars represent the standard error (P = 0.05).

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Table 4. In vitro ALS activity of R and S populations.[a]

Population	Total ALS activity	R/S	I_{50}(SE)[b]	RI[c]
	(nmol acetoin mg^{-1}protein min^{-1})		—g ai L^{-1}—	
S	10.24 (0.68)	–	0.015(0.004)	–
R	11.39 (0.59)	1.1	0.27(0.06)	18

[a]ALS, acetolactate synthase; R, resistant population; S, susceptible population.
[b]I_{50}, herbicide dose required to inhibit the ALS activity by 50% compared with the untreated control.
[c]RI (resistance index) was used to calculate the resistance level of the R population compared with the S population.

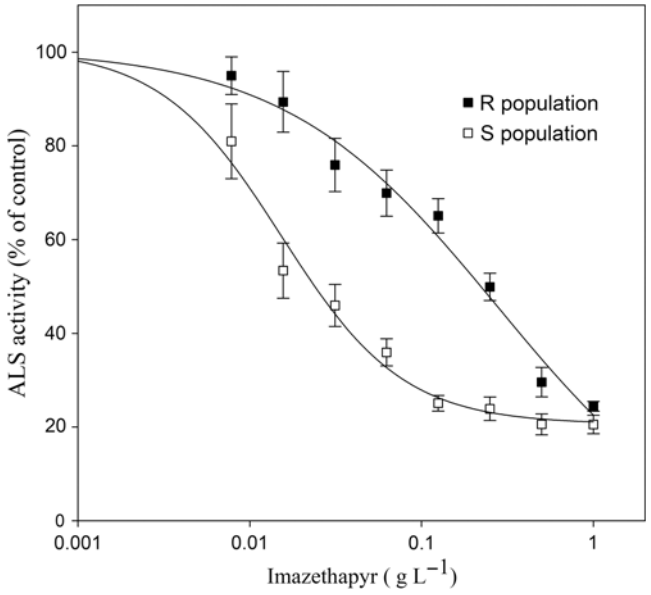

Figure 2. In vitro acetolactate synthase (ALS) activity assays in response to imazethapyr from resistant (R) and susceptible (S) populations. Each data point represents the mean of experiments repeated twice, with three replications per treatment, and vertical bars represent standard errors of the means (P = 0.05).

herbicide-resistant evolution. In most cases, the resistance mechanism to ALS-inhibiting herbicides is endowed by reduced target-enzyme sensitivity to the herbicide. In this study, the ALS activity assay showed that the ALS of the S *C. album* population was much more inhibited than that of the R population. This result was consistent with previous studies finding that many ALS inhibitor–resistant weed populations exhibit lower ALS sensitivity to the herbicide compared with susceptible populations (Tranel and Wright 2002; Yu et al. 2007, 2010). In our study, both dose–response tests and the in vitro ALS activity assay indicated that the R population was highly resistant to imazethapyr, indicating that target site–based resistance, such as an amino acid substitution in the ALS, was present in the R population.

Ala-122-Thr Substitution Found in Plants of the R Population

The *ALS* genes from plants of R and S *C. album* populations were cloned. Comparison of the *ALS* gene between R and S plants revealed a single nucleotide change of GCA to ACA, leading to an alanine to threonine substitution at amino acid position 122 (Figure 3). Moreover, no other known mutations were detected at Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, or Gly-654 in the R population.

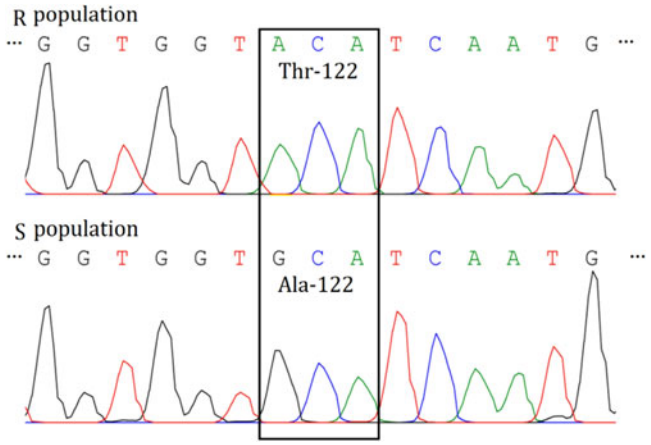

Figure 3. Comparison of partial sequences of *ALS* gene from resistant (R) and susceptible (S) populations. The amino acid position of ALS was based on the *Arabidopsis thaliana* sequence.

Resistance to ALS-inhibiting herbicides is mainly conferred by ALS mutation. To date, eight main sites with various substitutions in ALS have been reported to be resistant to ALS-inhibiting herbicides in different weed species (Tranel et al. 2019). In particular, there are at least nine weed species that carry the amino acid substitutions at position 122 of ALS, and five different substitutions have been reported, including Ala-122 mutations to Thr, Val, Tyr, Ser, and Asn (Tranel et al. 2019). In our study, ALS sequence analysis showed that the Ala-122-Thr substitution was present in the R population. This substitution was previously documented to endow herbicide resistance in common cocklebur (*Xanthium strumarium* L.) (Bernasconi et al. 1995), *S. ptychanthum* (Milliman et al. 2003), *A. retroflexus* (McNaughton et al. 2005), and Palmer amaranth (*Amaranthus palmeri* S. Watson) (Singh et al. 2019). Therefore, the Ala-122-Thr substitution in ALS likely conferred imazethapyr resistance in the R population.

Single-Dose Herbicide-Resistance Assay

To investigate the herbicide resistance status of the R *C. album* population, its response to different herbicides was assessed. The results indicated that the R population was 100% controlled by bispyribac-sodium (PTB) and flucarbazone (SCT), as well as herbicides with other sites of action, including glyphosate (EPSPS inhibitor), mesotrione (HPPD inhibitor), and fomesafen (PPO inhibitor), similar to the S population. However, the R population showed cross-resistance (>80% survival) to the thifensulfuron-methyl (SU) and flumetsulam (TP) (>90% survival) (Table 5).

Previous studies showed that plants with the Ala-122-Thr substitution in ALS exhibit cross-resistance to ALS-inhibiting herbicides. For example, the Ala-122-Thr substitution in *E. crus-galli* conferred resistance to IMI and TP herbicides, but did not confer cross-resistance to PTB herbicides (Riar et al. 2013). The same phenomenon was also observed in our study. Moreover, our results are consistent with the findings of McNaughton et al. (2005), who reported the Ala-122-Thr substitution endowing high-level resistance to an IMI herbicide in an *A. palmeri* population. However, in that study, the Ala-122-Thr mutation did not confer cross-resistance to SU herbicides, and the resistance to TP, PTB, and SCT herbicides was not determined. Furthermore, the Ala-122-Thr substitution has previously been reported to

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Table 5. Percentage survival of the R and S populations at 21 d after treatment.[a]

Target[b]	Herbicide	Number of plants	Survival rate of S	Survival rate of R
			—%—	—%—
ALS	Thifensulfuron-methyl	60	0	83
ALS	Flumetsulam	60	0	92
ALS	Bispyribac-sodium	60	0	0
ALS	Flucarbazone	60	0	0
EPSPS	Glyphosate	60	0	0
HPPD	Mesotrione	60	0	0
PPO	Fomesafen	60	0	0

[a]R, resistant population; S, susceptible population.
[b]ALS, acetolactate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; HPPD, hydroxyphenylpyruvate dioxidase; PPO, protoporphyrinogen oxidase.

confer resistance to IMI herbicides but not to SU herbicides (Tranel and Wright 2002). This is the first report of resistance to sulfonylureas provided by Ala-122-Thr in dicots. Our results would suggest that another mechanism might be present in the R *C. album* population to explain the resistance discrepancy. More research into the pattern of resistance conferred by the Ala-122-Thr mutation is required.

Different resistance patterns can potentially be explained by ALS 3D or crystal structure studies (Duggleby et al. 2008; McCourt et al. 2006), which are helpful for understanding the effect of crucial amino acids in herbicide binding to ALS in plants. The Ala-122-Thr substitution is commonly associated with IMI herbicide resistance, likely because Thr-122 would affect IMI herbicide binding and have low affinities for IMI herbicides. The R population in our study displayed cross-resistance to thifensulfuron-methyl (SU) and flumetsulam (TP); it may pose a large agronomic threat and increase weed management challenges. Fortunately, it did not show resistance to glyphosate (EPSPS inhibitor), mesotrione (HPPD inhibitor), or fomesafen (PPO inhibitor). Hence, herbicides with other sites of action should be selected to achieve successful control of the R population.

In summary, this *C. album* population from China with evolved imazethapyr resistance is the first confirmed case of *C. album* resistance to ALS-inhibiting herbicides. Sequence analysis and an ALS activity assay indicated that the resistance to imazethapyr was conferred by an Ala-122-Thr substitution in the ALS enzyme. This study will assist in planning sustainable weed management programs. To our knowledge, this is the first report of the molecular basis of ALS-inhibitor resistance conferred by an Ala-122-Thr substitution in *C. album*. Thus, herbicides with another site of action should be employed to control this *C. album* population. In addition, growers should integrate chemical control with other tools, such as nonchemical weed management options, to achieve a sustainable weed management program.

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