agronomy

MDPI

Article

A^{205}V, D^{376}E, W^{574}L, S^{653}T, and S^{653}N Substitutions in Acetohydroxy Acid Synthase from *Amaranthus retroflexus* L. Show Different Functional Impacts on Herbicide Resistance

Zhonghua Sun [1,†], Jianan Cong [1,†], Wenli Cao [1,†], Guang Yuan [1], Zhen Meng [1], Shen Wang [1], Chunjie Li [2] and Chunhong Teng [1,*]

[1] College of Plant Protection, Northeast Agricultural University, Harbin 150030, China; osunzhonghua@163.com (Z.S.); 18746385968@163.com (J.C.); 19970041860@163.com (W.C.); 15647439706@163.com (G.Y.); 17341062832@163.com (Z.M.); ws18346518997@163.com (S.W.)
[2] State Key Laboratory of Black Soils Conservation and Utilization, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China; lichunjie@iga.ac.cn
* Correspondence: tengch@neau.edu.cn
† These authors contributed equally to this work.

Abstract: *Amaranthus retroflexus* L. is a troublesome dicot weed in crop fields and has developed high resistance to nicosulfuron in China. The objective of this study was to determine the effects of specific resistance mutations (A^{205}V, D^{376}E, W^{574}L, S^{653}T, and S^{653}N) of the acetohydroxy acid synthase enzyme (AHAS) on the resistance of *A. retroflexus* to nicosulfuron. The resistance mutations in *A. retroflexus* not only conferred 17.17- to 31.70-fold resistance to nicosulfuron but also greatly decreased AHAS sensitivity and increased AHAS binding affinity to substrate pyruvate, which mechanisms were primarily responsible for the observed *A. retroflexus* resistance. Molecular docking results indicated that these resistance mutations altered AHAS binding free energy with nicosulfuron. All the resistance mutations showed less sensitivity to feedback inhibition by branched-chain amino acids, but the mutations did not necessarily affect biosynthesis in *A. retroflexus*. This report to compares the various mutations of ArAHAS in vitro and contributes to understanding herbicide resistance in this field weed.

Keywords: *Amaranthus retroflexus* L.; acetohydroxy acid synthase; resistance mutation; cross resistance

Citation: Sun, Z.; Cong, J.; Cao, W.; Yuan, G.; Meng, Z.; Wang, S.; Li, C.; Teng, C. A^{205}V, D^{376}E, W^{574}L, S^{653}T, and S^{653}N Substitutions in Acetohydroxy Acid Synthase from *Amaranthus retroflexus* L. Show Different Functional Impacts on Herbicide Resistance. *Agronomy* **2024**, *14*, 2148. https://doi.org/10.3390/agronomy14092148

Academic Editors: Judith Wirth, Agnieszka Lejman and Shouhui Wei

Received: 27 July 2024
Revised: 11 September 2024
Accepted: 19 September 2024
Published: 20 September 2024

1. Introduction

Herbicide resistance causes economic loss, threatens food safety, disrupts ecological equilibrium, and has been a severe global challenge [1,2]. Resistance mechanisms to acetohydroxy acid synthase (AHAS)-inhibiting herbicides can be classified into two main types, namely, target site-based resistance (TSR) and non-target site-based resistance (NTSR). TSR mechanisms primarily include amino acid mutations in the herbicide target protein and overexpression of the target enzyme gene. NTSR is generally characterized by reduction in translocation/uptake and enhanced vacuolar metabolism or sequestration of the herbicide [3–5].

Amaranthus retroflexus L. is a common, malignant, annual dicot weed in China. *A. retroflexus* exhibits strong environmental adaption as a monoecious C_4 species, is highly self-fertilized with occasional outcrossing, and produces a large number of seeds. It can constantly compete with crops for CO_2, water, light, and nutrients from emergence through to harvest [6]. Crop production in fields has considerably decreased because of the inability to have timely control of this weed, thereby affecting soybean (losses of approximately 58%) and corn (losses of 5–34%) production [7]. In the last thirty years, an AHAS-inhibiting herbicide represented by nicosulfuron with low cost and low toxicity to humans has been the preferred herbicide for controlling weeds in crop fields in northeast China. At

present, it has a poor controlling effect on *A. retroflexus* due to resistances evolved during long-term application.

AHAS (enzyme classification code, EC 2.2.1.6) is an enzyme in the first step of the biosynthesis of branched-chain amino acids (BCAAs), responsible for the production of L-isoleucine, L-leucine, and L-valine in microorganisms and plants but not in humans and animals. AHAS is composed of a catalytic subunit (CSU) and a regulatory subunit (RSU). The RSU enhances not only the activity of the CSU but also activities of the feedback inhibitors of BCAAs [8–10]. Due to their importance in protecting plants, AHAS inhibitors have been used in 58 commercial herbicides of the following five chemical families: sulfonylureas, triazolopyrimidine (TP), sulfonyl-aminocarbonyl-triazolinone (SCT), pyrimidinyl-thiobenzoate (PTB), and imidazolinone (IMI) [11,12]. As AHAS inhibitors are highly efficacious herbicides, have low toxicity in mammals, and have a broad spectrum of activity, they have been widely used for weed control in corn and soybean fields in China. Masses of weed species have evolved resistance to AHAS inhibitors due to persistent and intensive use of these herbicides in the late 20th century [13,14]. Currently, the following resistance mutation sites have been identified in AHAS in more than 50 weed species: Ala_{122}, Pro_{197}, Ala_{205}, Phe_{206}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654} [15]. Gene mutation probably leads to amino acid substitution, which influences AHAS-binding capacity with herbicide molecules and results in evolutionary adaption of weed species to herbicides [16,17]. Recently, effects of different AHAS-resistance mutation sites on competitiveness, growth, and pigments of *A. retroflexus* have been reported [18,19]. However, the resistance substitutions' effects on AHAS characterizations are still unknown in *A. retroflexus*. In this study, we aimed to characterize AHAS in *A. retroflexus* (enzyme kinetics, catalytic activity, and feedback inhibition by BCAAs), conduct a computer-based analysis of the binding of AHAS with nicosulfuron, and understand the effects of the resistance substitutions on cross resistance to other AHAS-inhibiting herbicides.

2. Materials and Methods

2.1. Sources of Plant Material

A. retroflexus seeds were collected from different cities across the Heilongjiang province in northeast China (Table 1). The seeds were soaked in 10% hydrogen peroxide solution for 22 min, rinsed with distilled water 6 times, allowed to germinate at 27 °C for 24 h, and transferred to garden pots (diameter: 12 cm, height: 10 cm). Each pot had approximately 5–6 seeds that were 1-cm deep under the Phaeozem. The pots were placed in the heliogreenhouse at Northeast Agricultural University in Harbin, Heilongjiang Province, from May to October each year from 2020 to 2023, with daytime temperatures averaging 25.2 ± 5.1 °C and nighttime temperatures averaging 14.8 ± 3.3 °C. A micro sprayer (EIDOLON ED-WC-GYPB, spray volume: 40 mL, spray caliber: 0.5 mm, pressure: 0.3 MPa) was used to treat *A. retroflexus* at the uniform 4-leaf stage with 75% nicosulfuron WDG (Anhui Fengle Agrochemical Inc., Hefei, China) at the recommended field dosage of 67.5 g.a.i. ha^{-1}, and the seeds were collected from surviving individuals.

Table 1. *A. retroflexus* geographical coordinates and AHAS-inhibiting herbicides application histories.

Name	Collection Locations	Latitude	Longitude	Application Years
S	Shitouhezizhen, Shangzhishi	N44.8559722	E128.6916972	0
R1	Youyinongchang, Shuangyasha	N46.7815583	E131.8107139	15
R2	Meilisi, Qiqihaer	N47.3095000	E123.7528111	18
R3	Zhaoguangnongchang, Heihe	N50.2521000	E127.5004806	17
R4	Dongfanghongshuiku, Hailun	N47.6190306	E127.1472167	20
R5	Yanganxiang, Beian	N48.2414611	E126.4910806	19

2.2. Identification of ArAHAS Gene Mutations

Weed genomic DNA was extracted from about 1.5 g of fresh tissues from each purified plant genotype following a previous experimental procedure described in Doyle and Doyle [20,21] and quantified using a NanoReady FC-3100 spectrophotometer (Life Real Inc., Hangzhou, China). The ArAHAS gene primer was designed following the sequences of *A. retroflexus* (AF363369.1). The final volume of the polymerase chain reaction (PCR) was 25 µL, which contained 1 µL of genomic DNA, 1 µL each of the forward/reverse primers, and 2 µL of 2× PrimeSTAR® Max DNA Polymerase (TaKaRa Bio. Inc., Osaka, Japan). DNA amplification was performed using a BIOER 1000 PCR with denaturation temperature at 98 °C for 11 s, followed by 36 cycles of a denaturation step at 60 °C for 16 s and an extension step at 75 °C for 12 s (Table 2). The PCR products were purified using a TaKaRa Mini BESTA agarose Gel DNA Extraction kit and identified using 1% agarose gel electrophoresis. The bidirectional sequencing of the PCR fragments (Genewiz Inc., Beijing, China) were aligned using SnapGene version 2.6.2 software (Snap Gene Inc., Chicago, IL, USA).

Table 2. Gene-specific primers for *A. retroflexus* AHAS gene amplification.

Primer	Sequence	Amplification Size (bp)	Mutation Sites
Forward 1	CAATCATCCATTTACGCTAT	782	122.197.205
Reverse 1	AGACTCACCCACTAACCTTAC		
Forward 2	GAGTCTAAGAGACCTGTGCT	1111	376.377.574.653.654
Reverse 2	GCTTCTCCTCTATAAGGATC		

2.3. Whole-Plant Dose Responses to Nicosulfuron

The resistant genotypes were treated with nicosulfuron at a concentration of 60, 30, 15, 7.5, 3.75, 1.88, and 0 g.a.i. ha^{-1}, and the wild type was sprayed at a concentration of 15, 7.5, 3.75, 1.88, 0.94, 0.47, and 0 g.a.i. ha^{-1} (Table 3). Each treatment was performed in triplicate. After 21 d, the aboveground plant tissues were dried at 75 °C for 75 h, and then dry biomass was measured for further data analysis.

Table 3. List of AHAS-inhibiting herbicides dosages.

Herbicides	Chemical Type	Rate (g.a.i.ha^{-1})	Rate Range of Dose (g.a.i. ha^{-1})	
			S	R
Nicosulfuron	SU	60	0, 0.47, 0.94, 1.88, 3.75, 7.5, 15	0, 1.88, 3.75, 7.5, 15, 30, 60
Flumetsulam	TP	30	0.94, 1.89, 3.75, 7.5, 15, 30	7.5, 15, 30, 60, 120, 240
Bispyribac-sodium	PTB	27	1.69, 3.38, 6.75, 13.5, 27, 54	1.69, 3.38, 6.75, 13.5, 27, 54
Imazethapyr	IMI	100.5	3.14, 6.28, 12.56, 25.13, 50.25, 100.5	25.13, 50.25, 100.5, 201, 402, 603
Flucarbazone-sodium	SCT	31.5	0.98, 1.97, 3.94, 7.88, 15.75, 31.5	7.88, 15.75, 31.5, 63, 126, 252

Chemical type: SU: sulfonylurea, TP: triazolopyrimidine, PTB: pyrimidinylthiobenzoate, IMI: imidazolinone, SCT: sulfonyl-aminocarbonyl-triazolinone.

2.4. In Vitro ArAHAS Activity Assay

Seedlings were grown from seeds collected from surviving individuals as described in Section 2.1. Fresh leaf tissues were collected at the 3–4-leaf stage from 6 genotypes and homogenized with a mortar and pestle in liquid nitrogen. ArAHAS enzyme extraction and herbicide-inhibition assays were performed as described by Yu [22] and Sun [23]. For the resistant and wild-type genotypes, 98% analytical-grade nicosulfuron was used at concentrations of 2000, 200, 20, 2, 0.02, 0.002, 0.0002, and 0 µM. The Bradford assay was used to quantify protein content in extracts [24]. ArAHAS activity was detected by determining acetoin production at 530 nm using a microplate spectrophotometer (BioTek Inc., Burlington, VT, USA). The assay was performed in triplicate with independent ArAHAS extractions.

2.5. Vmax and Km (Pyruvate) Assay for ArAHAS

The V_{max} and K_m (pyruvate) assay for ArAHAS was performed as described in Section 2.4. The difference for sodium pyruvate assay was a lack of elution and extraction buffers, and nicosulfuron was replaced with sodium pyruvate at concentrations of 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 mM in the reaction mixtures. Michaelis–Menten enzyme kinetics was used to calculate the V_{max} and K_m (pyruvate) as described in Equation (3). Each experiment was performed 3 times [25,26].

2.6. ArAHAS Feedback-Regulation Assays

In the feedback-regulation assays, nicosulfuron was replaced with isoleucine, valine, and leucine. The final concentrations of each branched-chain amino acid were 10, 2, 0.4, 0.08, 0.016, 0.0032, and 0 mM in the reaction mixtures and each experiment was performed 9 times.

2.7. Determination of Free Amino Acids in A. retroflexus

The offspring of surviving individuals were cultured 30 days and, the over-ground tissues were harvested at the 5–6-leaf stage. The leaves were dried at 48 °C for 52 h, powdered with a mortar and pestle, and homogenized in liquid nitrogen. Next, 100 mg of the powder was added to 10 mL of 10% sulfosalicylic acid solution and kept at 60 °C for 1 h in a water bath. After centrifuging at 10,000 rpm for 21 min, the mixture was allowed to stand overnight at 4 °C. Then 1 mL supernatant was filtered through a 0.22-μM membrane (Agilent Technologies Inc., Pola Alto, CA, USA) before determining the free amino acid concentration. An automatic amino acid analyzer (LA8080, HITACHI Inc., Tokyo, Japan) equipped with a 4.6 mm ID × 60 mm analytical column was used for free amino acid concentration analysis. Lithium citrate tetrahydrate buffers with pH ranging from 2.8–4.1 were used as a mobile phase in gradient elution mode at a flow rate of 0.35 mL/min. Ninhydrin solution was used for post-column reaction of analytes at a flow rate of 0.2 mL/min. All free amino acids were identified using an ultraviolet detector at 570 nm, apart from proline and asparagine for which a wavelength of 440 nm was used. Standard amino acids (Alta scientific Inc., Tianjin, China) were used for quantification and identification, and the concentration units of mg/g were used [27,28].

2.8. Determination of Cross Resistance

All genotypes were sprayed with AHAS inhibitors at the recommended field dosages to determine the AHAS mutations causing cross resistance toward the AHAS-inhibiting herbicides. The herbicides were applied at the following dosages: flumetsulam (TP) (10% SC, Liaoning Haijia Agrochemical Inc., Fushun, China) at 30 g.a.i. ha^{-1}, bispyribac-sodium (PTB) (20% OD, Anhui Golden Land Biotechnology Inc., Bengbu, China) at 27 g.a.i. ha^{-1}, imazethapyr (IMI) (10% AS, Jiangsu Ruibang Agrochemical Inc., Nantong, China) at 100.5 g.a.i. ha^{-1}, and flucarbazone-sodium (SCT) (70% WDG, Anhui Jiuyi Agriculture Inc., Bozhou, China) at 31.5 g.a.i. ha^{-1}. The recommended dosages of flumetsulam (TP), bispyribac-sodium (PTB), imazethapyr (IMI), and flucarbazone-sodium (SCT) are listed in Table 3. Whole-plant dose responses to AHAS-inhibiting herbicides were determined following the method described in Section 2.3. All data points were selected to obtain a sigmoidal dose–response curve. Each treatment was performed in triplicate, and all experiments were performed 3 times.

2.9. Molecular Binding Mode Analysis for ArAHAS and Nicosulfuron

The molecular structure of nicosulfuron was constructed using ChemDraw (CambridgeSoft Corporation, Cambridge, MA, USA). The ArAHAS enzyme amino acid sequence of *A. retroflexus* shows 90% and 86% homology with *Chenopodium album* (Model ID: AF-A0A5B9A0D2-F1) and *Arabidopsis thaliana* AHAS $P^{197}T$ mutant in complex with bispyribac-sodium (PDB ID: 7TZZ). Therefore, 3D models of the wild type and 5 substituted ArAHASs ($A^{205}V$, $D^{376}E$, $W^{574}L$, $S^{653}T$, and $S^{653}N$) were constructed using the above protein homol-

ogy modeling and using the Swiss-Model online server (https://swissmodel.expasy.org, accessed on 1 January 2024) [29,30]. Molecular docking was operated using Autodock 4.0 (Scripps Research Institute, San Diego, CA, USA), and docking consequences were visualized using PyMOL (DeLano Scientific LLC, San Francisco, CA, USA).

2.10. Data Analysis

Whole-plant responses and in vitro ArAHAS-activity assay datasets were analyzed by analysis of variance using SPSS 21.0 (IBM, Armonk, New York, NY USA). The *t*-test ($p < 0.05$) was applied to evaluate the significance of regression parameters. Dose–response curves (sigmoidal logistic, 4 parameters) were executed using SigmaPlot 12.5 [31].

In the whole-plant response single-sigmoid curve (Equation (1)), x is the log of AHAS-inhibiting herbicide dosage, y represents the percentage of *A. retroflexus* dry weight (% control), GR_{50} (50% growth inhibition) is the herbicide rate, b is the slope at the half-maximal effective dosage, and D and C are the upper and lower limits of the response equation, respectively.

$$y = C + (D - C)/[1 + \left(x/GR_{50}\right)^{b}\Big], \quad (1)$$

The dose–response curves from the in vitro ArAHAS assays and ArAHAS feedback inhibition by BCAAs assays were performed as double-sigmoid curves (Equation (2)), where y is the percentage of ArAHAS activity (% control), x is the log of the herbicide dosage, p represents the synthetic ratio of f(x) and g(x), a referred to as f(x) represents the log of the I_{50} of one sigmoid curve, and b referred to as g(x) represents the log of the I_{50} of the other sigmoid curve located in higher concentration regions than f(x) ($b > a$) [32–34].

$$y = p \times f(x) + (1 - p) \times g(x), \quad (2)$$

$$f(x) = 100/\Big[1 + 10^{(a-x)}\Big],$$

$$g(x) = 100/\Big[1 + 10^{(b-x)}\Big],$$

V_{max} and K_m (pyruvate) assay of ArAHAS recognized as the Michaelis–Menten model (Equation (3)) were prepared using GraphPad 8 (GraphPad Software, Boston, MA, USA). S is the content of sodium pyruvate, and V represents reaction velocity [35,36].

$$V = V_{max} \times S/(K_m + S) \quad (3)$$

3. Results

3.1. Dose–Response Assays

The levels of resistance to nicosulfuron of the five *A. retroflexus* resistance-mutation genotypes and the wild type were evaluated using dose–response experiments. The resistant genotypes were collected from the surviving individuals after treatment with nicosulfuron at the recommended field dose. All the resistant genotypes exhibited extremely high resistance to nicosulfuron; however, the wild type exhibited significant inhibition of growth (Figure 1). The GR50 of the wild type was 1.29 g.a.i. ha^{-1} nicosulfuron, while the resistant genotypes were highly resistant with >10-fold values of the resistance index (Table 4).

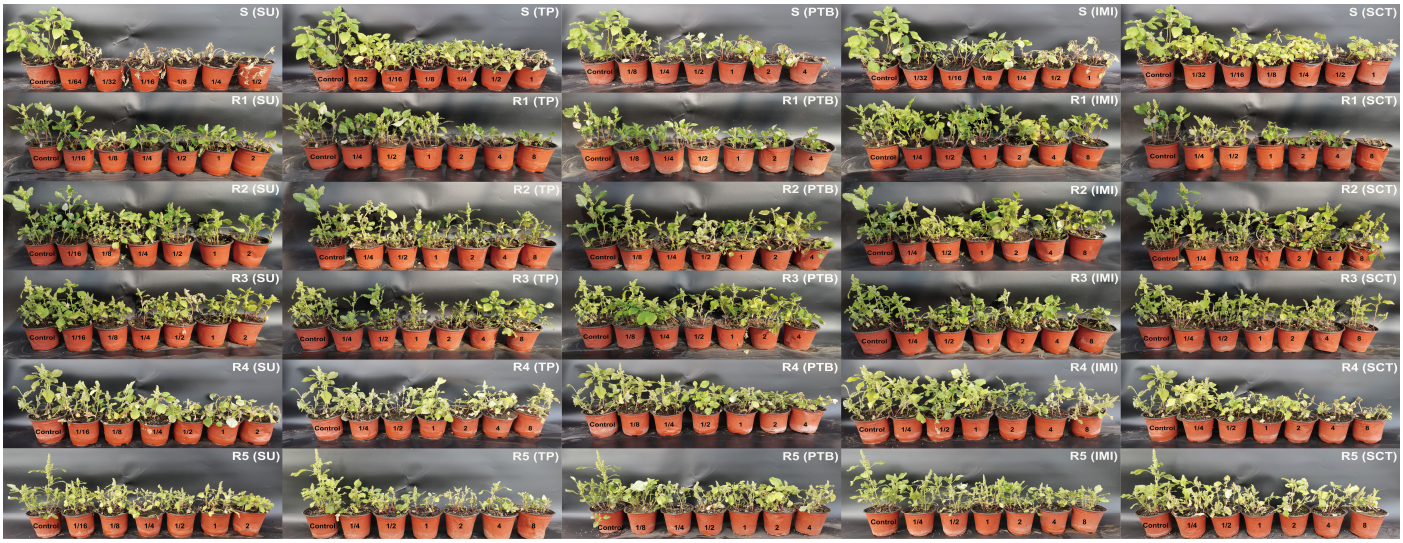

Figure 1. The different genotypes (S, R1, R2, R3, R4, and R5) of *A. retroflexus* 21 days after AHAS-inhibiting herbicides treatments at doses indicated by labels superimposed on the pots.

Table 4. GR_{50} and I_{50} values of nicosulfuron for wild-type and resistance-mutation genotypes of *A. retroflexus*.

Name	Mutation	GR_{50} (g.a.i.ha^{-1})	RI	Total ALS Activity (nmol Acetoin mg^{-1} Protein min^{-1})	I_{50} (μM)	RI
S	Wild type	1.28 ± 0.18 c		48.75 ± 1.47 d	0.99 ± 0.34	
R1	Ala-205-Val	23.18 ± 5.44 b	18.11	52.38 ± 0.65 d	12.31 ± 5.23	12.43
R2	Asp-376-Glu	21.98 ± 3.72 c	17.17	63.41 ± 1.46 c	24.70 ± 4.36	24.95
R3	Trp-574-Leu	40.55 ± 8.36 a	31.70	151.29 ± 7.38 a	42.83 ± 12.69	43.26
R4	Ser-653-Thr	24.96 ± 4.40 b	19.50	110.56 ± 1.64 b	32.62 ± 11.43	32.95
R5	Ser-653-Asn	22.44 ± 1.04 b	17.53	109.22 ± 1.21 b	24.35 ± 7.45	24.60

GR_{50}: Herbicide rate causing 50% growth reduction of plant. I_{50}: Herbicide rate causing 50% inhibition of AHAS enzyme activity. RI (Resistance index) = R-GR_{50}/S-GR_{50}. Different letters represents significance test of difference.

3.2. ArAHAS Gene Sequence Analyses

Two fragments of the *ArAHAS* gene were identified, and results of the BLAST sequence analysis showed the fragments were 99.91% and 98.76% identical to the known *ArAHAS* sequence (GenBank: AF363369. 1). The first fragment was about 782 bp and covered Ala_{122}, Pro_{197}, Ala_{205}, and Phe_{206}, whereas the second fragment was approximately 1111 bp, encompassing Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654}. *ArAHAS* in R1 had an $A^{205}V$ mutation, Ala (GCT) substituted with Val (GTT); in R2 had a $D^{376}E$, Asp (GAT) substituted with Glu (GAG); in R3 had a $W^{574}L$ mutation, Trp (TGG) substituted with Leu (TTG); and in R4 and R5 the amino acid at site 653, Ser (AGC), was replaced with Thr (ACC) and Asn (AAC), respectively (Figure 2).

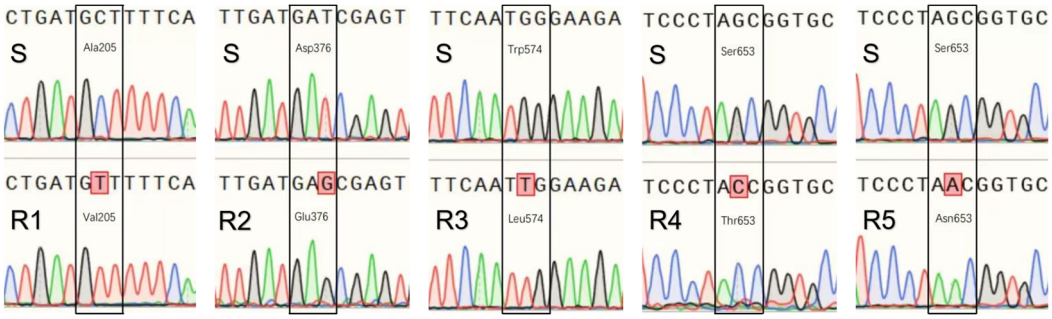

Figure 2. Comparison of partial amino acid sequences of AHAS genes from the wild-type and resistance-mutation genotypes of *A. retroflexus*.

3.3. In Vitro ArAHAS-Activity Assays

The results of the in vitro ArAHAS-activity assays showed decreases in ArAHAS sensitivity to nicosulfuron in the five resistant genotypes compared with the wild type (Figure 3). There were significant variations in the I_{50} values of the ArAHAS extracts derived from the resistant genotypes versus the wild type. The resistance indexes of the resistant genotypes were 10- to 40-fold higher than that of the wild type. The R3 genotype had significantly higher resistances than the other genotypes (Table 4).

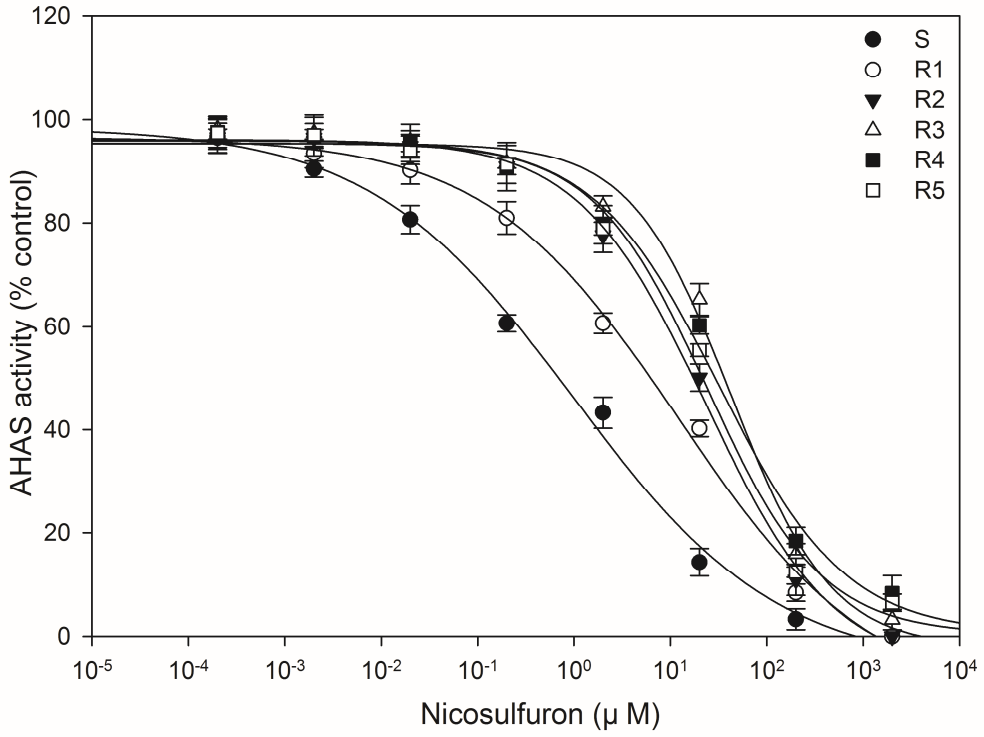

Figure 3. In vitro ArAHAS activity of the wild-type and resistant genotypes treated with nicosulfuron. Each point represents the mean ± SE of three replicates.

3.4. Km (Pyruvate) and Vmax Assays for ArAHAS

The V_{max} values for all the mutated ArAHASs decreased significantly compared with those of the wild type, whereas the K_m values of the mutated ArAHASs exhibited no significant decrease versus those of the wild type (Table 5). This indicated that all the resistance mutations increased the ArAHAS binding affinity with the pyruvate substrate.

Table 5. V_{max} values and K_m (pyruvate) of pyruvate for wild type and mutations.

Name	Mutation	*Vmax* (nmol Acetoin mg^{-1} Protein min^{-1})	R/S	*Km* ± SE (mM)	R/S
R1	Ala-205-Val	117.91 ± 1.20 c	0.20	0.0076 ± 0.0025 b	0.03
R2	Asp-376-Glu	37.82 ± 1.62 e	0.06	0.0767 ± 0.0174 b	0.31
R3	Trp-574-Leu	301.84 ± 16.52 b	0.52	0.1886 ± 0.0317 a	0.85
R4	Ser-653-Thr	49.67 ± 5.80 e	0.09	0.1795 ± 0.0534 a	0.81
R5	Ser-653-Asn	78.92 ± 7.75 d	0.14	0.203 ± 0.0318 a	0.92
S	Wild type	582.33 ± 23.26 a	1.00	0.2216 ± 0.0244 a	1.00

Within a column, means with different letters are significantly different ($p < 0.05$). Data are means ± standard error of three repetitions.

3.5. ArAHAS Feedback-Regulation Assay

All the ArAHAS enzyme activity was inhibited by valine, isoleucine, and leucine. The R3-mutated ArAHAS enzyme exhibited reduced sensitivity to feedback inhibition by each BCAA. The least sensitivity to feedback inhibition for the ArAHAS enzyme was to inhibition by isoleucine and the most sensitivity was to inhibition by leucine (Figure 4).

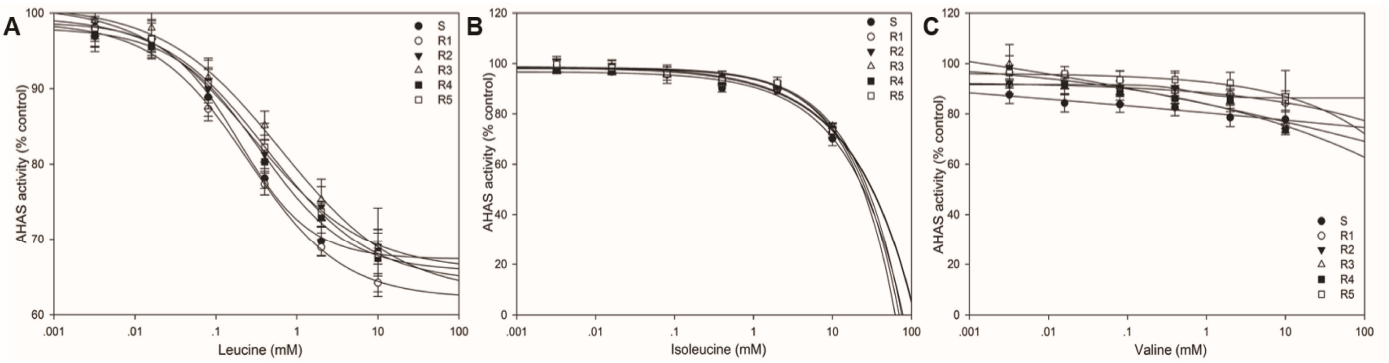

Figure 4. Feedback inhibition effects of branched amino acids on ArAHAS activity in wild-type and resistant genotypes treated with a range of leucine (**A**), isoleucine (**B**), and valine (**C**). Each point represents the mean ± SE of three replicates.

3.6. Determination of Free Amino Acids in A. retroflexus

Determination of the free amino acid and BCAA concentrations in the different genotypes of *A. retroflexus* assessed the possible physiological consequences of the altered ArAHAS sensitivities to feedback inhibition. The concentrations of free BCAAs in the R2 ($D^{376}E$) and R5 ($S^{653}N$) genotypes were significantly higher compared to the other genotypes. The BCAA concentrations were not significantly different among the wild type, R1 ($A^{205}V$), R3 ($W^{574}L$), and R4 ($S^{653}T$). The concentrations of free amino acids in R2 ($D^{376}E$) were higher than in the other genotypes (Figure 5).

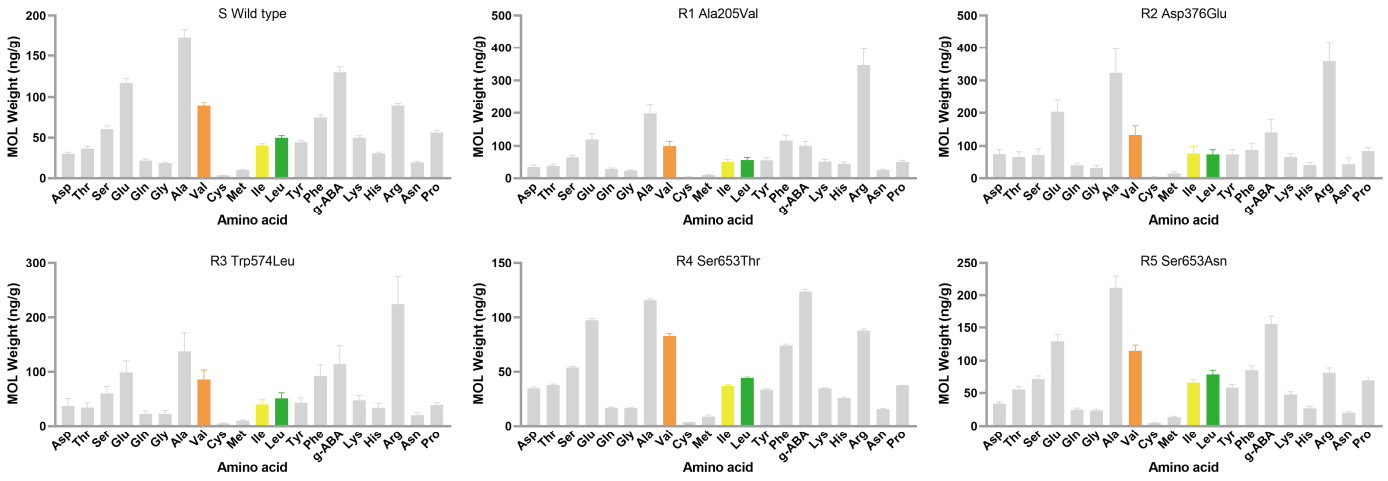

Figure 5. The concentrations of free amino acids in wild-type and resistant genotypes. Each value represents the mean ± SE of three replicates.

3.7. Computational Analysis of AHAS Binding with Nicosulfuron

The molecular docking results showed that each resistance mutation had a different effect on the ArAHAS-binding affinity with nicosulfuron (Figure 6). All the mutations of the *A. retroflexus* genotype reduced the binding affinity of AHAS with nicosulfuron, with the number of hydrogen bonds ranging from three (R1) to one (R3), and the binding capacity gradually decreasing from −1.68 (S) to −0.37 (R3) (Table 6). The W574L mutation had little effect on the binding capacity of AHAS.

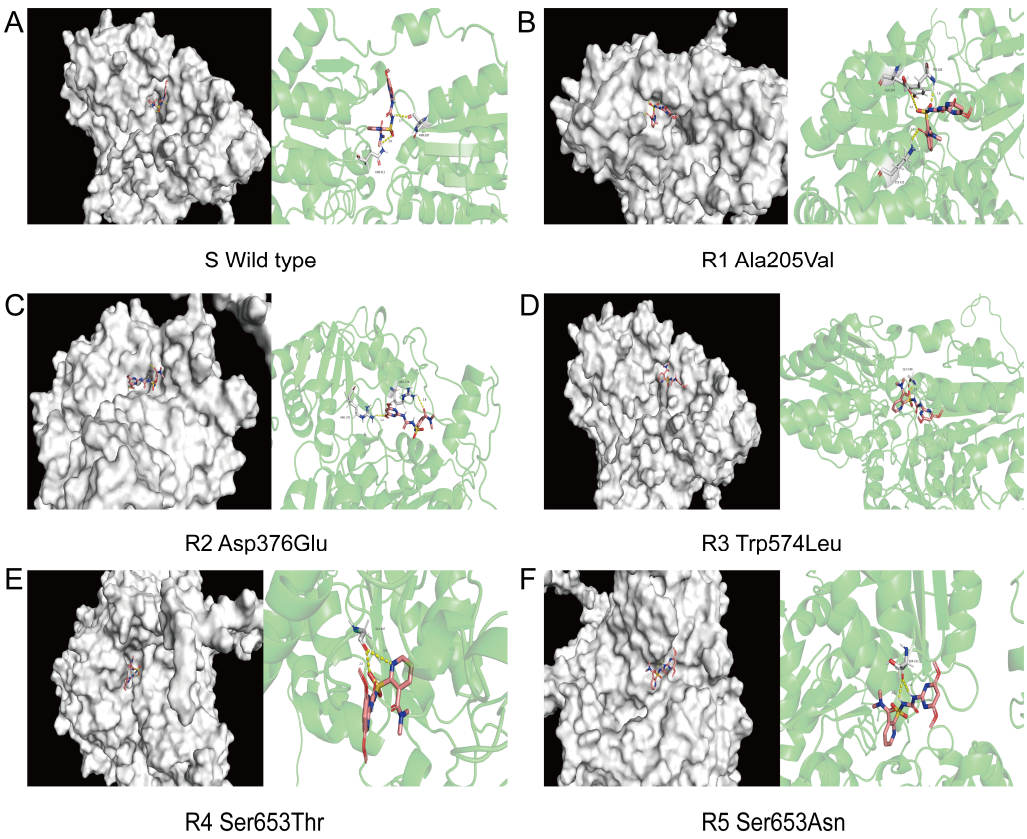

Figure 6. Analysis of the docking mode between nicosulfuron and the wild-type (**A**) or resistant genotypes carrying ArAHAS mutations of Ala205Val (**B**), Asp376Glu (**C**), Trp574Leu (**D**), Ser653Thr (**E**), Ser653Asn (**F**).

Table 6. Computational analyses of the binding free energy and the interactions between nicosulfuron and wild type or mutated receptors of AHAS enzyme.

Name	Mutation	Binding Free Energy (kcal/mol)	Hydrogen Bonds
S	wild type	−1.68	Asn-238, Asn-311
R1	Ala-205-Val	−1.58	Try-280, Glu-285, Lys-423
R2	Asp-376-Glu	−1.53	Arg-372, Arg-376
R3	Trp-574-Leu	−0.37	Gly-240
R4	Ser-653-Thr	−1.38	Gly-507, Gly-507
R5	Ser-653-Asn	−1.14	Ser-212, Ser-212

3.8. Determination of Cross Resistance

Four different chemical-type herbicides with the same modes of action were applied to the purified genotypes to study cross resistance toward AHAS inhibitors caused by the specific AHAS substitutions. The R3 ($W^{574}L$) genotype showed high levels of resistance to imazethapyr (IMI), and the GR_{50} value was 70-fold higher than that for the wild type. All the mutated genotypes had nearly similar levels of resistance to bispyribac-sodium (PTB), whereas R3 exhibited significantly higher levels of resistance to flucarbazone-sodium (SCT). The R1 ($A^{205}V$) and R2 ($D^{376}E$) phenotypes did not exhibit significantly different levels of resistance to flumetsulam (TP). All the mutated genotypes exhibited cross resistance to the four different chemical-type herbicides (Figure 7).

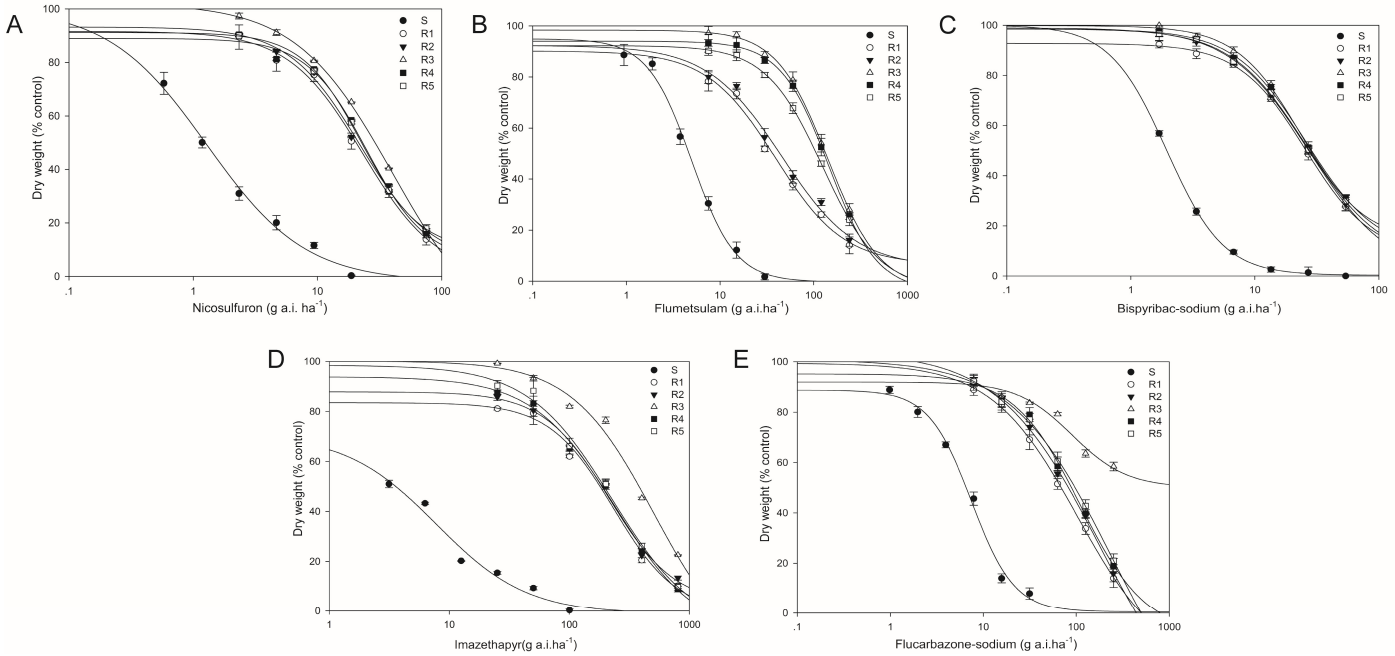

Figure 7. Dose–response curves for aboveground dry weights of the susceptible S (●) and resistant genotypes R1 (○), R2 (▼), R3 (△), R4 (■), and R5 (□) of *A. retroflexus* treated with five different chemical-type herbicides. SU: nicosulfuron (**A**), TP: flumetsulam (**B**), PTB: bispyribac-sodium (**C**), IMI: imazethapyr (**D**), SCT: flucarbazone-sodium (**E**). Each point represents the mean ± SE of three replicates, and the experiment was repeated with similar results.

4. Discussion

AHAS-inhibiting herbicides are highly efficacious, safe for crops, have a wide spectrum of control, exhibit low toxicity, and are cost effective. Thus, these herbicides are used for weed management in field crops in China. For comprehensive, economic, and effective weed control, farmers have repetitively increased the nicosulfuron application dose instead of using herbicides with different modes of action. Unfortunately, many weed populations have developed resistance to AHAS-inhibitor herbicides as these herbicides have been

applied continuously to large areas [37]. The high level of nicosulfuron resistance in the five mutations of *A. retroflexus* genotype studied here is likely due to altered AHAS enzyme activity (Table 4). Studies have shown that the mechanism of resistance to AHAS inhibitors is increased target enzyme activity to overcome the herbicide, which is similar to the findings in our study [38]. Many resistant weeds have exhibited reduced AHAS sensitivity compared with susceptible populations [39]. However, total AHAS activity of many other resistant weeds has been identical to that of susceptible populations [40].

Amino acid substitution effects weed resistance due to the 3-dimensional structure of AHAS, which can influence AHAS binding with the pyruvate substrate of herbicides. Decreased AHAS-binding energy with herbicide molecules is mainly responsible for resistance to AHAS inhibitors and is the most important mechanism of resistance. In this study, all five mutations in *A. retroflexus* significantly reduced the binding affinity of nicosulfuron with the ArAHAS enzyme (Table 6). In addition, increasing the AHAS-binding ability with the pyruvate substrate can confer herbicide resistance, which can help weeds survive even after herbicide application. The effects of herbicide-resistance substitutions on AHAS enzyme binding affinity with pyruvate substrates should be evaluated on a specific basis. In *L. rigidum*, mutations $P^{197}Q$ and $P^{197}A$ significantly increased AHAS binding ability with pyruvate, $P^{197}R$ mutation led to an obvious decrease, and no change was noted with mutations $W^{574}L$ and $P^{197}S$ [41]. In other work, $P^{197}S$, $P^{197}H$, $P^{197}L$, and $P^{197}T$ mutations significantly reduced AHAS activity, $D^{376}E$ mutation obviously increased AHAS activity, and $W^{574}L$ mutation did not lead to any changes [42]. In the present study, the five observed mutations each caused an obvious increase in the total ArAHAS enzyme activity, and $W^{574}L$ significantly increased ArAHAS activity (Table 4).

The molecular docking results showed that the resistance substitutions affected the binding capacity by changing the binding free energy and the number of hydrogen bonds between AHAS and nicosulfuron (Table 6). The mutations $S^{653}T$ and $S^{653}N$ led to lower nicosulfuron binding ability compared to the $A^{205}V$ and $D^{376}E$ mutations; thus, nicosulfuron resistance was higher (Table 4). However, the binding ability of the $S^{653}T$ mutation was lower than that of to the $S^{653}N$ mutation, and the nicosulfuron resistance was higher. The $S^{653}T$ mutation probably led to nontarget resistance. Although the number of hydrogen bonds in the $W^{574}L$ mutation was less than the number in the others, this had little effect on the ArAHAS binding free energy with nicosulfuron; the R3 ($W^{574}L$) genotype showed high resistance, and ArAHAS in R3 was insensitive to nicosulfuron. Our findings are consistent with those reported previously and confirm that the resistant *A. retroflexus* Trp_{574} mutations increase resistance to the five types of AHAS inhibitors (Figure 7). In addition, Trp_{574} mutations in *Descurainia sophia*, *Raphanus raphanistrum*, and *Sagittaria trifolia* lead to high resistance to AHAS-inhibiting herbicides [22,41,42]. Resistance mutations in *A. retroflexus* may change the ArAHAS-binding ability not only with herbicides but also with the pyruvate substrates. Increases in AHAS binding ability with pyruvate may compensate herbicide inhibition [42]. In the current study, the five resistance substitutions reduced the K_m for pyruvate, indicating increased AHAS-binding ability with pyruvate. Therefore, using homologous modeling and molecular docking, we could successfully visualize herbicide binding to the target proteins in the weeds and better understand the mechanism of herbicide resistance (Figure 6).

The regulatory mechanism of AHAS in vivo activity involves feedback inhibition of the end product to maintain dynamic equilibrium of the metabolites. Decreased sensitivity to BCAA feedback inhibition would tend to promote BCAA synthesis as a consequence of the weed surviving treatment with AHAS inhibitors [42–45]. In fact, AHAS sensitivity to feedback inhibition by RSUs containing the binding sites for BCAAs and their regulation is more complicated than what is known to us. In plants, RSUs have two ACT domains in a single polypeptide, whereas microbes have only one [10,14]. Our research indicated decreased ArAHAS sensitivity to feedback inhibition by the three BCAAs. Hence, the five resistance mutations probably altered the ArAHAS-binding sites for the three types of BCAA.

The potential physiological consequences of altered sensitivity of ArAHAS to feedback inhibition remain unclear. Although decreased ArAHAS sensitivity to BCAAs can lead to BCAA accumulation in certain weed species, the effect of specific resistance-endowing mutations on BCAA yields should be evaluated based on concrete analyses of concrete results. Our findings indicated that the $W^{574}L$ genotype slightly decreased the accumulation of isoleucine and valine in *A. retroflexus*. In contrast, the $S^{653}T$ genotype decreased the ArAHAS sensitivity to leucine, isoleucine, and valine, which led to no BCAA accumulation in those plants. In the meantime, the $S^{653}T$ genotype significantly reduced the accumulation of in vivo free amino acids compared to the other genotypes.

5. Conclusions

Taken together, all the resistance mutations ($A^{205}V$, $D^{376}E$, $W^{574}L$, $S^{653}T$, and $S^{653}N$) endowed *A. retroflexus* with high cross resistance to AHAS-inhibiting herbicides either by significantly increasing the ArAHAS-binding ability with the pyruvate substrate or by reducing the ArAHAS enzyme-binding capacity with the herbicide. The ArAHAS sensitivity to BCAA feedback inhibition was dependent on the specific amino acid substitutions, but alteration had little effect on free branched chain amino acids in resistant *A. retroflexus*. Further studies are therefore warranted to investigate multiple resistances to other herbicides and to determine the possibility of target and nontarget site-based mechanisms endowing herbicide resistance in the $S^{653}T$ genotype.

Author Contributions: Z.S., J.C. and W.C. performed the experiments, wrote the paper, and prepared figures and tables; G.Y. performed the experiments and prepared figures; S.W. and Z.M. performed the graphing, analyzed the data, and completed some of the experiments; C.L. collected the materials and analyzed the analyses and graphs data; and C.T. conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper, and approved the final draft. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDA28070305), the "Young Talents" Project of Northeast Agricultural University (Grant No. 22QC04), and the Domestic post-training excellent program of Northeast Agricultural University (Grant No. 23ZYZZ0706).

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Acknowledgments: We are grateful to Fangfang Wu and Wenyu Li for help with amino acid analysis.

Conflicts of Interest: The authors declares no conflicts of interest.

References

1. Ash, C. Meeting resistance. *Science* **2018**, *360*, 726–727. [CrossRef] [PubMed]
2. Gould, F.; Brown, Z.S.; Kuzma, J. Wicked evolution: Can we address the sociobiological dilemma of pesticide resistance? *Science* **2018**, *360*, 728–732. [CrossRef] [PubMed]
3. Yu, Q.; Powles, S.B. Resistance to AHAS inhibitor herbicides: Current understanding. *Pest Manag. Sci.* **2014**, *70*, 1340–1350. [CrossRef] [PubMed]
4. Gaines, T.A.; Duke, S.O.; Morran, S.; Rigon, C.A.G.; Tranel, P.J.; Küpper, A.; Dayan, F.E. Mechanisms of evolved herbicide resistance. *J. Biol. Chem.* **2020**, *295*, 10307–10330. [CrossRef] [PubMed]
5. Duke, S.O.; Dayan, F.E. The search for new herbicide mechanisms of action: Is there a 'holy grail'? *Pest Manag. Sci.* **2022**, *78*, 1303–1313. [CrossRef]
6. Huang, Z.; Chen, J.; Zhang, C.; Huang, H.; Wei, S.; Zhou, X.; Chen, J.; Wang, X. Target-site basis for resistance to imazethapyr in redroot amaranth (*Amaranthus retroflexus* L.). *Pestic. Biochem. Physiol.* **2015**, *128*, 10–15. [CrossRef]
7. Wang, H.; Wang, H.; Zhao, N.; Zhu, B.; Sun, P.; Liu, W.; Wang, J. Multiple resistance to PPO and ALS inhibitors in redroot pigweed (*Amaranthus retroflexus*). *Weed Sci.* **2020**, *68*, 19–26. [CrossRef]
8. McCourt, J.A.; Pang, S.S.; King-Scott, J.; Guddat, L.W.; Duggleby, R.G. Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 569–573. [CrossRef]
9. Lonhienne, T.; Garcia, M.D.; Pierens, G.; Mobli, M.; Nouwens, A.; Guddat, L.W. Structural insights into the mechanism of inhibition of AHAS by herbicides. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E1945–E1954. [CrossRef]

10. Lonhienne, T.; Low, Y.S.; Garcia, M.D.; Croll, T.; Gao, Y.; Wang, Q.; Brillault, L.; Williams, C.M.; Fraser, J.A.; McGeary, R.P.; et al. Structures of fungal and plant acetohydroxyacid synthases. *Nature* **2020**, *586*, 317–321. [CrossRef]
11. Chen, J.; Huang, Z.; Zhang, C.; Huang, H.; Wei, S.; Chen, J.; Wang, X. Molecular basis of resistance to imazethapyr in redroot pigweed (*Amaranthus retroflexus* L.) populations from China. *Pestic. Biochem. Physiol.* **2015**, *124*, 43–47. [CrossRef] [PubMed]
12. Garcia, M.D.; Nouwens, A.; Lonhienne, T.G.; Guddat, L.W. Comprehensive understanding of acetohydroxyacid synthase inhibition by different herbicide families. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E1091–E1100. [CrossRef] [PubMed]
13. Liu, X.; Merchant, A.; Xiang, S.; Zong, T.; Zhou, X.; Bai, L. Managing herbicide resistance in China. *Weed Sci.* **2021**, *69*, 4–17. [CrossRef]
14. Garcia, M.D.; Chua, S.M.H.; Low, Y.S.; Lee, Y.T.; Agnew-Francis, K.; Wang, J.G.; Nouwens, A.; Lonhienne, T.; Williams, C.M.; Fraser, J.A.; et al. Commercial AHAS-inhibiting herbicides are promising drug leads for the treatment of human fungal pathogenic infections. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E9649–E9658. [CrossRef]
15. Heap, I. The International Herbicide-Resistant Weed Database. 2024. Available online: www.weedscience.org (accessed on 1 June 2024).
16. Neve, P.; Vila-Aiub, M.; Roux, F. Evolutionary-thinking in agricultural weed management. *New Phytol.* **2009**, *184*, 783–793. [CrossRef]
17. Délye, C.; Jasieniuk, M.; Le Corre, V. Deciphering the evolution of herbicide resistance in weeds. *Trends Genet.* **2013**, *29*, 649–658. [CrossRef]
18. Cao, Y.; Zhou, X.; Huang, Z. Amino acid substitution (Gly-654-Tyr) in acetolactate synthase (ALS) confers broad spectrum resistance to ALS-inhibiting herbicides. *Pest Manag. Sci.* **2022**, *78*, 541–549. [CrossRef]
19. Wang, R.; Han, Y.; Sun, Y.; Huang, H.; Wei, S.; Huang, Z. Growth and competitiveness of ALS-inhibiting herbicide-resistant *Amaranthus retroflexus* L. *Plants* **2022**, *11*, 2639. [CrossRef]
20. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
21. Doyle, J.J.; Doyle, J.L. A rapid total DNA preparation procedure for fresh plant tissue. *Focus* **1990**, *12*, 13–15.
22. Yu, Q.; Han, H.; Vila-Aiub, M.M.; Powles, S.B. AHAS herbicide resistance endowing mutations: Effect on AHAS functionality and plant growth. *J. Exp. Bot.* **2010**, *61*, 3925–3934. [CrossRef] [PubMed]
23. Sun, Z.; Li, X.; Wang, K.; Zhao, P.; Li, J.; Wang, W.; Ahmed, M.; Shafi, J.; Zhao, B.; Fu, D.; et al. Molecular basis of cross-resistance to acetohydroxy acid synthase-inhibiting herbicides in *Sagittaria trifolia* L. *Pestic. Biochem. Physiol.* **2021**, *173*, 104795. [CrossRef] [PubMed]
24. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
25. Zhao, N.; Yan, Y.; Du, L.; Zhang, X.; Liu, W.; Wang, J. Unravelling the effect of two herbicide resistance mutations on acetolactate synthase kinetics and growth traits. *J. Exp. Bot.* **2020**, *71*, 3535–3542. [CrossRef] [PubMed]
26. Du, Y.; Wang, M.; Chen, Y.; Deng, Y.; Zhang, L.; Bai, T.; Ji, M. Occurrence and mechanism of target-site resistance to bensulfuron-methyl in Monochoria korsakowii from China. *Pestic. Biochem. Physiol.* **2023**, *191*, 105379. [CrossRef] [PubMed]
27. Zeng, Y.; Cai, W.; Shao, X. Quantitative analysis of 17 amino acids in tobacco leaves using an amino acid analyzer and chemometric resolution. *J. Sep. Sci.* **2015**, *38*, 2053–2058. [CrossRef]
28. Ta, H.Y.; Collin, F.; Perquis, L.; Poinsot, V.; Ong-Meang, V.; Couderc, F. Twenty years of amino acid determination using capillary electrophoresis: A review. *Anal. Chim. Acta* **2021**, *1174*, 338233. [CrossRef] [PubMed]
29. Guo, Y.; Liu, C.; Long, W.; Gao, J.; Zhang, J.; Chen, S.; Pu, H.; Hu, M. Development and molecular analysis of a novel acetohydroxyacid synthase rapeseed mutant with high resistance to sulfonylurea herbicides. *Crop J.* **2022**, *10*, 56–66. [CrossRef]
30. Torsten, S. SWISS-MODEL. 2023. Available online: https://swissmodel.expasy.org/ (accessed on 27 November 2023).
31. Seefeldt, S.S.; Jensen, J.E.; Fuerst, E.P. Log-Logistic analysis of herbicide dose-response relationships. *Weed Technol.* **1995**, *9*, 218–227. [CrossRef]
32. Lipovetsky, S. Double logistic curve in regression modeling. *J. Appl. Stat.* **2010**, *37*, 1785–1793. [CrossRef]
33. Kawamata, S. NLR Ana v6.6d. 2012. Available online: https://itcweb.cc.affrc.go.jp/affrit/web-links-en (accessed on 27 November 2023). (In Japanese).
34. Yamato, S.; Sada, Y.; Ikeda, H. Characterization of acetolactate synthase from sulfonylurea herbicide-resistant *Schoenoplectus juncoides*. *Weed Biol. Manag.* **2013**, *13*, 104–133. [CrossRef]
35. Michaelis, M.; Menten, M.L. Die kinetik der invertinwirkung. *Biochem. J.* **1913**, *49*, 333–369.
36. Peletier, L.A.; Gabrielsson, J. Impact of enzyme turnover on the dynamics of the Michaelis-Menten model. *Math. Biosci.* **2022**, *346*, 108795. [CrossRef] [PubMed]
37. Huang, Z.; Cui, H.; Wang, C.; Wu, T.; Zhang, C.; Huang, H.; Wei, S. Investigation of resistance mechanism to fomesafen in *Amaranthus retroflexus* L. *Pestic. Biochem. Physiol.* **2020**, *165*, 104560. [CrossRef] [PubMed]
38. Huang, Z.; Huang, H.; Chen, J.; Chen, J.; Wei, S.; Zhang, C. Nicosulfuron-resistant *Amaranthus retroflexus* L. in northeast China. *Crop Prot.* **2019**, *122*, 79–83. [CrossRef]
39. Li, M.; Yu, Q.; Han, H.; Vila-Aiub, M.; Powles, S.B. ALS herbicide resistance mutations in *Raphanus raphanistrum*: Evaluation of pleiotropic effects on vegetative growth and ALS activity. *Pest Manag. Sci.* **2013**, *69*, 689–695. [CrossRef]
40. Deng, W. The Resistance Mechanisms of Tribenuron-methyl Resistant Fixweed (*Desurainia sophia* L.) and Effects of Resistance-Endowing Mutations on ALS Functionality. Ph.D. Thesis, China Agricultural University, Beijing, China, 2017.

41. Yu, Q.; Han, H.; Li, M.; Purba, E.; Walsh, M.J.; Powles, S.B. Resistance evaluation for herbicide resistance–endowing acetolactate synthase (ALS) gene mutations using *Raphanus raphanistrum* populations homozygous for specific ALS mutations. *Weed Res.* **2012**, *52*, 178–186. [CrossRef]
42. Yang, Q.; Deng, W.; Wang, S.; Liu, H.; Li, X.; Zheng, M. Effects of resistance mutations of Pro197, Asp376 and Trp574 on the characteristics of acetohydroxyacid synthase (AHAS) isozymes. *Pest Manag. Sci.* **2018**, *74*, 1870–1879. [CrossRef]
43. Ashigh, J.; Tardif, F.J. An Ala205Val substitution in acetohydroxyacid synthase of eastern black nightshade (*Solanum ptychanthum*) reduces sensitivity to herbicides and feedback inhibition. *Weed Sci.* **2007**, *55*, 558–565. [CrossRef]
44. Ashigh, J.; Corbett, C.A.L.; Smith, P.J.; Laplante, J.; Tardif, F.J. Characterization and diagnostic tests of resistance to acetohydroxyacid synthase inhibitors due to an Asp376Glu substitution in *Amaranthus powellii*. *Pestic. Biochem. Physiol.* **2009**, *95*, 38–46. [CrossRef]
45. Bansal, A.; Karanth, N.M.; Demeler, B.; Schindelin, H.; Sarma, S.P. Crystallographic Structures of IlvN·Val/Ile complexes: Conformational selectivity for feedback inhibition of Aceto Hydroxy Acid Synthases. *Biochemistry* **2019**, *58*, 1992–2008. [CrossRef] [PubMed]