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Target-site basis for resistance to imazethapyr in redroot amaranth (*Amaranthus retroflexus* L.)

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ABSTRACT

Experiments were conducted to confirm imazethapyr resistance in redroot amaranth (*Amaranthus retroflexus* L.) and study the target-site based mechanism for the resistance. Whole-plant response experiments revealed that the resistant (R) population exhibited 19.16 fold resistance to imazethapyr compared with the susceptible (S) population. *In vitro* ALS activity assay demonstrated that the imazethapyr I_{50} value of the R population was 21.33 times greater than that of the S population. However, qRT-PCR analysis revealed that there is no difference in ALS gene expression between the R and S populations. Sequence analysis revealed an Asp-376-Glu substitution in ALS in the R population. In order to verify that the imazethapyr resistance was conferred by Asp-376-Glu mutation, the *ALS-R* and *ALS-S* genes were fused to the CaMV 35S promoter and introduced into Arabidopsis respectively. The expression of *ALS-R* in transgenic Arabidopsis plants exhibited 13.79 fold resistance to imazethapyr compared to *ALS-S* transgenic Arabidopsis.

1. Introduction

Acetolactate synthase (ALS, EC 2.2.1.6) is the critical enzyme in the biosynthetic pathway of the branched-chain amino acids, valine, leucine and isoleucine [1]. It is a plastidic enzyme that is found in plants, fungi and bacteria [2]. ALS is also the common target enzyme for five classes of herbicides including sulfonylureas (SU) [3], imidazolinones (IMI) [4], pyrimidinylthio-benzoates (PTB) [5], triazolopyrimidines (TP) [6] and sulfonylamino-carbonyltriazolinones (SCT) [7]. ALS-inhibiting herbicides are used in various crops worldwide because of their high efficacy, low application rates and low mammalian toxicity [8]. However, repeated applications of these herbicides have resulted in the selection of resistant weeds. Since prickly lettuce (*Lactuca serriola* L.) was identified as the first case of ALS-resistant weed selected by ALS-inhibiting herbicides in 1987 [9], 156 weed species, including dicots and monocots, have evolved with resistance to ALS-inhibiting herbicides [10].

Target-site modification and/or enhanced metabolism can both confer resistance to ALS inhibitors [11–14]; however, an altered ALS enzyme is the most common mechanism. The positions reported conferring resistance in ALS include Ala_{122}, Pro_{197}, Ala_{205}, Trp_{574}, Ser_{653} [15,16], Asp_{376} [17–19], Gly_{654} [20] and Arg_{377} [21]. The substitutions in ALS prevent or reduce herbicide binding while the ALS enzyme is still functional. Resistance and cross-resistance level can vary by different substitutions [16]. Generally, the Ala-122-Val substitution endows resistance to the IMIs and POBs but not SU herbicides [22,23]. Most mutations at Pro_{197} confer resistance to SU but not IMI, while plants with Trp-574-Leu substitution showed high level resistance to all the five ALS-inhibiting herbicides [23–25]. Furthermore, mutations at Ala_{205} conferred much lower resistance levels compared with Trp_{574} [19].

Redroot amaranth (*Amaranthus retroflexus* L.), a common self-pollinated annual dicot weed, is distributed widely in farmlands. It is considered a strong initial competitor and a prolific seed producer [26]. The first ALS inhibitor resistant redroot amaranth was discovered in 1997 in Ontario [27]. To date, four target-site mutations (Ala122, Ala205, Trp574, Pro197) have been reported in redroot amaranth [28,29]. In China, redroot amaranth is distributed widely in fields of maize, cotton, soybean and sweet potato, causing substantial crop yield reduction [30–32]. One redroot amaranth population cannot be controlled by imazethapyr in a soybean field at Nenjiang, Heilongjiang province of China. The objective of this research was to evaluate response to imazethapyr and determine the molecular basis of resistance mechanism in redroot amaranth.

2. Materials and methods

2.1. Plant materials and growth conditions

Redroot amaranth seeds with suspected resistance to ALS-inhibiting herbicides were collected from a private land in Nenjiang, Heilongjiang

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province of China, with a history of at least 5 years of imazethapyr use. The owner of the land gave us permission to conduct this study. Seeds from three susceptible populations were collected in the same region from the road side with no history of herbicide use. Seeds were immersed in gibberellin solution (200 mg/L) for 12 h to break dormancy, and rinsed thoroughly with distilled water before planting. Seedlings were planted in potting mix containing a 1:1 (*V*/*V*) peat: sand sterile in 5 cm radius pots. Plants were placed outdoors with temperature range 21–35 °C and watered as required.

Arabidopsis thaliana ecotype Col-0 were used either as a recipient for plant transformation or as a wild type control. Plants were raised on MS medium supplemented with 30 g/l sucrose, 8 g/l agar and adjusted to pH 5.8. The plants were cultivated in a growth chamber with a 16/8 h light/dark photoperiod at 22 °C.

2.2. Whole-plant dose–response tests

Redroot amaranth seedlings at 5–6 leaf stage were treated with imazethapyr (Pursuit, 50%W, BASF, Shanghai, China). Imazethapyr was applied at 0, 1/32, 1/16, 1/8, 1/4, 1/2 and 1 fold the recommended dose (90 g ai ha^{-1}) for the S population, and 0, 1/4, 1/2, 1, 2, 4 and 8 fold the recommended dose for the R population. Herbicide treatments were applied with a compressed air, moving nozzle cabinet sprayer, 3WPSH-500D, equipped with one Teejet XR8003 flat fan nozzle calibrated to deliver 450 L ha^{-1} at 0.3 MPa. Plants were returned outdoors after treatments. All aboveground plant material was harvested 21 days after herbicide treatments and put in drying oven with the temperature at 60 °C. 3 days later, dry weight was measured for further analysis. All treatments were replicated three times, and the experiment was conducted twice.

2.3. In vitro ALS activity assay

Redroot amaranth leaves were harvested at 5–6 leaf stage and 5 g of leaf tissue was used for a crude enzyme extraction. The ALS enzyme extraction and assay were based on the method of Ray [33] with modifications. Frozen leaf tissues from plants of each population were ground in liquid nitrogen with a chilled mortar and pestle. The powder was homogenized and 10% polyvinylpolypyrrolidone (PVPP) [*wt*/*V*] and 0.2 mM dithiothreitol (DTT) were added just before extraction. Extraction buffer [100 mM potassium phosphate (pH 7.5), 10 mM sodium pyruvate, 5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 100 μM flavine adenine dinucleotide (FAD) and 10% glycerol (*V*/*V*)] were added. The homogenate was filtered and centrifuged at 20,000 *g* for 20 min. The supernatant was then 50% saturated with $(NH_4)_2SO_4$ on ice and covered. After stirring for 60 min, the solution was centrifuged at 27 000 g for 20 min. The supernatant was discarded and the pellets were suspended in 1–6 mL of an elution buffer [50 mM potassium phosphate buffer (pH 7.0), 20 mM sodium pyruvate and 0.5 mM $MgCl_2$]. ALS activity was assayed after diluting the extract to 1.5 mg protein mL^{-1}. Protein (200 μL) and 100 μL of herbicide solution or water was added to 700 μL of reaction buffer [50 mM potassium phosphate (pH 7.0), 100 mM sodium pyruvate, 1 mM TPP, 10 mM $MgCl_2$, 1 μM FAD]. The assay mixture was incubated for 60 min at 37 °C. The reaction was stopped by adding 100 μL of 6 N H_2SO_4, followed by incubation for 30 min at 60 °C to form acetoin. Next, 100 μL of 50% NaOH, 300 μL of a solution of creatine (0.9%) plus α-napthol (9%) in 2.5 N NaOH (*wt*/*V*) were added to each tube. Tubes were incubated for 30 min at 60 °C and centrifuged at 10,000 *g* for 2 min.

The imazethapyr was applied at active ingredient concentrations of 0, 0.0001, 0.0003 0.0012, 0.0195, 0.0781, 0.3125, 0.65, 1.25 g/L for the S population and 0, 0.0781, 0.1562, 0.3125, 0.625, 1.25, 2.5, 5 and 10 g/L for the R population. Protein concentration of the crude extract was measured by the Bradford method [34]. The experiments were conducted according to a completely randomized block design with three replications per treatment. The ALS activity assay was repeated three times.

2.4. Statistical analysis

Data from the whole-plant response tests and *in vitro* ALS activity assays were analyzed by ANOVA, and t-test ($P = 0.05$) was used to test the significance of the regression parameters. Non-linear regression was used to estimate the herbicide rate causing 50% reduction in plant growth (GR_{50}) and the herbicide dose to inhibit 50% ALS activity (I_{50}) using SigmaPlot software (v. 12.0). The data were fitted by the log-logistic model [35]:

$$y = C + \left[\frac{D-C}{1+(x/ED_{50})^{b}}\right]$$

where *C* is the lower limit, *D* is the upper response limit, b is the slope of the curve and ED_{50} is the imazethapyr concentration required for GR_{50} or I_{50}. The resistance index (RI) was calculated as the ratio of the GR_{50} or I_{50} value of the R population to that of the S population to indicate the level of resistance.

2.5. ALS gene expression analysis

Redroot amaranth seedlings at the 5–6 leaf stage from the R and S populations were treated with imazethapyr (90 g ai ha^{-1}). Shoot materials from treated and untreated control plants were harvested 48 h after treatment. Total RNA was extracted and pretreated extensively with an RNase-free DNase to eliminate any contaminating genomic DNA. It was reverse-transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen).

The expression of *ALS* was determined by quantitative real-time PCR (qRT-PCR) in an Applied Bio-systems 7500 Real-Time PCR system using the SYBR Green I master mix. The following primers were used: ALS-Qf × ALS-Qr and Actin-Qf × Actin-Qr for amplification of *ALS* and *Actin* (Table 1). To determine the specificity of the primers for target *ALS* and *Actin* genes, melting curve analysis was performed before qRT-PCR. The two-step thermal cycling profile used was 15 s at 95 °C and 25 s at 62 °C. The cycle at which PCR amplification curves crossed a determined threshold was recorded (Ct), and the relative expression of *ALS* gene expression level was calculated with the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta CT = Ct_{ALS} - Ct_{Actin}$), and the error bars indicate SD (n = 3). *ALS* relative expression level was normalized to reference gene (*Actin*). Two experiments on independently grown plant materials were performed to confirm the reproducibility of the results.

2.6. ALS gene sequencing

Young shoot tissue of the R and S populations was harvested respectively and stored at −80 °C. DNA was extracted from 100 mg shoot tissue using the CTAB method [36].

Table 1
Primers used in study.

Primers	Sequence (5′ → 3′)	Purpose of primers
ALS-1f	TTTCTCCATTCTCGCTTAG	Amplify the full length of *ALS*
ALS-1r	CATTCCACCACTTTACATTCT	
ALS-2f	CCGGATCCATGGCGTCCACTTCTTCAAAC	Amplify the coding region
ALS-2r	GGCCCGGGCTAATAAGCCCTTCTTCCATCA	of *ALS*
ALS-Qf	TGCCTAAACCCACTTATTCTGC	qRT-PCR analysis of *ALS* gene
ALS-Qr	ACCGGAATACCTGTCAATTCG	
Actin-Qf	ATGTGGCAATTCAGGCTGTTCTT	qRT-PCR analysis of *ACTIN*
Actin-Qr	AAGGATGGCGTGTGGGAGAG	gene