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# Multiple resistance to PPO and ALS inhibitors in redroot pigweed (*Amaranthus retroflexus*)

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## Research Article

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## Abstract

A redroot pigweed (*Amaranthus retroflexus* L.) population (HN-02) collected from Nenjiang County, Heilongjiang Province, exhibited multiple resistance to fomesafen and nicosulfuron. The purposes of this study were to characterize the herbicide resistance status of an HN-02 population for both acetolactate synthase (ALS) and protoporphyrinogen oxidase (PPO) inhibitors and the response to other herbicides and to investigate the target site-based mechanism governing fomesafen and nicosulfuron resistance. Three mutations, Ala-205-Val and Trp-574-Leu mutations in the *ALS* gene and an Arg-128-Gly mutation in the *PPX2* gene, were identified in individual resistant plants. An HN-02F1-1 subpopulation homozygous for the Ala-205-Val and Arg-128-Gly mutations was generated, and whole-plant experiments confirmed multiple resistance to PPO inhibitors (fomesafen, fluoroglycofen-ethyl, and acifluorfen) and ALS inhibitors (imidazolinones [IMI], sulfonylureas [SU], and triazolopyrimidines [TP]) in the HN-02F1-1 plants, which presented resistance index values ranging from 8.3 to 110; however, these plants were sensitive to flumioxazin, fluroxypyr-meptyl, and 2,4-D butylate. In vitro ALS enzyme activity assays revealed that, compared with ALS from susceptible plants, ALS from the HN-02F1-1 plants was 15-, 28- and 320-fold resistant to flumetsulam, nicosulfuron, and imazethapyr, respectively. This study confirms the first case of multiple resistance to PPO and ALS inhibitors in *A. retroflexus* and determines that the target-site resistance mechanism was produced by Ala-205-Val and Arg-128-Gly mutations in the *ALS* gene and *PPX2* gene, respectively. In particular, the Ala-205-Val mutation was found to endow resistance to three classes of ALS inhibitors: TP, SU, and IMI.

## Introduction

Redroot pigweed (*Amaranthus retroflexus* L.), an annual dicotyledonous weed species that undergoes partial or full self-fertilization and occasional outcrossing, is widely distributed in many farmlands (Scarabel et al. 2007). $C_4$ plants produce large amounts of seed (up to 1.5 million seeds per plant) and are highly adaptable (capable of germinating under a variety of environmental conditions), which makes them highly competitive with crop species in terms of acquiring light, water, and nutrients, especially in soybean [*Glycine max* (L.) Merr.]–corn (*Zea mays* L.) rotation systems (Bensch et al. 2003; Francischini et al. 2014; Ghanizadeh et al. 2014; Sheibany et al. 2009). Typically, *A. retroflexus* control is still heavily dependent on chemical herbicides, particularly protoporphyrinogen oxidase (PPO; EC 1.3.3.4) and acetolactate synthase (ALS; EC 2.2.1.6) inhibitors.

PPO is an important enzyme involved in the biosynthesis of chlorophyll and heme groups. Plants have two different PPO orthologues that are located in the chloroplast (*PPX1*) and mitochondria (*PPX2*) (Lermontova et al. 1997). Several herbicides, including diphenylethers and oxidiazoles, inhibit PPO enzymes, resulting in the accumulation of protogen IX (a substrate of the PPO enzyme) (Jacobs and Jacobs 1993). Protogen IX overflows from these organelles and is oxidized by catalase to proto-IX in the cytoplasm, but proto-IX is in turn oxidized to singlet oxygen in the presence of sunlight (Beale and Weinstein 1990). As a result, the amount of both chlorophyll and carotene decreases, and membrane leakage occurs; this phenomenon causes cells to lose water, and organelles rapidly disintegrate, eventually causing plant death (Duke et al. 1991; Lee et al. 2000).

ALS, which is also referred to as acetohydroxyacid synthase, is a key enzyme that is essential for plant growth and is involved in the biosynthesis of branched-chain amino acids (Leu, Ile, and Val) (Duggleby et al. 2008; Liu et al. 2015). ALS is the target of five commercially used herbicides including sulfonylurea (SU) (Chaleff and Mauvais 1984), imidazolinone (IMI) (Shaner et al. 1984), triazolopyrimidine (TP) (Gerwick et al. 1990), pyrimidinylthiobenzoate

(Stidham 1991), and sulfonylaminocarbonyltriazolinone (Santel et al. 1999). ALS inhibitors can control many weed species, present low toxicity to mammals, and are selective in major crops around the world. Therefore, these types of herbicides are widely used in weed management worldwide. In China, fomesafen and nicosulfuron have been registered for use to control broadleaf annual weeds in corn–soybean rotation fields since the 1990s. Unfortunately, in northeast China, especially in Heilongjiang Province, high-level resistance to these two herbicides has been observed in *A. retroflexus* populations.

With respect to herbicide resistance in weed species, the mechanisms responsible can be grouped into two main types: target-site resistance (TSR) and non–target site resistance (NTSR) (Délye et al. 2013; Powles and Yu 2010). In most cases, TSR is caused by one or more point mutations in the target gene that reduce the sensitivity of the target enzyme to herbicides (Liu et al. 2015). The substitution of different amino acids at the same position or gene mutations at different locations usually results in different cross-resistance to herbicides that have the same target (Beckie and Tardif 2012). To date, it has been reported that 13 weed species (Heap 2019) have evolved resistance to PPO inhibitors; the resistance mechanisms have been identified as a Gly-210 deletion or an Arg-128 or Gly-399 substitution within the PPO (*PPX2*) enzyme (Patzoldt et al. 2006; Rangani et al. 2019; Rousonelos et al. 2012). Compared with PPO, ALS is a target site that is more resistance prone (Yu and Powles 2014b). To date, within the *ALS* gene, eight positions providing 29 amino acid substitutions leading to resistance have been discovered in 162 weed species (Tranel et al. 2019).

The role of NTSR mechanisms in the emergence of multiple resistance to herbicides has received increased amounts of attention (Yu and Powles 2014a). In contrast to TSR, metabolic resistance employs the activity of multiple genes, such as those encoding ATP-binding cassette transporters, glutathione *S*-transferases, glucosyltransferases, and cytochrome P450 monooxygenases, to detoxify herbicides (Yuan et al. 2007). In addition, regardless of the herbicide mode of action, NTSR can cause unpredictable resistance in weed species (Petit et al. 2010).

In September 2017, the plants of an *A. retroflexus* population (HN-02) that had withstood the field-recommended application rate of fomesafen were collected from a soybean field in Nenjiang County, Heilongjiang Province. Single-dose tests revealed that HN-02 had evolved resistance to fomesafen and nicosulfuron, and TSR gene sequencing revealed three amino acid substitutions, Ala-205-Val (65%) and Trp-574-Leu (20%) mutations in the *ALS* gene and a rare Arg-128-Gly mutation in the *PPX2* gene, that were simultaneously present in the individual HN-02 plants. A purified subpopulation of HN-02, designated HN-02F1-1, in which the individual members are homozygous for both the Ala-205-Val and Arg-128-Gly substitutions, was generated to characterize its response to different herbicides and the underlying resistance mechanism. This study aimed to (1) determine the resistance levels to fomesafen and nicosulfuron within the HN-02F1-1 population, (2) characterize the susceptibility of HN-02F1-1 to many other herbicides exhibiting different modes of action, (3) assay the in vitro ALS activity of HN-02F1-1 plants in response to three groups of ALS inhibitors, and (4) clarify the molecular basis of TSR to fomesafen and nicosulfuron.

## Materials and Methods

### Plant Materials

In September 2017, ripe seeds ($F_0$ generation) of a suspected resistant (R, HN-02) *A. retroflexus* population were collected from at least 100 plants in a soybean field in Nenjiang County (49.11°N, 124.94°E), Heilongjiang Province, where fomesafen and nicosulfuron have failed to control *A. retroflexus* since 2014. The sampled field was approximately 0.5 ha and had been under a repeated soybean–corn rotation since 1980. A herbicide-susceptible population (S, ST-1) was collected from Mount Tai (36.05°N, 117.03°E), Shandong Province, and had never been exposed to herbicides. After being cleaned and dried, the seeds from individual plants were collected and pooled into a single composite sample for each field and then stored at 4 C in paper bags for later use.

### Single-dose Herbicide Resistance Testing

Before planting, both populations of seeds ($F_0$ generation, approximately 110) were germinated in petri dishes that contained two layers of filter paper (Whatman No. 1) moistened with 6 ml water and incubated in a growth chamber (33/23 C, 12/12 h day/night). The germinated seedlings were then transplanted into plastic pots (15-cm diameter, 12-cm height) containing loam; the final quantity of plants that were treated with herbicide was 10 per pot. The plants were grown to the 3- to 4-leaf stage in a controlled greenhouse environment (30/20 C, day/night) and watered every other day to maintain soil moisture. For each population, 50 plants were treated with fomesafen at 225 g ai $ha^{-1}$ (the field-recommended rate), another 50 plants were treated with nicosulfuron at 48 g ai $ha^{-1}$ (the field-recommended rate), and 10 plants were maintained as untreated controls. All herbicides were sprayed using a moving-nozzle cabinet sprayer equipped with one TeeJet® 9503EVS (Greenman Machinery Company, Beijing, China) flat-fan nozzle (450 L $ha^{-1}$ at 280 kPa). The percentage of surviving plants was visually measured at 21 d after treatment. The plants were considered dead if their leaf tissue was brittle and necrotic and if no new growth point had developed. Afterward, approximately 100 mg of young leaf tissue of each individual surviving plant was removed and used for total RNA extraction via TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

### Gene Sequencing and Acquisition of Homozygous TSR Resistant Seeds

The isolated RNA was immersed in 70% ethanol and stored at −80 C. RNA was treated with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) to remove any gDNA contamination. A pair of primers (forward primer [ALS-F]: 5′-TGCGATGTTCTCGTTG AAGC-3′; reverse primer [ALS-R]: 5′-CCCTTCTTCCATCAC CCT-3′; 1,711 bp) previously reported by Liu et al. (2015) was used to amplify the *ALS* sequence. To amplify the *PPX2* sequence, primers (PPX2-F: 5′-GCCATCGCCATTGTCAGTTTCA-3′; PPX2-R: 5′-ATGGATGAGAAGACCGCGTAA-3′; 1,450 bp) were designed (Primer Premier 5.0, Biosoft, Palo Alto, CA, USA) according to GenBank accession no. KY882137.1 and a study by Patzoldt et al. (Patzoldt et al. 2006). Both primer pairs were designed to amplify all reported mutation sites with PPO and ALS resistance, respectively. The *PPX2* sequence for *A. retroflexus* has been published and is available in the NCBI database (MK716317-MK716318). Polymerase chain reaction (PCR) was performed in a final reaction volume of 25 µl, which consisted of 1 µl of cDNA, 1 µl of each primer (synthesized by Sangon Biotech, Shanghai, China), 12.5 µl of TransTaq HiFi PCR SuperMix II (TransGen Biotech, Beijing, China), and 9.5 µl of nuclease-free water. PCR was performed on a T100 thermal cycler

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**Table 1.** Herbicides and herbicide application rates used in the whole-plant experiments.[a]

| Site of action | Group | Herbicide[b] | Application rate[c] | |
|---|---|---|---|---|
| | | | ST-1 (S) | HN-02F1-1 (R) |
| | | | g ai ha$^{-1}$ | |
| PPO | Diphenyl ethers | Fomesafen | 0.3, 1.5, 7.5, 37.5, **187.5**, 937.5 | 2.3, 11.3, 56.3, **281.3**, 1406.3, 7031.3 |
| | | Fluoroglycofen-ethyl | 3.75, 7.5, 15, 30, **60**, 120 | 45, **90**, 180, 360, 720, 1440 |
| | | Acifluorfen | 22.5, 44.9, 89.9, 179.8, **359.5**, 719 | 240.8, **481.5**, 963, 1926, 3852, 7704 |
| | N-phenylphthalimides | Flumioxazin | 0.036, 0.18, 0.9, 4.5, **22.5**, 112.5 | 0.048, 0.24, 1.2, 6, **30**, 150 |
| ALS | Sulfonylureas | Nicosulfuron | 0.0768, 0.384, 1.92, 9.6, **48**, 240 | 0.48, 2.4, 12, **60**, 300, 1500 |
| | | Thifensulfuron-methyl | 0.72, 3.6, **18**, 90, 450, 2250 | 1.01, 5.04, **25.2**, 126, 630, 3150 |
| | Triazolopyrimidines | Cloransulam-methyl | 0.04, 0.20, 1.0, 5.0, **25.0**, 125.0 | 0.25, 1.26, 6.3, **31.5**, 157.5, 787.5 |
| | | Flumetsulam | 0.03, 0.15, 0.75, **3.75**, 18.75, 93.75 | 0.20, 1.0, **5.0**, 25.0, 125.0, 625.0 |
| | Imidazolinones | Imazethapyr | 0.12, 0.6, 3.0, 15.0, **75.0**, 375.0 | 4.2, 21.0, **105.0**, 525.0, 2625.0, 13125.0 |
| | | Imazamox | 0.072, 0.36, 1.8, 9.0, **45.0**, 225.0 | 0.40, 2.0, 10.0, **49.8**, 249.0, 1245.0 |
| Synthetic Auxins | Arylpicolinate | Fluroxypyr-meptyl | 0.29, 1.4, 7.2, 36.0, **180.0**, 900.0 | 1.7, 8.4, 42.0, **210.0**, 1050, 5250.0 |
| | Phenoxy-carboxylic-acids | 2,4-D butylate | 3.3, 16.5, 82.6, **412.8**, 2064.0, 10320.0 | 5.0, 25.0, 124.8, **624.0**, 3120.0, 15600.0 |

[a]Abbreviations: R, resistant; S, susceptible; ALS, acetolactate synthase; PPO, protoporphyrinogen oxidase; AS, aqueous solution; ME, microemulsion; WP, water power; OF, oil miscible suspension; WG, water dispersible granule; SC, suspension concentrate; EC, emulsifiable concentrate.
[b]Fomesafen (250 g L$^{-1}$ AS, BrightMart CropScience, Foshan, China), fluoroglycofen-ethyl (10% ME, Huifeng Biological Agriculture, Jiangsu, China), acifluorfen (21.4% AS, Hanshen Biotechnology, Qindao, China), flumioxazin (50% WP, Sumitomo Chemical Corp, Japan), nicosulfuron (40 g L$^{-1}$ OF, Zhongshan Chemical Group, Huzhou, China), thifensulfuron-methyl (75% WG, FMC Corporation, Shanghai, China), cloransulam-methyl (84% WG, Dow AgroSciences, Beijing, China), flumetsulam (80% WG, Dow AgroSciences, Beijing, China), imazethapyr (5% AS, Lvbang Crop Science, Jinan, China), imazamox (4% AS, Jiangsu Agricultural Hormone Engineering Technology Research Center, Nanjing, China), fluroxypyr-meptyl (50% SC, Qingyuan Crown Resistant Weed Control, Qingdao, China), 2,4-D butylate (57% EC, Songliao Chemical, Liaoning, China).
[c]The bold values represent the field-recommended rates.

**Table 2.** Herbicides and herbicide application rates used in in vitro ALS activity assays.[a]

| Herbicide | Purity | Supplier | Application rate | |
|---|---|---|---|---|
| | | | ST-1 (S) | HN-02F1-1 (R) |
| | | | μM | |
| Flumetsulam | 97% | Jiangsu Agricultural Hormone Engineering Technology Research Center, Nanjing, China | 0.001, 0.01, 0.1, 1, 10, 100 | 0.01, 0.1, 1, 10, 100, 1000 |
| Imazethapyr | 98% | Runfeng Chemical, Weifang, China | 0.2, 1, 5, 25, 125, 625 | 0.4, 2, 10, 50, 250, 1250 |
| Nicosulfuron | 95% | Lvba Chemical, Weifang, China | 0.002, 0.02, 0.2, 2, 20, 200 | 0.02, 0.2, 2, 20, 200, 2000 |

[a]Abbreviations: ALS, acetolactate synthase; R, resistant; S, susceptible.

(Bio-Rad, USA) with the following amplification conditions of the target gene: 94 C; 35 cycles of 30 s at 94 C, 30 s at X C (56 C and 58 C for the *PPX2* and *ALS* genes, respectively), and 1 min 30 s at 72 C; and then 10 min at 72 C. The PCR products were separated by a 1.0% agarose gel run in 1X TAE buffer at 120 V and then purified by an EasyPure Quick Gel Extraction Kit (TransGen Biotech, Beijing, China). The purified products were subsequently sequenced in both the forward and reverse directions by a commercial sequencing company (Sangon Biotech, Shanghai, China).

DNAMAN v. 5.2.2 software (Lynnon Biosoft, Quebec, Canada) was used to align the nucleotide sequence positions of the *PPX2* and *ALS* genes with those of the *PPX2* gene of Palmer amaranth (*Amaranthus palmeri* S. Watson) (GenBank accession no. KY882137.1) and the *ALS* gene of *A. retroflexus* (GenBank accession no. AF363369.1), respectively. On the basis of results of the sequencing analysis, plants homozygous for both the *ALS* and *PPX2* gene mutations were identified and grown in isolation in a growth chamber (33/23 C, 12/12 h day/night) to ensure that no other pollen was introduced. Subsequently, whole-plant herbicide-sensitivity bioassays and in vitro ALS activity assays were performed only for seeds collected from this separated R population, which was designated HN-02F1-1. For the HN-02F1-1 resistance test, 20 seeds were randomly selected for single-dose herbicide-resistance tests (following methods described earlier), and five plants were randomly selected for gene sequencing.

### Whole-Plant Herbicide Sensitivity Bioassays

The plant germination and growth conditions of the R (HN-02F1-1) and S (ST-1) populations followed the methods described earlier. When the seedlings reached the 3- to 4-leaf stage, herbicides (four PPO inhibitors, six ALS inhibitors, and two synthetic auxins) were sprayed at a series of rates (Table 1) in accordance with the methods described earlier. The aboveground *A. retroflexus* materials were harvested at 21 d after herbicide treatment and then oven-dried for 72 h at 75 C, after which their dry weights were recorded. Each treatment was repeated three times, and the entire experiment was carried out twice.

### In Vitro ALS Activity Assays

Seedlings of R (HN-02F1-1) and S (ST-1) populations were germinated and grown under the same conditions as those described earlier. At the 3- to 4-leaf stage, young leaf tissue was harvested and stored at –80 C. In accordance with the methods of Yu et al. (2010) and Han et al. (2012), ALS was extracted, and an in vitro assay of inhibitory activity was performed. The ALS activity was determined via colorimetry (530 nm) with a UV spectrophotometer (Thermo Fisher Scientific) by measuring the production of acetoin. The herbicide concentrations used for the activity assays are listed in Table 2. Three replications per assay were carried out, and the entire experiment was conducted twice, using independent ALS extraction.

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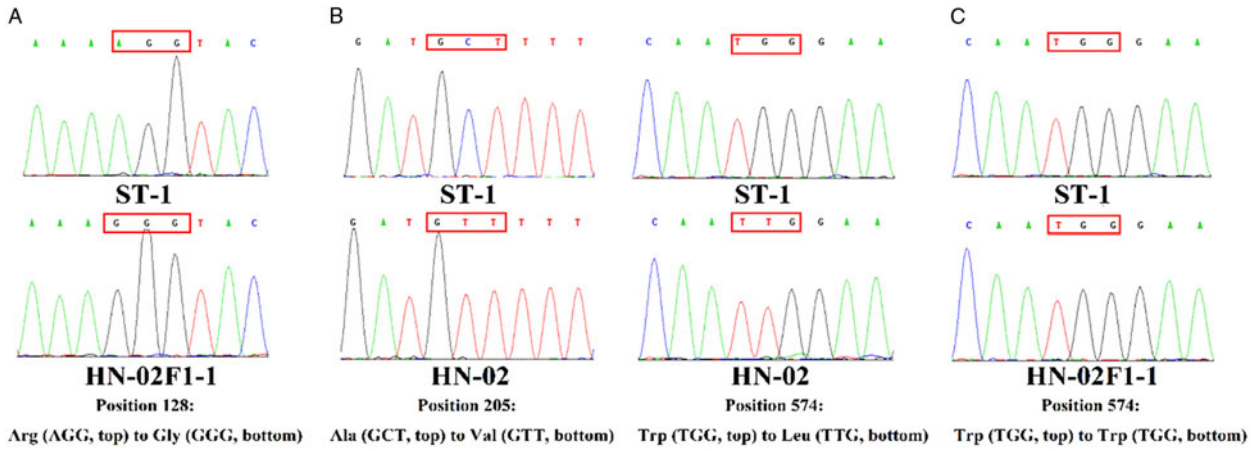


**Figure 1.** cDNA sequencing results indicating (A) the Arg-128-Gly mutation in the *PPX2* gene, (B) the Ala-205-Val (frequency of 65%) and Trp-574-Leu mutations (frequency of 20%) in the *ALS* gene in resistant *Amaranthus retroflexus* plants (HN-02, bottom), and (C) no Trp-574-Leu mutation in the *ALS* gene in the subpopulation HN-02F1-1 (bottom), compared with the susceptible plants (ST-1, top).

### *Statistical Analyses*

The data from both the whole-plant bioassays and the in vitro ALS activity assays were analyzed via ANOVA (SPSS v. 19.0, IBM, Armonk, NY, USA). The results indicated that there was no statistically significant ($P > 0.05$) interaction between repeats of either experiment, and thus, the combined data were analyzed using a four-parameter log-logistic curve via SigmaPlot (v. 12.5; SigmaPlot Software) as follows:

$$y = c + (d - c)/\{1 + \exp[b(\log x - \log \mathrm{GR}_{50})]\} \qquad [1]$$

where $c$ represents the lower limit, $d$ represents the upper limit, $x$ represents the herbicide application rate, $b$ represents the slope of the curve for the dose causing a 50% dry weight reduction in the aboveground growth (at $\mathrm{GR}_{50}$), and $y$ represents the growth response (percentage of the untreated controls). The resistance index (RI) was calculated as the ratio of the $\mathrm{GR}_{50}$ value or the $\mathrm{I}_{50}$ value (herbicide concentration required for 50% ALS activity inhibition) of the resistant and susceptible populations.

## Results and Discussion

### *Single-Dose Testing, Gene Sequencing, and Resistant Population Purification*

With respect to the single-dose testing, as expected, all plants of the HN-02 (R) population survived fomesafen at 225 g ai $ha^{-1}$, and 46 plants survived the nicosulfuron treatment; however, all plants of the ST-1 (S) population died.

Generally, all known target point mutations (ΔGly210, Arg-128-Leu, Arg-128-Gly/Met, Gly-399-Ala) causing PPO-inhibitor resistance in weed species are reported to occur in *PPX2* instead of in *PPX1* (Giacomini et al. 2017; Patzoldt et al. 2006; Rangani et al. 2019; Rousonelos et al. 2012). Thus, a 1,437-bp fragment that encompasses all three known mutation sites within the *A. retroflexus PPX2* gene was amplified from 40 individual plants (randomly selected from 96 surviving plants) of HN-02 and from the 10 control plants of ST-1. The results indicated that the similarity of the *PPX2* gene sequences between HN-02 and ST-1 was approximately 99.6%. The change of a single nucleotide from AGG to GGG resulting in an Arg-128-Gly substitution was revealed when the *PPX2* gene fragments were compared between the R and S samples. Furthermore, the corresponding sequence chromatograms revealed a single peak at all amino acid 128 mutation sites (Figure 1A), which indicated that only one allele is expressed, and the analyses of cDNA indicate that it is homozygous.

Furthermore, *ALS* gene fragments that include all eight known mutation sites (Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654) (Tranel et al. 2019) were also amplified from the abovementioned 40 *A. retroflexus* plants. The gene-sequencing results of the 40 individual plants from HN-02 revealed a Trp-574-Leu substitution produced by the TGG-574-TTG mutation in 8 plants and an Ala-205-Val substitution produced by the GCT-205-GTT mutation in 26 plants, while 6 plants had no ALS-resistance mutations. However, no plants had the two abovementioned mutations coexisting in the *ALS* gene. The corresponding sequence chromatograms revealed a single peak in all 205 or 574 mutation sites (Figure 1B), indicating that all 205 and 574 mutants were homozygous. This phenomenon may have occurred because *A. retroflexus* is diploid and is mainly self-fertilized (Mandák et al. 2011; Yu and Powles 2014b). Six plants had no ALS-resistance mutations but were resistant to nicosulfuron, indicating that NTSR is also present within the HN-02 population. All sequences of the 10 ST-1 individuals were identical, and no known mutations providing resistance to ALS or PPO inhibitors were found.

On the basis of the TSR gene-sequencing analysis, all 26 plants homozygous for both the *PPX2* gene Arg-128-Gly mutation and the *ALS* gene Ala-205-Val mutation were crossed, producing an HN-02F1-1 subpopulation. To verify the purity of the HN-02F1-1 subpopulation, 20 seeds were randomly selected for cultivation, and five plants for sequencing. The results showed that all plants survived the field-recommended rates of fomesafen and nicosulfuron. The five individuals selected for sequencing were found to have identical sequences and to be homozygous for the Ala-205-Val mutation in the *ALS* gene and Arg-128-Gly mutation in the *PPX2* gene (data not shown). Neither the Trp-574-Leu mutation (Figure 1C) nor other herbicide-resistance mutations were detected in the *ALS* gene or *PPX2* gene.

In the past 20 yr, several PPO inhibitors, including oxidiazoles and diphenyl ethers, and ALS inhibitors, including SU and IMI, have been continuously used to control *A. retroflexus* in northeast China. Owing to unique planting patterns (soybean–corn rotations) and the complex history of herbicide treatment (the

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**Table 3.** Parameter values of the four-parameter log-logistic equationfor calculating the $GR_{50}$values of *Amaranthus retroflexus* for multiple herbicides with standard errors in parentheses.[a]

| Herbicide | Population[b] | Regression parameters | | | | $GR_{50}$ | RI[c] |
|---|---|---|---|---|---|---|---|
| | | *c* | *d* | *b* | $r^2$ | | |
| Cloransulam-methyl | R | 1.49 (2.91) | 94.61 (1.61) | −1.02 (0.10) | 0.9991 | 38.56 (3.98) | 12.05 |
| | S | 0.83 (2.56) | 96.41 (2.07) | −0.87 (0.08) | 0.9992 | 3.20 (0.33) | |
| Flumetsulam | R | 21.70 (1.47) | 92.65 (1.46) | −1.61 (0.16) | 0.9988 | 11.93 (1.04) | 8.28 |
| | S | 14.60 (0.89) | 86.34 (0.91) | −1.69 (0.11) | 0.9995 | 1.44 (0.08) | |
| Nicosulfuron | R | 4.02 (6.60) | 94.54 (3.85) | −1.11 (0.29) | 0.9943 | 70.67 (16.90) | 11.25 |
| | S | 10.65 (2.11) | 98.45 (1.68) | −0.91 (0.08) | 0.9993 | 6.28 (0.59) | |
| Thifensulfuron-methyl | R | 9.07 (7.08) | 94.58 (4.62) | −1.04 (0.31) | 0.9923 | 19.15 (5.53) | 9.62 |
| | S | 20.04 (3.61) | 94.18 (6.46) | −1.66 (0.65) | 0.9891 | 1.99 (0.52) | |
| Imazethapyr | R | 12.59 (2.98) | 93.47 (2.98) | −2.07 (0.38) | 0.9960 | 230.82 (36.83) | 113.15 |
| | S | 16.83 (1.84) | 101.14 (3.78) | −1.02 (0.14) | 0.9984 | 2.04 (0.29) | |
| Imazamox | R | 7.19 (7.94) | 91.67 (3.90) | −1.22 (0.38) | 0.9912 | 81.89 (23.78) | 85.30 |
| | S | 9.45 (1.78) | 99.58 (3.34) | -1.79 (0.25) | 0.9980 | 0.96 (0.11) | |
| Fomesafen | R | 9.22 (4.04) | 90.12 (2.52) | −1.83 (0.58) | 0.9959 | 340.51 (47.54) | 53.29 |
| | S | 5.88 (0.76) | 99.77 (1.19) | −1.73 (0.13) | 0.9997 | 6.39 (0.23) | |
| Fluoroglycofen-ethyl | R | 10.93 (5.84) | 103.39 (7.43) | −1.98 (0.55) | 0.9936 | 215.54 (28.75) | 14.80 |
| | S | 17.56 (4.60) | 100.00 (7.37) | −2.62 (0.87) | 0.9906 | 14.56 (1.87) | |
| Acifluorfen | R | 6.25 (9.34) | 98.80 (10.18) | −1.61 (0.58) | 0.9913 | 1289.69 (243.77) | 17.02 |
| | S | 24.86 (1.45) | 95.77 (2.52) | −6.55 (2.30) | 0.9979 | 75.78 (5.15) | |
| Flumioxazin | R | 4.21 (0.87) | 96.88 (1.22) | −1.79 (0.16) | 0.9997 | 1.67 (0.04) | 1.95 |
| | S | 16.28 (2.41) | 84.09 (4.19) | −2.84 (1.77) | 0.9934 | 0.60 (0.17) | |
| Fluroxypyr-meptyl | R | 14.55 (2.38) | 96.04 (4.15) | −2.61 (1.07) | 0.9958 | 27.77 (5.56) | 1.89 |
| | S | 23.06 (2.31) | 96.41 (2.42) | −1.04 (0.14) | 0.9981 | 14.67 (2.06) | |
| 2,4-D butylate | R | 7.73 (2.11) | 92.33 (3.68) | −3.23 (0.72) | 0.9969 | 55.19 (10.41) | 1.23 |
| | S | 16.64 (5.12) | 92.49 (9.17) | −2.35 (1.32) | 0.9971 | 44.98 (8.81) | |

[a] $y = c + (d - c)/\{1 + \exp[b(\log x - \log ED_{50})]\}$, where *b* represents the relative slope, *c* represents the lower limit, *d* represents the upper limit, *x* represents the herbicide dose, and *y* represents the growth response (percentage of the untreated control); $GR_{50}$, dose required to reduce the plant dry weight by 50%.
[b] R, resistant population HN-02F1-1; S, susceptible population ST-1.
[c] Resistance index (RI) = $GR_{50}$ (HN-02F1-1)/$GR_{50}$ (ST-1).

long-term use of PPO and ALS inhibitors) in this region, the specific crop-planting system and herbicide application history have played an important role in driving the evolution of multiple herbicide resistance in the HN-02 populations collected in the area. Therefore, investigation of the resistance mechanism against fomesafen and nicosulfuron in this specific *A. retroflexus* population will facilitate the understanding of the evolution of the multiple herbicide resistance in this weed species.

Molecular analysis of the surviving plants confirmed that three amino acid substitutions, Arg-128-Gly in the *PPX* gene and both Ala-205-Val and Trp-574-Leu in the *ALS* gene, were present in the plants from the HN-02 population. The most common amino acid substitution sites in *ALS* genes are Ala-205-Val and Trp-574-Leu, which perhaps reflect herbicide use patterns (SU + IMI select mostly for Trp-574-Leu, whereas IMI herbicides select mostly for Ala-205), selection pressure, and fitness costs correlated with *ALS* genetic mutations (Yu and Powles 2014b). Imazethapyr is one of the most widely used herbicides in soybean fields (Li et al. 2008), while nicosulfuron is the main herbicide applied in cornfields in northeast China. These facts may explain why there are mainly Ala-205-Val mutations and relatively few Trp-574-Leu mutations in the HN-02 population. The Val mutation, in which Val is the most common substituted amino acid at the Ala-205 position in the *ALS* gene, has been documented in many weed species, including *A. retroflexus* (Ashigh and Tardif 2009; Matzrafi et al. 2015; McNaughton et al. 2005). The evolution of PPO-inhibitor resistance has been relatively rarely documented; to date, only 13 weed species have evolved resistance to PPO inhibitors (Heap 2019). Moreover, the Arg-128 substitution has rarely been found in common ragweed (*Ambrosia artemisiifolia* L.) and *A. palmeri* (Dayan et al. 2018). On the basis of our phenotype and sequencing results, the detection of the Arg-128-Gly mutation in the *PPX2* gene and the Ala-205-Val mutation in the *ALS* gene may be related to resistance to fomesafen and nicosulfuron, respectively, in HN-02F1-1 plants. Many studies have shown that the frequency of herbicide-resistance mutations is correlated with specific weed species, geographic environment, herbicide selection pressure, and suitability (Délye et al. 2010). Therefore, additional studies on the difference in mutation frequencies between amino acids 205 and 574 in the HN-02 population will be important to understand the multiple resistance of *A. retroflexus*.

### Whole-Plant Herbicide Bioassays

The resistance profile of *A. retroflexus* to 12 herbicides was characterized in this study. The results indicated that the S population (ST-1) was sensitive (RI < 2.0) to all tested herbicides and that the R population (HN-02F1-1) displayed different levels of resistance to the herbicides tested. On the basis of RI values (Table 3), compared with the ST-1 population, the HN-02F1-1 population was 53-fold resistant to fomesafen, 15-fold resistant to fluoroglycofen-ethyl, 17-fold resistant to acifluorfen, and 1.9-fold resistant to flumioxazin. Therefore, the HN-02F1-1 population has evolved a high level of resistance to fomesafen, fluoroglycofen-ethyl, and acifluorfen while still being sensitive to flumioxazin. Whole-plant bioassays also revealed that HN-02F1-1 was highly resistant (RI > 10) to cloransulam-methyl (TP), nicosulfuron (SU), imazethapyr (IMI), and imazamox (IMI) and moderately resistant (RI, 5.0~10) to flumetsulam (TP) and thifensulfuron-methyl (SU). On the basis of the $GR_{50}$ values and the RI values (Table 3), compared with ST-1, HN-02F1-1 was 8.3- to 113-fold more resistant to all tested ALS inhibitors. Moreover, compared with those of

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**Table 4.** Parameter values of the four-parameter log-logistic equationfor calculating the $I_{50}$values of the in vitro ALS activity at different herbicide doses.[a]

| Herbicide | Population[b] | Regression parameters | | | | $I_{50}$ | |
|---|---|---|---|---|---|---|---|
| | | *c* | *d* | *b* | $r^2$ | —μM— | RI[c] |
| Flumetsulam | R | 10.69 (3.62) | 101.12 (5.87) | −1.21 (0.45) | 0.9933 | 0.74 (0.19) | 14.51 |
| | S | 9.31 (2.56) | 95.24 (4.55) | −1.54 (0.44) | 0.9956 | 0.051 (0.013) | |
| Nicosulfuron | R | 9.66 (4.30) | 100.98 (4.55) | −0.63 (0.12) | 0.9966 | 2.08 (0.84) | 27.55 |
| | S | 10.58 (6.23) | 107.15 (15.56) | −0.65 (0.29) | 0.9912 | 0.076 (0.006) | |
| Imazethapyr | R | 8.77 (4.48) | 88.79 (3.74) | −2.62 (0.34) | 0.9945 | 27.35 (5.97) | 319.88 |
| | S | 8.95 (2.36) | 93.22 (4.26) | −1.51 (0.33) | 0.99861 | 0.086 (0.0201) | |

[a]$y = c + (d - c)/1 + \exp[b(\log x - \log ED_{50})]]$, where *b* represents the slope of the curve at $I_{50}$ (herbicide dose required to inhibit ALS activity by 50% compared with that of the untreated control), *c* represents the lower limit, *d* represents the upper limit, *x* represents the herbicide dose, and *y* represents the in vitro ALS activity response (as a percentage of the untreated control). The total acetolactate synthase (ALS) activity for HN-02F1-1 and ST-1 was 16.78 ± 0.53 nmol acetoin $mg^{-1}$ protein $min^{-1}$ and 15.11 ± 0.48 nmol acetoin $mg^{-1}$ protein $min^{-1}$, respectively.
[b]R, resistant population HN-02F1-1; S, susceptible population ST-1.
[c]Resistance index (RI) = $I_{50}$ (HN-02F1-1)/ $I_{50}$ (ST-1).

the S group, the RI values of the R group for fluroxypyr-meptyl and 2,4-D butylate were lower than 2, indicating that the HN-02F1-1 population was still sensitive to those herbicides.

Resistance to PPO inhibitors has been well represented in waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer], the first weed species identified to have evolved resistance to these herbicides. The ΔGly210 mutation in the *PPX2* gene provides *A. tuberculatus* with broad cross-resistance to most PPO inhibitors (Wuerffel et al. 2015). The Arg-128 mutation was subsequently shown to confer cross-resistance against PPO inhibitors in both *A. artemisiifolia* and *A. palmeri* (Giacomini et al. 2017; Rousonelos et al. 2012). The Ala-205-Val substitution mutation is commonly considered to confer high IMI resistance to plants rather than resistance to SU herbicides (Ashigh and Tardif 2007). However, cross-resistance has also been reported to depend on specific mutations, on chemical groups of ALS inhibitors, and on specific herbicides within a particular group, and sometimes on weed species (Yu and Powles 2014b). For instance, the Ala-205-Phe mutation in the *ALS* gene provides broad-spectrum resistance to ALS inhibitors in annual bluegrass (*Poa annua* L.) (Brosnan et al. 2016). NTSR also plays an important role in ALS-inhibitor resistance. Délye et al. (2011) reported that different corn poppy (*Papaver rhoeas* L.) individuals with identical *ALS* genotypes exhibited different levels of herbicide resistance, which was the result of NTSR. Therefore, in this study, the possibility of a metabolic resistance mechanism also cannot be ruled out in HN-02F1-1 with respect to the patterns of cross-resistance to PPO inhibitors and ALS inhibitors. The HN-02F1-1 population was still sensitive to flumioxazin, fluroxypyr-meptyl, and 2,4-D butylate. Therefore, flumioxazin, fluroxypyr-meptyl, and 2,4-D butylate can be used to prevent and manage the resistance of *A. retroflexus*, while crop rotation and/or mixtures of herbicides with different mechanisms of action as well as integrated pest management strategies are encouraged.

### *In Vitro Assays of ALS Activity*

To confirm the ALS-inhibitor resistance related to the Ala-205-Val substitution of target resistance observed in the R population, ALS enzymes from the HN-02F1-1 and ST-1 populations were extracted and assayed. In the absence of ALS inhibitors, the HN-02F1-1 and ST-1 populations presented similar (RI = 1.1) activity of extracted ALS (17 nmol acetoin $mg^{-1}$ protein $min^{-1}$ and 15 nmol acetoin $mg^{-1}$ protein $min^{-1}$, respectively). Moreover, some of the same ALS inhibitors used for the whole-plant studies were also used for in vitro ALS activity assays in both R and S plants. As shown in Table 4, the $I_{50}$ of imazethapyr (IMI) for ST-1 was 0.86 μM, while it was 27 μM for HN-02F1-1. In addition, the $I_{50}$ of flumetsulam (TP) for ST-1 and HN-02F1-1 was 0.051 μM and 0.74 μM, respectively. The $I_{50}$ of nicosulfuron (SU) $I_{50}$ for the ST-1 population was 0.076 μM, while it was 2.0 μM for HN-02F1-1. On the basis of the RI value at $I_{50}$, compared with the ST-1 population, the HN-02F1-1 population was highly resistant to imazethapyr (320-fold), flumetsulam (15-fold), and nicosulfuron (28-fold). These results indicate that the Ala-205-Val substitution within ALS resulting in insensitivity provides cross-resistance to at least three types of ALS inhibitors (TP, SU, and IMI).

The results of the in vitro ALS activity inhibition assays of HN-02F1-1 plants were consistent with the results of the whole-plant herbicide bioassays. This finding further confirmed that the Ala-205-Val mutation plays an essential role in SU and IMI herbicide resistance, which is similar to the conclusions of other reports (Ashigh and Tardif 2006; Chen et al. 2015). Moreover, the HN-02F1-1 plants were highly resistant to TPs; this is the first reported case in which the Ala-205-Val mutation provides resistance to TP herbicides (Tranel et al. 2019). The reduced susceptibility of herbicide target enzymes in vitro typically translates into herbicide resistance in whole plants (Li et al. 2017). Hence, the results of this study are consistent with those of previous research on SU and IMI resistance due to the Ala-205-Val mutation in horseweed (*Erigeron canadensis* L.) (Matzrafi et al. 2015).

In most cases, amino acid substitutions in ALS provide resistance to ALS inhibitors (Shaner 1999). Here, we did not evaluate the role of the NTSR mechanism; thus, the possibility of NTSR cannot be excluded. In addition, the RIs did not completely correlate with the $GR_{50}$ and $IC_{50}$, which may due to a variety of reasons. NTSR may contribute to resistance in some HN-02F1-1 individuals, and the overexpression of the *ALS* gene was not ruled out. NTSR has recently been confirmed to be responsible for fomesafen resistance in *A. palmeri* populations from Arkansas (Varanasi et al. 2018). Given that many weed species develop NTSR to ALS inhibitors (Zhao et al. 2017), it is reasonable to deduce that NTSR is present in *A. retroflexus*. Further investigations should focus on this aspect.

*Amaranthus retroflexus* is one of the main troublesome weed species in both soybean and cornfields (Wang et al. 2017). In China, most farmers prefer to use one herbicide repeatedly as a single weed control method in a crop field. Additionally, farmers tend to increase the application rates of herbicides to ensure

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control efficacy. Given these factors, it is not surprising that the HN-02 population evolved multiple resistance to PPO inhibitors and ALS inhibitors. Evolution of severe herbicide resistance to photosystem II inhibitors, ALS inhibitors, PPO inhibitors, and glyphosate in *A. tuberculatus* populations in the U.S. Midwest has also been reported (Tranel et al. 2010). Therefore, increased attention should be paid to investigating the resistance mechanism fully along with its fitness cost, biology, and ecology to control this notorious weed species.

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