Pesticide Biochemistry and Physiology 126 (2016) 22–27

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/ypest

Molecular basis of multiple resistance to ACCase- and ALS-inhibiting herbicides in *Alopecurus japonicus* from China

Yaling Bi [a,b], Weitang Liu [b], Wenlei Guo [b], Lingxu Li [b,c], Guohui Yuan [b], Long Du [b], Jinxin Wang [b,*]

[a] College of Agronomy, Anhui Science and Technology University, Anhui, Fengyang 233100, PR China
[b] Key Laboratory of Pesticide Toxicology and Application Technique, College of Plant Protection, Shandong Agricultural University, Shandong, Tai'an 271018, PR China
[c] College of Chemistry and Pharmaceutical Science, Qingdao Agricultural University, Shandong, Qingdao 266109, PR China

ARTICLE INFO

Article history:
Received 14 March 2014
Received in revised form 12 June 2015
Accepted 10 July 2015
Available online 16 July 2015

Keywords:
Acetyl-CoA carboxylase (ACCase)
Acetolactate synthase (ALS)
Amino acid substitution
Multiple resistance
Alopecurus japonicus

ABSTRACT

Fenoxaprop-P-ethyl-resistant *Alopecurus japonicus* has become a recurring problem in winter wheat fields in eastern China. Growers have resorted to using mesosulfuron-methyl, an acetolactate synthase (ALS)-inhibiting herbicide, to control this weed. A single *A. japonicus* population (AH-15) resistant to fenoxaprop-P-ethyl and mesosulfuron-methyl was found in Anhui Province, China. The results of whole-plant dose–response experiments showed that AH-15 has evolved high-level resistance to fenoxaprop-P-ethyl (95.96-fold) and mesosulfuron-methyl (39.87-fold). It was shown via molecular analysis that resistance to both fenoxaprop-P-ethyl and mesosulfuron-methyl was due to an amino acid substitution of Ile1781 to Leu in acetyl-CoA carboxylase (ACCase) and a substitution of Trp 574 to Leu in ALS, respectively. Whole-plant bioassays indicated that the AH-15 population was resistant to the ACCase herbicides clodinafop-propargyl, clethodim, sethoxydim and pinoxaden as well as the ALS herbicides pyroxsulam, flucarbazone-Na and imazethapyr, but susceptible to the ACCase herbicide haloxyfop-R-methyl. This work reports for the first time that *A. japonicus* has developed resistance to ACCase- and ALS-inhibiting herbicides due to target site mutations in the ACCase and ALS genes.

1. Introduction

Alopecurus japonicus, an abundant seed producer, is an aggressive annual grass weed that can substantially reduce crop yield in winter wheat and canola fields in eastern China [1]. Fenoxaprop-P-ethyl, an acetyl-CoA carboxylase (ACCase; EC 6.4.1.2)-inhibiting herbicide, was registered in China in 1988. It is applied post-emergence routinely by farmers to control *A. japonicus* and other grass weeds in winter wheat fields [2,3]. Unfortunately, the widespread use of this herbicide over the last 20 years has led to strong selection for resistant populations. Starting in 2007 it was observed that fenoxaprop-P-ethyl was no longer controlling *A. japonicus* when used at the recommend dose: initially in the provinces of Anhui and Jiangsu, but this has now been observed in at least five provinces in China [4].

Once fenoxaprop-P-ethyl resistance has been observed farmers will generally start to use mesosulfuron-methyl, an acetolactate synthase (ALS) inhibitor herbicide, as an alternative for control of *A. japonicus*. This herbicide was commercially introduced into China in 2003, and was very effective at controlling fenoxaprop-P-ethyl-resistant *A. japonicus* initially. However, after several years of successful control, growers in some regions of Anhui Province observed that applications of mesosulfuron-methyl at the recommended rates could no longer control *A. japonicus* as effectively as previously.

As resistance to single herbicide becomes more widespread in weed species, some species are developing multiple resistance to herbicides with various modes of action. Multiple resistance to herbicides with a range of modes of action has been reported in many weed species, such as *Lolium rigidum*, *Lolium multiflorum*, *Alopecurus myosuroides*, *Kochia scoparia*, *Avena fatua*, *Amaranthus tuberculatus*, *Amaranthus retroflexus*, *Echinochloa phyllopogon* and *Lolium perenne* [5]. The incidence of multiple herbicide resistance can lead to major practical problems, particularly because this type of resistance significantly reduces the availability of herbicides for controlling weeds.

In *A. japonicus*, the two herbicides to which resistance has developed have different target sites. ACCase is a key enzyme in fatty acid biosynthesis and oxidation that catalyzes the formation of malonyl-CoA [6], and ACCase-inhibiting herbicides include three different chemical families, aryloxyphenoxypropionic acids (APP), cyclohexanediones (CHD) and phenylpyrazolines (PPZ) [7]. ALS is the first enzyme in the pathway for the biosynthesis of the branched chain amino acids valine, leucine and isoleucine [8]. ALS inhibitors involve five different chemical classes, sulfonlylureas (SU), imidazolinones (IMI), pyrimidinylthiobenzoates (PTB), sulfonylaminocarbonyltriazolinones (SCT) and triazolopyrimidines (TP) [9–13].

* Corresponding author.
E-mail address: wangjx@sdau.edu.cn (J. Wang).

http://dx.doi.org/10.1016/j.pestbp.2015.07.002
0048-3575/

Resistance to ACCase inhibitors has been recorded in 46 grass weed species worldwide [5], and this resistance is based on two mechanisms: target-site resistance (TSR) and non-target site resistance (NTSR) [14–16]. TSR usually occurs due to a single key-point mutation in the carboxyl transferase (CT) domain of ACCase. Thirteen conserved amino acid substitutions in seven sites are known to endow ACCase-inhibitor resistance in various weed species: Ile 1781 to Leu or Val or Thr, Trp 1999 to Cys or Leu or Ser, Trp 2027 to Cys, Ile 2041 to Asn or Val, Asp 2078 to Gly, Cys 2088 to Arg and Gly 2096 to Ser or Ala [17–19]. Various resistance patterns in the ACCase inhibitors are generated by the amino acid substitutions at these seven positions [17]. NTSR is caused by a reduction in the amount of active herbicide that reaches the target, including a reduced rate of herbicide translocation, enhanced metabolism and sequestration of the herbicide from the target-site [7,17].

Currently, 145 weed species show resistance to ALS inhibitors worldwide [5]. In many cases, the resistance is due to the decrease in sensitivity of the target-site to the herbicides caused by amino acid substitution within the ALS gene [20–22]. To date, 26 amino acid substitutions involving ALS-inhibitor resistance have been identified at eight positions (Ala 122 (3), Pro 197 (12), Ala 205 (1), Asp 376 (1), Arg 377 (1), Trp 574 (3), Ser 653 (3) and Gly 654 (2)) in the ALS codons of various weed species [5,17]. As with ACCase inhibitors, different mutations in the ALS gene will result in a range of resistance patterns.

As far as we are aware, there have been no reports of multiple herbicide resistance from China previously. From China there have been eight examples of grass weed species with ACCase inhibitors reported and 11 species with ALS inhibitors [5]. Mohamed et al. reported that Chinese *A. japonicus* had evolved resistance to fenoxaprop and pinoxaden [4]. Moreover, Xu et al. [23] found that the Trp 2027 to Cys substitution confers resistance to several APP herbicides and a PPZ inhibitor, but not to CHD and ALS herbicides in *A. japonicus*. Tang et al. [24] and Yang et al. [25] reported that *A. japonicus* has evolved high resistance to haloxyfop-R-methyl. Bi et al. [1] reported that the Pro to Thr substitution at amino acid 197 of ALS endowed resistance to mesosulfuron-methyl in *A. japonicus* from Jiangsu Province, China. These studies present plenty of evidence for individual resistance to ACCase-inhibitor herbicides or ALS inhibitors in *A. japonicus* but none for multiple resistance to ACCase and ALS herbicides. Since 2011, farmers in Anhui Province, China, have observed that mesosulfuron-methyl at recommended rates cannot control fenoxaprop-P-ethyl-resistant *A. japonicus* in wheat fields any longer, after several years of successful control. Therefore, this study aims to: (1) investigate and quantify the resistance in *A. japonicus* to fenoxaprop-P-ethyl and mesosulfuron-methyl; (2) explore the molecular basis for the resistance in *A. japonicus* to fenoxaprop-P-ethyl and mesosulfuron-methyl; and (3) characterize the resistance patterns of the resistant population of *A. japonicus* to other ACCase- and ALS inhibitors.

2. Materials and methods

2.1. Plant materials

Seeds from an *A. japonicus* population that was suspected of being resistant (R) to fenoxaprop-P-ethyl and mesosulfuron-methyl, AH-15, were used in this study; these seeds were obtained in 2011 from wheat field in Longji, Tianchang county, Anhui Province, China (32°83′80.48″N, 119°4′74.44″E). A susceptible (S) *A. japonicus* population, AH-7, was collected from uncultivated hills to which no herbicide had been applied, in Fengyang county, Anhui Province, China (32°87′47.35″N, 117°53′16.23″E). All the seeds from 50 randomly selected plants were collected by hand and combined into one sample each for the AH-15 and AH-7 populations. All seeds were air-dried and stored in paper bags at 4 °C until use.

2.2. Herbicides and chemicals

The herbicides used for the dose–response experiments were: fenoxaprop-P-ethyl (69 g/L EW, Bayer, Beijing, China), mesosulfuron-methyl (30 g/L OF, Bayer), clodinafop-propargyl (15% WP, Syngenta, Beijing, China), haloxyfop-R-methyl (108 g/L EC, Dow AgroSciences, Beijing, China), clethodim (120 g/L EC, Longdeng Agrochemical, Jiangsu, China), sethoxydim (12.5% EC, Binnong Technology, Shandong, China), pinoxaden (5% EC, Syngenta), pyroxsulam (7.5% WG, Dow AgroSciences), imazethapyr (10% AS, Binnong Technology) and flucarbazone-Na (70% WG, Heben Pesticide, Shanghai, China). In the dose–response experiment, herbicides with a range of different modes of action were used at various doses (Table 1).

2.3. Sensitivity to ACCase- and ALS-inhibiting herbicides

Seeds were pre-germinated and 20 were sown in moist loam soil in 12 cm diameter pots. The pots were placed in an artificial climate chamber maintained at 25/20 °C (day/night temperatures) with a 12 h cycle, 434.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 75% relative humidity. Once the seedlings had emerged they were reduced to 10 similar sized examples per pot, and water and fertilizer were applied as required. Herbicide treatments were applied to *A. japonicus* plants at the three-to-four-leaf stage using a compressed air moving nozzle cabinet sprayer equipped with one Teejet 9503EVS flat fan nozzle delivering 450 L ha^{-1} at 275 kPa. All shoots were harvested by cutting at the soil surface 21 d after treatment (DAT) and the dry weight was determined. There were three replicates of each treatment and the experiment was performed twice.

2.4. Statistical analyses

Data from the repeated experiments were analyzed by ANOVA (SPSS v17.0; SPSS Inc.). Regression analyses were conducted using Sigma Plot (V10.0). The dose–response was obtained by non-linear regression analysis using the log-logistic equation (Eq. (1)) proposed by Seefeldt et al. [26]:

$$Y = c + (d-c)/(1 + x/GR_{50})^b \quad (1)$$

where c = the lower limit, d = the upper limit, b = the slope at GR_{50} and GR_{50} = the herbicide dose required for a 50% inhibition of growth reduction. In this regression equation, the herbicide rate was the independent variable (x) and the growth response was the dependent variable (y). The fitted equations were used to estimate the amount of herbicide required for a 50% growth reduction (GR_{50} value). The resistance levels were determined from the resistance ratio (*R/S*), which was calculated as the GR_{50} of the R population divided by the GR_{50} of the S population.

2.5. Genomic DNA extraction

Approximately 100 mg of young shoot tissue was selected from each a single plant at the 3–4-leaf stage from the AH-15 and AH-7 populations and stored at −80 °C. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method [27].

2.6. Plastidic ACCase gene CT domain sequencing

A pair of primers was designed to amplify the CT domain of the plastidic ACCase gene of *A. japonicus* (Table 2), and a 1437-base pair (bp) ACCase gene fragment encompassing codons Ile1781 to Gly2096, numbered according to the ACCase gene sequence of *A. myosuroides* (GenBank: AJ310767.1), was amplified and comparisons were made between the AH-15 and AH-7 populations.