Resistance level and target-site mechanism to fenoxaprop-*p*-ethyl in *Beckmannia syzigachne* (Steud.) Fernald populations from China

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ABSTRACT

Beckmannia syzigachne (Steud.) Fernald is one of the main grass weeds severely harming wheat (*Triticum aestivum* L.) production in rice-wheat areas in China. Fenoxaprop-*p*-ethyl is the main herbicide used to selectively control grass weed in China. *Beckmannia syzigachne* has evolved resistance to fenoxaprop-*p*-ethyl due to continuous application. To investigate fenoxaprop-*p*-ethyl resistant level and mechanism in *B. syzigachne* in a portion of the rice-wheat area in China, samples from 31 field populations were collected and treated with fenoxaprop-*p*-ethyl. The results show that 10 of the 31 tested field populations evolved a high level of resistance to fenoxaprop-*p*-ethyl. A portion of the acetyl-coenzyme A carboxylase (*ACCase*) gene was amplified, sequenced and aligned. The known Ile-1781-Leu, Ile-1781-Val, Ile-2041-Asn, Asp-2078-Gly and Gly-2096-Ala mutations were identified in five resistant populations. None of the known resistant substitutions was identified in the other five resistant populations, which means the resistance to fenoxaprop-*p*-ethyl in these populations is likely endowed by non-target-site resistance mechanism.

Key words: ACCase, American slough grass, amino acid substitution, resistant level, resistant mechanism.

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important grain crops in China. Due to the various temperatures and rainfall levels, wheat is rotated with different crops in China (Wang et al., 2009). The rice (*Oryza sativa* L.)-wheat system is popular in the middle and lower Yangtze River as well as southwestern China. The total rice-wheat area is estimated to cover approximately 7.4 Mha and is one of the main grain product areas in China (Timsina and Connor, 2001; Dawe et al., 2004). *Beckmannia syzigachne* (Steud.) Fernald (American slough grass), a diploid and annual grass weed, distributes all over China and is more popular in the middle and lower Yangtze River and southwestern China. In these area *B. syzigachne* is predominant in wheat fields rotated with rice and severely harms winter wheat product (Li, 1998; Rao et al., 2008).

Acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides kill grass weed by inactivating ACCase and blocking fatty acid biosynthesis, this type of herbicide is safe to broadleaved weeds (Devine, 1997). ACCase inhibitor selectivity is based on the different ACCase forms. Two forms of ACCase have been identified in plants; one is located in the plastid, and the other is in the cytosol (Konishi et al., 1996). The plastid ACCase is essential for the *de novo* fatty acid synthesis, and the cytosolic ACCase is involved in synthesizing long chain fatty acids (Kaundun et al., 2013a). The cytosolic ACCase is homomeric and contains the biotin carboxylase (BC) domain, the biotin carboxyl carrier protein (BCCP) domain, and the carboxyltransferase (CT) domain in a single polypeptide. The plastid ACCase is a heterodimeric enzyme that carries the three domains in four subunits encoded by a nuclear gene and a chloroplastic gene in most plants. Most plants include the two forms of ACCase described above. The Poaceae family is special because it includes a homomeric plastid ACCase (Konishi et al., 1996). The homomeric plastid ACCase in grass weed is sensitive to the ACCase-inhibiting herbicides, while the heterodimeric enzyme in broadleaved weed is insensitive (Konishi and Sasaki, 1994).

The ACCase-inhibiting herbicides that contain three dissimilar classes of herbicides, aryloxyphenoxypropionates (APPs), cyclohexanediones (CHDs) and phenylpyraxolins (DENs), target the plastid ACCase CT domain in grass weed (Yu et al., 2010). ACCase-inhibiting herbicides are widely used to selectively control gramineous weeds in wheat fields. However, continuous application has increased resistance to the herbicides. An insensitive target enzyme and enhanced metabolism are the main mechanisms that result in herbicide resistance (Délye,

2005; Powles and Yu, 2010). An insensitive enzyme typically results from a single point mutation that confers an amino acid change in the target enzyme and prevents herbicide binding (Powles and Yu, 2010). Seven amino acid substitutions in the ACCase CT domain confer ACCase-inhibitor resistance in grass weed: Ile-1781-Leu/Val/Thr (Délye et al., 2002; 2005; Kaundun et al., 2013b), Trp-1999-Cys/Ser (Liu et al., 2007; Kaundun et al., 2013a), Trp-2027-Cys (Délye et al., 2005; Xu et al., 2013), Ile-2041-Asn/Val (Délye et al., 2003; Scarabel et al., 2014), Asn-2078-Gly (Délye et al., 2005), Cys-2088-Arg (Yu et al., 2007; Kaundun et al., 2012), and Gly-2096-Ala/Ser (Délye et al., 2005; Cruz-Hipolito et al., 2012). Metabolic resistance is mainly due to enhanced detoxifying enzymes, such as glutathione-S-transferases and cytochrome P450s (Délye et al., 2011).

ACCase-inhibiting herbicide fenoxaprop-*p*-ethyl (ethyl (*R*)-2-[4-(6-chloro-1,3-benzoxazol-2-yloxy) phenoxy]propionate) is one of the main herbicides used to selectively control *B. syzigachne* in China. Over-reliance on fenoxaprop-*p*-ethyl has generated resistance to this herbicide in *B. syzigachne* (Li et al., 2013; 2014). In order to sustain herbicide efficacy to achieve effective weed controlling, it is necessary to identify the resistant level and the underlying resistance mechanisms. This paper aims to determine the resistance level to fenoxaprop-*p*-ethyl in *B. syzigachne* from the rice-wheat area in China and investigate the resistance mechanism to fenoxaprop-*p*-ethyl in *B. syzigachne*.

Table 1. Information on the collection location and herbicide history.

	Location			
Population	Province	County	Village	Time
A01	Anhui	Feixi	Kuahe	June 2011
A02	Anhui	Feidong	Wuji	June 2011
A03	Anhui	Shouxian	Chaan	June 2011
A04	Anhui	Shouxian	Zhangdun	June 2011
A05	Anhui	Dingyuan	Yihe	June 2011
A06	Anhui	Shouxian	Yanliu	June 2012
A07	Anhui	Shouxian	Anfengtang	June 2012
A08	Anhui	Dingyuan	Xiaogao	June 2012
A09	Anhui	Tianchang	Batian	June 2012
J01	Jiangsu	Danyang	Hujia	June 2011
J02	Jiangsu	Danyang	Liangjia	June 2011
J03	Jiangsu	Danyang	Zheliu	June 2011
J04	Jiangsu	Siyang	Chengxiang	June 2011
J05	Jiangsu	Danyang	Hushu	June 2010
J06	Jiangsu	Shuyang	Longmiao	June 2012
J07	Jiangsu	Funing	Dayu	June 2012
J08	Jiangsu	Jiangyan	Qianye	June 2012
J09	Jiangsu	Jurong	Guozhuang	June 2012
J10	Jiangsu	Danyang	Xingtang	June 2012
J11	Jiangsu	Danyang	Maojia	June 2012
J12	Jiangsu	Liyang	Xiaojinsha	June 2012
J13	Jiangsu	Jintan	Houzhuang	June 2012
J14	Jiangsu	Jintan	Houzhuang	June 2012
J15	Jiangsu	Peixian	Xiaozhuang	June 2012
S01	Shandong	Yutai	Tanzhuang	June 2011
S02	Shandong	Yutai	Xihua	June 2011
S03	Shandong	Yutai	Zhangnian	June 2011
S04	Shandong	Weishan	Hanzhuang	June 2012
S05	Shandong	Yutai	Qisuolou	June 2012
S06	Shandong	Yutai	Xiaoli	June 2012
S07	Shandong	Yutai	Chenzhuang	June 2012
TS	Shandong	Taishan	Daqiao	June 2012

MATERIALS AND METHODS

Plant material

Thirty-one field populations were collected from winter wheat fields rotated with rice in Anhui, Jiangsu and Shandong provinces from June 2011 to June 2012 and are coded as A××, J×× and S××, respectively. All the fields have been sprayed by fenoxaprop-*p*-ethyl in the last 5 yr. A known susceptible population, TS was collected from the Mountain Taishan scenic spot, Taishan District, Shandong Province. Seeds from at least 20 mature plants were randomly collected by hand and bulked. The seeds were air-dried and stored in paper bags at room temperature until use. The collecting position is shown in Table 1.

Fenoxaprop-*p*-ethyl single-rate test

The experiment was conducted from September 2013 to December 2013. The herbicide sensitivity assessment procedure was the same for every sample. Seeds were germinated and planted in plastic pots (d = 12 cm) as previously described (Li et al., 2014). Next, the seedlings were transferred to a greenhouse (temperature was maintained at approximately 15 to 25 °C with 75% RH and natural sunlight) and watered as needed. The plants were thinned to 10 evenly sized plants per pot at the two-leaf stage. The plants were sprayed with 62.1 g ai ha^{-1} (1×) fenoxaprop-*p*-ethyl (69 g L^{-1} EW, Bayer, Hangzhou, China) at the three- to four-leaved-stage using a compressed air, moving-nozzle cabinet sprayer equipped with one flat fan nozzle (9503EVS, Teejet Technologies, Springfield, Illinois, USA) and calibrated to deliver 450 L ha^{-1} at 0.28 MPa. The plants treated with water were selected as a control. Every treatment contained one pot and all treatments were replicated three times. All plants were returned to the greenhouse after the herbicide treatment. Plant survival was assessed visually 21 d after the herbicide was applied. Severely injured plants were classified as sensitive, while surviving plants that expanded new green leaves were classified as resistant.

Fenoxaprop-*p*-ethyl dose-response experiment

To determine the level of resistance in resistant populations, dose-response experiments were performed using 11 populations from December 2013 to May 2014. The plant and spray procedure were as described above except each pot only contained five seedlings. The rates of herbicide application are described in Table 2. The plants were cut at the soil surface, and the fresh weight were recorded 21 d after treatment (DAT) after the plant survival was assessed. The treatments were replicated three times, and the experiments were performed twice.

Table 2. Herbicide dose used to test the resistance index.

Populations	Herbicide dose					
	g ai ha^{-1}					
J11, J13, J14, S03	62.10	186.30	558.90	1676.70	5030.10	15090.30
J05, J09	62.10	124.20	248.40	496.80	993.60	1987.20
A01, J01	31.05	62.10	124.20	248.40	496.80	-
J02	20.70	41.40	82.80	165.60	331.20	662.40
J03	20.70	41.40	82.80	165.60	331.20	-
TS	0.77	2.30	6.90	20.70	62.10	186.30

Note: The recommended dose of fenoxaprop-*p*-ethyl for use in fields is 62.1 g ai ha^{-1}.

Statistics

All regression analyses were performed using Sigma Plot 12.0 (Systat Software, Chicago, Illinois, USA). Fresh-weight data were subjected to a nonlinear regression analysis using the log-logistic equation (Seefeldt et al., 1995):

$$y = C + (D - C)/ + (x/\mathrm{GR}_{50})^{b}$$

where C is the lower limit, D is the upper limit, b is the relative slope around the herbicide dose that yields 50% growth inhibition (GR_{50}), x is the herbicide rate, and y is the growth response (percentage of the untreated control). The significances of the regression parameters were determined using the t-test method ($P = 0.05$). The fitted equations were used to estimate the GR_{50} value. The resistance index (RI) was calculated as the GR_{50} of the resistant population divided by the GR_{50} of the susceptible population to indicate the level of resistance for the resistant population. Data sets from repeated experiments were analyzed by ANOVA (IBM SPSS Statistics for Windows, Version 20.0; IBM Corp., Armonk, New York, USA). The data were pooled for subsequent analyses as the variance between repeated experiments was nonsignificant. Similarly, the median lethal concentration (LC_{50}) was attained according to the plant survival rate.

Identification of resistant amino acid substitution

The seeds were germinated and planted as described above except each pot only included one seedling. The pots were placed in the same greenhouse and watered as needed. The seedlings were managed as described above. Approximately 50 mg shoot tissue from each individual plant at the three- to four-leaf stage was cut and immediately frozen in liquid nitrogen for further analyses. The total DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1990). The forward primer 5'--TTTCCCAGCGGCAGACAGAT—3', and reverse primer 5'--TCCCTGGAGTCTTGCTTTCA—3' were used to amplify a 1437-bp fragment that coded 479 amino acids containing all the seven known substitutions that confer resistance to ACCase-inhibiting herbicides (Bi et al., 2015). The PCR experiments were performed in a final volume of 25 µL, containing 1 µL genomic DNA (approximately 30 ng), 1 µL each primer (10 µM), 2.5 µL 10× Trans EasyTaq buffer (Mg2+ Plus, TransGen Biotech, Beijing, China), 2 µL deoxynucleotide triphosphate mixture (2.5 mM, TransGen Biotech), 0.25 µL Trans EasyTaq DNA polymerase (2.5 units), and 17.25 µL distilled deionized H_2O. The PCR experiments were performed using a thermal cycler (T100, Bio-Rad Laboratories, Hercules, California, USA) programmed for an initial denaturation step of 94 °C for 5 min followed by 35 cycles of 50 s at 94 °C, 50 s at 58 °C, and 100 s at 72 °C. A final extension cycle for 10 min at 72 °C was also included.

The PCR products were visualized by electrophoresis on 1.0% agarose gel running in 1× Tris-acetate-ethylenediaminetetraacetic acid buffer. The intended bands were extracted from 1.0% agarose gel using the EasyPure Quick Gel Extraction kit (TransGen Biotech) and then cloned using pEASY-T1 vector (TransGen Biotech). The recombinant plasmids were introduced into competent *Escherichia coli* (*Trans1*-T1 Phage Resistant Chemically Competent Cell, TransGen Biotech) in accordance with the manufacturer's instructions. The positive clones were sequenced on an ABI PRISM 3730 DNA sequencer (Shanghai Sangon Biological Engineering Technology & Services Co., Shanghai, China). In total, 10 individual plants from each population were sequenced. At least five clones for each biological replicate were sequenced. The sequence data for the resistant and susceptible populations were compared to determine whether a nucleotide substitution was associated with resistance. Sequence data for each population were aligned and compared using DNAMAN version 5.2.2 software (Lynnon Biosoft, Quebec, Canada).

RESULTS AND DISCUSSION

Fenoxaprop-*p*-ethyl single-rate test

For the single-rate test, 21 populations were severely injured, and no new leaves expanded after treatment with fenoxaprop-*p*-ethyl at the recommended dose (62.1 g ai ha^{-1}) compared with the untreated control. Certain populations, such as TS and A02, were completely killed. Eight of nine populations from Anhui province were efficiently controlled by 1× fenoxaprop-*p*-ethyl, and the number from Jiangsu province and Shandong province were 7/15 and 6/7 respectively. Fenoxaprop-*p*-ethyl did not efficiently control 10 populations (A01, J01, J02, J03, J05, J09, J11, J13, J14 and S03) at the same dose, even completely failed to control several populations, such as J11, J13, J14 and S03.

Fenoxaprop-*p*-ethyl dose-response experiments

The two whole-plant experiments did not show different results. Therefore, data were combined for the subsequent analyses. In the dose-response experiment, all field populations tested showed high-level resistance to fenoxaprop-*p*-ethyl. The LC_{50} value for J03 was 91.28 g ai ha^{-1}, which was the lowest in all resistant populations, but still was much higher than the recommended rate 62.1 g ai ha^{-1}. The GR_{50} values varied from 91.55 to 2798.87 g ai ha^{-1} with RI values from 14.6 to 445.0, which were compared to the susceptible TS population (Table 3).

Table 3. The level of resistance to fenoxaprop-*p*-ethyl in 10 *Beckmannia syzigachne* populations.

Population	GR_{50} (g ai ha^{-1}) (SE)	RI
A01	> 496.8	> 79.0
J01	> 496.8	> 79.0
J02	104.56 (16.78)	16.6
J03	91.55 (11.92)	14.6
J05	990.26 (265.43)	157.4
J09	1640.53 (229.70)	260.8
J11	3554.08 (110.22)	565.0
J13	1619.74 (61.31)	257.5
J14	2798.87 (483.28)	445.0
S03	1488.39 (646.53)	236.8
TS	6.29 (0.13)	-

GR_{50}: Herbicide dose required to decrease plant fresh weight by 50% compared with the untreated control. Each value represents the mean ± standard error (SE).
RI: Resistance index calculated by dividing the GR_{50} value of the resistant population by the susceptible population

Fenoxaprop-*p*-ethyl is one of the main herbicides that have been used in wheat fields to selectively control grass weed in China since the 1990s. Widespread and continuous use of fenoxaprop-*p*-ethyl has produced resistant weeds. *Beckmannia syzigachne* field populations resistant to fenoxaprop-*p*-ethyl were first identified in 2008 in Danyang county, Jiangsu province (Liu and Zhang, 2008). Next, *B. syzigachne* field populations that are resistant to fenoxaprop-*p*-ethyl were successively identified in Jurong county Jiangsu province and Lujiang county Anhui province (Lv et al., 2012; Li et al., 2014). In this research, 31 *B. syzigachne* field populations were treated with fenoxaprop-*p*-ethyl to investigate their resistance to fenoxaprop-*p*-ethyl. The results show that 10 of the 31 field populations have evolved a high level of resistance to fenoxaprop-*p*-ethyl (Table 3). Eight of the 10 resistant populations were collected in Jiangsu province. The data indicates that agriculture in the Jiangsu province has been more severely disturbed by fenoxaprop-*p*-ethyl resistant *B. syzigachne*.

Identification of resistant amino acid substitution

A portion of the CT gene was amplified and sequenced to compare the sequence and determine the resistance mechanisms (the gene information is deposited in National Center for Biotechnology Information [NCBI] with the accession number KT291176-KT291186). The sequence comparison showed several key amino acid substitutions in the CT region of the resistant populations (Table 4). Ile-1781-Leu, Ile-1781-Val, Ile-2041-Asn, Asp-2078-Gly and Gly-2096-Arg substitutions were identified in the J09, J11, J13, J14 and S03 populations, respectively. The key substitution is the target-site-resistant mechanism to fenoxaprop-*p*-ethyl in the corresponding populations.

Moreover, no known substitutions conferring resistance to ACCase-inhibiting herbicides were identified in the A01, J01, J02, J03 and J04 populations. Resistance to fenoxaprop-*p*-ethyl in these populations is highly suspected to be conferred by non-target-site resistance mechanism.

Table 4. Key amino acids in the carboxyltransferase domain of fenoxaprop-*p*-ethyl-susceptible *Alopecurus myosuroides* and fenoxaprop-*p*-ethyl-resistant and -susceptible *Beckmannia syzigachne*.

	Amino acid position and relative nucleotide and amino acid sequence						
Population	1781	1999	2027	2041	2078	2088	2096
A01	Ile	Trp	Trp	Ile	Asp	Cys	Gly
J01	Ile	Trp	Trp	Ile	Asp	Cys	Gly
J02	Ile	Trp	Trp	Ile	Asp	Cys	Gly
J03	Ile	Trp	Trp	Ile	Asp	Cys	Gly
J05	Ile	Trp	Trp	Ile	Asp	Cys	Gly
J09	**Leu**	Trp	Trp	Ile	Asp	Cys	Gly
J11	**Val**	Trp	Trp	Ile	Asp	Cys	Gly
J13	Ile	Trp	Trp	**Asn**	Asp	Cys	Gly
J14	Ile	Trp	Trp	Ile	**Gly**	Cys	Gly
S03	Ile	Trp	Trp	Ile	Asp	Cys	**Ala**
TS	Ile	Trp	Trp	Ile	Asp	Cys	Gly
Alopecurus myosuroides	Ile	Trp	Trp	Ile	Asp	Cys	Gly

Note: The known resistance amino acid substitution is in bold for the resistant populations.

To date, 12 substitutions at seven positions in the CT domain have been identified as the basis for resistance to ACCase-inhibiting herbicides in grass weed (Beckie and Tardif, 2012; Kaundun et al., 2013b). Five substitutions at four positions (Table 4) have been identified in this research, which contain the less reported Ile-1781-Val and Gly-2906-Ala. The Trp-2027-Cys substitution was also identified in a fenoxaprop-*p*-ethyl-resistant *B. syzigachne* population in our previous research (Li et al., 2014). These results indicate that *B. syzigachne* exhibits rich diversity target-site resistance mechanisms for fenoxaprop-*p*-ethyl resistance even though it is a diploid, self-pollinated grass weed. Due to the different cross-resistance patterns associated with different ACCase substitutions, this diversity will make it more difficult to control fenoxaprop-*p*-ethyl resistant *B. syzigachne*.

Other mechanisms except the resistant substitution are also likely involved in fenoxaprop-*p*-ethyl-resistant *B. syzigachne* field populations. Currently, little is known about other resistant mechanisms except the insensitive target enzyme in *B. syzigachne*, especially the NTSR mechanism. Compared to the single-gene encoded TSR mechanism, NTSR mechanism likely encoded by multiple genes has unpredictable cross-resistance patterns and more complex genetic patterns (Délye et al., 2011; 2013).

CONCLUSIONS

In this research, single-dose treatments and dose-response experiments were employed to determine fenoxaprop-*p*-ethyl resistance in 31 field populations collected from a portion of the rice-wheat areas in China. The results show that 10 of the populations have evolved a high level of resistance to fenoxaprop-*p*-ethyl. Ile-1781-Leu, Ile-1781-Val, Ile-2041-Asn, Asp-2078-Gly and Gly-2096-Ala substitutions were identified in five resistant populations. None of the known substitutions that confer resistance were identified in other resistant populations.

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