Fenoxaprop-p-ethyl Susceptibility and Mutation Point Detection of Acetyl-CoA Carboxylase (ACCase) in Different Wild oat (*Avena fatua* L.) Populations from China

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Abstract

To explore resistant mechanism of wild oat to fenoxaprop-p-ethyl, the susceptibility of Acetyl-CoA Carboxylase (ACCase) from 24 wild oat populations to fenoxaprop-p-ethyl, the level of gene expression, and mutation site of ACCase were conducted. *In vitro* ACCase activities were solated and measured by enzyme-linked immunosorbent assay kit (ELISA) assays, the results indicated that the IC_{50} value of the ACCase of the most unsusceptible to fenoxaprop-p-ethyl in the wild oat population from Yexian2017 (W24) was 7206.557 -fold compared to that of the ACCase of most susceptible to fenoxaprop-p-ethyl in the wild oat population from Queshan (W11). The differential expression of genes in wild oat treated by the IC_{50} fenoxaprop-p-ethyl concentration (6.9 mg/L) for 24 hours using RNA-seq, digital gene expression (DGE) profling was conducted. We found that 8 unigenes annotated as ACCase genes, 0 up-regulaed expression and 3 down-regulated expression were observed. The down-regulaed expressed *ACCase* was selected for qPCR in the relative susceptible population were significantly more suppressed than the three relative resistant populations. The mutations point of ACCase, Ile-1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Asp-2078-Gly, Cys-2088-Arg published were not found in the populations tested by multiple sequence alignment with a model complete ACCase sequence of *Alopecurus myosuroides*. These findings suggest that ACCase plays a critical role in the development of wild oat resistance to fenoxaprop-p-ethyl.

Keywords: *Avena fatua*, Acetyl-coa Carboxylase (ACCase), fenoxaprop-p-ethyl resistance, mutation points

Wild oat (*Avena fatua* L.) also named oat grass, belongs to the Gramineae Avena and matures one or two years (Christoffers *et al.* 2002). Wild oat is as malignant weeds that harms wheat, oilseed rape, and other crops in China, and has developed serious resistance to herbicides all over the world (Cavan *et al.* 2001).

At present, wild oat is controlled using chemical herbicides. The herbicide varieties that effectively control wild oat are few. The only inhibitors are metsulfuron-methyl and fenoxaprop-p-ethyl. Fenoxaprop-p-ethyl is a kind of aryloxyphenoxypropionate herbicide (AOPP). Its mode of action is by inhibiting the activity of acetyl-coenzyme A carboxylase (ACCase) in grassy weeds and then blocking normal fatty acid synthesis in plants (Devine & Shukla 2000). As a highly effective herbicide, it has been widely used in wheat fields to prevent grassy weeds, such as wild oat. However, both long-term and single uses of fenoxaprop-p-ethyl in wheat fields have caused resistance to wild oat. In addition, fenoxaprop-p-ethyl only acts on ACCase, so weeds are easily able to develop resistance (Delye 2005). Fenoxaprop-p-ethyl belongs to the high-risk level in the resistant risk classification, in which weeds will typically develop resistance after continuous use for a year (Chen 2018; Pornprom 2006). Recently, wild oat had been difficult to control in wheat fields using only fenoxaprop-p-ethyl. However, the resistance of wild oat to fenoxaprop-p-ethyl has been seldom reported, especially the resistance mechanism.

Carboxylation of acetyl-CoA includes mainly two processes which is involved with carboxylation of biotin and transfer of carboxylation reaction respectively (Konishi *et al.* 1996), the ACCases of plants comprise homogeneity (ACCase I) and heterogeneity (ACCase II), they are both responsible for catalysing acety-CoA to malony-CoA (*Schulte et al.* 1997). Homomeric ACCase localizes in cytosol and it is a pivotal enzyme to the step of fatty acid synthesis reaction. Heteromeric ACCase used to be in cytosol, its production malony-CoA is mainly utilized for elongating fatty acid chain and for synthesizing metabolite associated with flavonoids, resistance-endowing (Shorrosh *et al.* 1994). However, there are two special cases in cellular distribution of the two isoenzymes. The first one is that the chloroplast of rape has not only homomeric ACCase and heteromeric ACCase. The second one is that the ACCases of gramineous plants are all classified as homogeneity whether they present in cytosol or in plastid (Sasaki *et al.* 1997).

Target-site herbicide resistance (TSR) and non-target-site herbicide resistance (NTSR) are the main mechanisms of weed resistance to herbicides. TSR involves a

target-site mutation that affects the target enzyme sensitivity and results in much lower or higher expression levels of the target enzyme. Studies of NTSR have mainly included osmosis, reduced herbicide absorption and conduction, metabolic detoxification, shielding effects and buffer functioning to increased herbicide resistance herbicide heightening (Powles *et al.* 1997). ACCase is used as the target of AOPP. Weeds develop resistance to AOPP mainly due to mutations of ACCase; but the overexpression of ACCase in weeds also results in the development of resistance to herbicides (Zhang & Powles 2006). *Sorghum halepense* had developed resistance to enoxaprop-p-ethyl and sethoxydim due to the overexpression of *ACCase* gene (Parker *et al.* 1990; Bradley *et al.* 2015).

Previous studies of the resistance mechanism to ACCase herbicides in resistant Avena spp. populations revealed that ACCase gene mutation can confer the resistance to weed frequently (Maneechote *et al.* 1994, 1997; Seefeldt *et al.* 1996; Shukla *et al.* 1997; Cocker *et al.* 2000). Ile-1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Asp-2078-Gly and Cys-2088-Arg are now known mutation sites of ACCase to be associated with resistance to fenoxaprop-p-ethyl in wild oat (Christoffers *et al.* 2002; Liu *et al.* 2007; Cruz-Hipolito *et al.* 2011; Beckie *et al.* 2012). Therefore, the susceptibility of Acetyl-CoA Carboxylase (ACCase) from 24 wild oat populations to fenoxaprop-p-ethyl, digital gene expression (DGE) profling, the level of gene expression, and mutation site of ACCase were conducted in *Avena fatua*. It would provide a theoretical basis for understanding the molecular mechanisms of weed resistance.

MATERIALS AND METHODS

Seeds of wild oat

Seeds of wild oat collected from Henan Province were distributed in Xiangxian (Xuchang)=W1, Huojia (Xinxiang) =W2, Huixian (Xinxiang) =W3, Wuzhi (Jiaozuo) =W4, Xiuwu (Jiaozuo) =W5, Xunxian (Hebi)=W6, Fugou (Zhuokou) =W7, Wuyang (Luohe)=W8, Lankao (Kaifeng)=W9, Heshan (Hebi) =W10, Queshan (Zhumadian)=W11, Qixian (Hebi)=W12, Suiyang (Shangqiu)=W13, Zhaoling (Luohe)=W14, Huaiyang (Zhoukou)=W15, Xihua (Jiaozuo)=W16, Dengfeng

(Zhengzhou)=W17, Suiping (Zhumadian)=W18, Linying (Luohe)=W19, Sheqi (Nanyang)=W20, Xihua (Zhoukou) =W21, Yanling (Xuchang)=W22, Tangyin (Anyang)=W23, Yexian (Pingdingshan) =W24 in 2017 and Yexian (Pingdingshan) in 2016=W25.

Cultivation of seedlings

The greenhouse potting methods of were adopted (Li *et al.* 2010) . The seeds of different populations of wild oat were sown into pots with a surface area of 75 cm^2. The soil surface with the unused herbicide was mixed with a proportion of grass biochar, sifted and cultured in the greenhouse. Rearing conditions were 20°C in the daytime and 15°C at night, 75±5% relative humidity, and a 12:12 h light: dark photoperiod. Seeds of wild oat collected from Henan Province were distributed in 24 populations.

Chemicals

The test herbicide fenoxaprop-p-ethyl oil formulation (69%) and the active compound of fenoxaprop-p-ethyl (95.8%) were supplied by Noposion (Shenzhen, China). Flavine adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), phenylmethanesulfonyl fluoride (PMSF), sodium pyruvate ($C_3H_3NaO_3$), alpha naphthol (α-naphthol), Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) and polyvinylpyrrolidone (PVP) were obtained from Sigma-Aldrich (USA). Creatine ($C_4H_9N_3O_2$), sodium hydroxide (NaOH), Coomassie brilliant blue–G250, monopotassium phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$), ethylenediaminetetraacetate acid (EDTA), and bovine serum albumin (BSA) as well as an ACCase-ELISA kit were purchased from Beijing Tongzheng Biological Company (China). HiPure Plant RNA Mini Kit, HiPure Plant DNA Mini Kit and HiPure Gel Pure DNA Mini Kit produced by Magen were used to isolated total RNA, genomic DNA and purify the production of PCR, respectively. Taq-Plus PCR Forest Mix(2x) from NOVA (China) was the reagent for PCR. The kit HiScript® Ⅱ QRT SsuperMix for qPCR and chamQ™ SYBR® qPCR Master Mix, which are used for cDNA synthesis and qRT-PCR separately, are bought from company Vazyme (Nanjing, China). Peptone, yeast powder and agar powder purchased from AOBOX (Beijing, China) were used make LB culture medium. The Ampicillin purchased from Gentihold (Beijing, China) was applied in *Escherichia coli*

transformation experiment as the bacterial inhibitor.

Instruments and equipment

An ultraviolet and visible spectrophotometer (UV2102PC, Multi Wavelength Fluorescence Spectrophotometer, China), refrigerated centrifuge (5417R, Eppendorf, Germany), and a VMax Microplate Reader (Molecular Devices) with SoftMax Pro 5.4 software were used in the analyses. The primary herbicide sprayer was an atomizer with an auto spray device (Model: ASP-1098, Spray-head: ST110-01, Pressure: 0.2 MPa). The PCR program ran in the Analytik Jena Gradient PCR Instrument (9701 series, Germany) and productions of PCR were validated in the electrophoresis equipment (Beijin Junyi-dongfang Equipment Co.Ltd, Voltage:100v, time: 20 min). The Bio-Rad CFX 96 (USA) was used for qPCR. An constant temperature incubator shaker produced by Jie Rui Er (Jiangsu, China) was used to cultivate *Escherichia coli*.

In vitro ACCase susceptibility of different wild oat populations to fenoxaprop-p-ethyl

Enzyme preparations

Buffer solution: 100 mmol/L Tris-HCl (pH 8.3), 300 mmol/L glycerinum, 5 mmol/L DTT, 2 mmol/L EDTA, 0.5 mmol/L PMSF, and 0.01% (v/v) Triton X-100. First, take 3 g of aerial portions at the 3-leaf stage, tear them in pieces and put them into a prechilled mortar. Grind into a powder in liquid nitrogen, transfer the contents to a 50 mL centrifuge tube, wash the mortar with cold buffer solution in another 50-mL centrifuge tube and place the tube in the refrigerator at 4°C for two hours. Second, primary product to be disposed in two steps. First, put the abovementioned homogenate into a high-speed freezing centrifuge at 4000×g at 4°C for 30 min, discard the sediment, and then, retain liquid supernatant. Second, continue to centrifuge at 20000×g at 4°C for 30 min, discard the sediment, and then, retain the liquid supernatant containing the protein. Transfer the supernatant to a 1.5-mL centrifuge tube to standby for application. Regarding the sample treatment and requirement of the tissue sample, centrifuge the sample for 30 min (4000 r/min). Retain the liquid supernatant for immediate measurement or place it in a -20°C refrigerator for later use. Note that the sample does not contain NaN_3 because it affects the activity of haptoglobin-related protein (HRP).

The ACCase activity of wild oat

The test of the ACCase activity depends on the instruction of the ELISA-ACCase kit. The kit is based on the double antibody-sandwich method for testing the ACCase activity. The depurated ACCase antibody is used to parcel the microplate. Prepare a solid-phase antibody, add the ACCase to the parcel-antibody microplate in the proper order, combine with the ACCase antibody marked by HRP and generate antigen-antibody-enzyme-labelled antibody compounds. After washing the microplate, add the substrate TMB to make it change colours. The TMB turns blue under the catalytic action of the HRP enzyme. The TMB will turn yellow at the end because of the action of the terminating liquid. The depth of the colour is positive correlated with the concentration of ACCase. The spectrophotometric (OD) values are record by a Vmax microplate reader at a wavelength of 450 nm. The protein content was determined by the method of Bradford *et al*. (1976) using bovine serum albumin as a standard. ACCase activity was expressed as U/mg protein/h. The herbicide was applied at a dose rate of 1, 10, 100, 1000, and 10000 mg/L each sample according to preliminary experiments. The inhibition rate of ACCase activity was calculated. The IC_{50} values and the comparative analysis were calculated using a software SPSS 15.0.

Fenoxaprop-p-ethyl treatment

A whole-plant assay modified was conducted according to Ryan (1970). Plants thinned and planted in the field (20 plants per pot) at the 3-leaf stage (20 cm plant height), were treated with fenoxaprop-*p*-ethyl by atomizing with an auto spray device (Model: ASP-1098, Spray-head: ST110-01, Pressure: 0.2 MPa). The herbicide was applied at a concentration of 10 g ai/ha (IC_{50}) according to the results of a whole plant assay, the amount of spouting liquid was 450 L/ha. Water alone was used as a control. Each treatment was repeated three times. Foliar parts were collected for gene stability analyses at 24 h after treatment and stored at –80°C for RNA extraction.

DGE library preparation and sequencing

A total of 10 3-leaf stage, 5 from the control and 5 from fenoxaprop-p-ethyl treated *A. fatua* were collected separately according to the above description, each sample was prepared as 2 biological replicates. RNA extraction was done using TRIzol kit in

211 according to manufacturer instruction, and DNase I (Promega, Madison, WI) was also
212 used to remove genomic DNA from the samples. Approximately 30 μg RNA from each
213 sample was used to construct the DGE libraries, each sample was replicated two times.
214 The mRNA was treated as described in cDNA library construction and enriched by
215 PCR amplification. The library products were then ready for sequencing analysis via
216 Illumina HiseqTM 4000 (Beijing Novogene, Beijing, China) using paired-end
217 technology (150 bp) in a single run. DGE analysis was performed to obtain a global
218 view of wild oat transcriptome differences between the control and fenoxaprop-p-ethyl
219 treated wild oat. To compare differentially expressed genes between the libraries
220 (fenoxaprop-p-ethyl treatment vs. / the control), the level of gene expression was
221 determined by normalizing the number of unambiguous tags in each library to reads per
222 kilobase mapped (RPKM).

223 **The effects of fenoxaprop-p-ethyl on the expression of ACCase gene in some**
224 **populations**

225 Relative expression of ACCase gene in 3 resistant populations and 1 susceptible
226 population (W11) treated by fenoxaprop-p-ethyl at their own IC_{50} concentration in 3-
227 leaf stage were determined. Samples were corrected for qPCR, each treatment was
228 repeated for 3 times. *18S* was selected as the reference gene (Zhang *et al*. 2018), an
229 ACCase sequence from RNA-seq of wild oat was used to design primers by primer 3.0
230 (http://bioinfo.ut.ee/primer3-0.4.0/). The primers used were listed in Table 1.

231 **RNA extraction and cDNA synthesis**

232 To synthesize cDNA, RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad,
233 USA) according to the manufacturer's instructions. The concentration and quality of
234 RNA was test by NanoDrop 2000 (Thermo Scientific, USA). RNA samples with an
235 A260/A280 ratio ranging from 1.8 to 2.0 and an A260/A230 ratio >2.0 were used for
236 cDNA synthesis. Total RNA (1μg) was reverse transcribed into First-strand
237 complementary DNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara,
238 Dalian, China) according to the manufacturer's instructions and stored at –20°C.

239 ***Quantitative Real-Time PCR (qPCR)***

240 ROX's Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) was used for

qPCR and implementing on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The reactions were performed in a 20 µl volume of a mixture containing 1 µl of cDNA template, 10 µl of SYBR Green qPCR SuperMix-UDG, 0.3 µl of each primer, and 8.7 µl of nuclease-free water. The thermocycling program was as follows: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s, and 55°C for 30 s. To acquire a high specificity amplification, a melting curve analysis between 65 to 95°C, was constructed at the end of each PCR run. And it based on a 2-fold dilution series of cDNA (1:5, 1:10, 1:10, 1:10, 1:20, and 1:40). The corresponding qPCR efficiencies (E) were calculated refer to the formula E= $10^{-1/slope}$-1 (PFAFFL, 2001; Tellinghuisen 2014; Spiess *et al.* 2015, 2016). Each sample was prepared as 3 biological replicates, and each reaction was analysed with 2 technical replications.

Mutation point detection

DNA extraction, PCR amplification, and cloning

cDNA was isolated from 3-leaf stage issue of 1 relative susceptible populations and 7 relative unsusceptible populations using HiPure Plant DNA Mini Kit. 20 plants was tested in each population selected as mutation point detection. As the complete ACCase sequence is unknown, so PCR primers was designed from *Alopecurus myosuroides* (GeneBank accession No. AJ310767) and the region of PCR product in the wild oat plastidic ACCase carboxyl transferase domain with 5 known and potential ACCase resistance mutation sites, lle-1,781- Leu, Trp-1,999-Cys, Trp-2,027-Cys, lle-2041-Asn, and Asp-2,078-Gly. The PCR was conducted in 25 µL volume that consist of 12.5 µL Taq-Plus PCR Forest Mix(2x) (NOVE, Jiangsu, China), 0.5 µL forward primer: CTGAATGAAGAAGACTATGGTCG, 0.5 µL reverse primer: TCCTCTGACCTGAACTTGATCTC, 1 µL DNA, 10.5 µL dd H_2O. The PCR was run with the following profiles: 94℃ for 2 min; 25-35 cycles for 30 sec, 60℃ for 30 sec, 72℃ 2 min; 72℃ 5min. The PCR products was purified from agarose gels using HiPure Gel Pure DNA Mini Kit. The DNA that it had been purified was connected with pGEM-Teasy vector by T4 DNA Ligase as follows: 3 µL PCR product, 5 µL 2 × T4 Ligase

buffer, 1 μL pGEM-Teasy vector, 1 μL Ligase. All of operations above were on the ice and the action was at 4℃ overnight for the maximum number of transformants.

The transformation of ligation product consists mainly of following steps: First, centrifuging the ligation reactions briefly and adding 2 μL of each ligation reaction to a sterile 1.5 mL tube on ice; Second, placing the competent cells in an ice bath until just thawed (5 minutes) and mixing cells by gently flicking the tube; Third, transferring carefully 50 μL cells to the ligation reaction tubes from Step 1, then flicking gently the tubes and incubating on ice for 30 minutes; Fourth, heat-shocking the cells for 45-50 seconds in a water bath at exactly 42℃ without shaking and returning immediately the tubes to ice for 2-3 minutes; Fifth, adding 950 μL room temperature liquid LB medium (without ampicillin) to the ligation reaction transformations and incubating for 1.5 hours at 37℃ with shaking (150-200 rpm); Sixth, plating 100 μL transformation culture onto solid duplicate LB/ampicillin/ IPTG/X-Gal plates; Finally, incubating plates overnight at 37℃.

ACCase gene sequencing and alignment

Selecting white colonies and transferring it into liquid LB medium (including ampicillin) continue to incubate for 18 hours at 37℃ with shaking (220 rpm) , then it was sequenced by a gene corporation named Beijing Genomic Institute, BGI (Beijing, China). These sequences were analysis and alignment using a software, DNAMAN with a model ACCase sequence (*Alopecurus myosuroides* Gene Bank Accession No. AJ310767) due to the lack of complete wild oat ACCase sequence (Brown 1990)

Statistical analysis

Statistical and bioassay analyses were performed using Microsoft Excel (2010). IC_{50} values were calculated by SPSS (15.0). Statistical analysis was performed using one-way analysis of variance and Tukey's test ($P < 0{\cdot}05$) with InStat Version 3.0 software (GraphPad Software, San Diego, CA).

RESULTS

In vitro ACCase susceptibility of different wild oat populations to fenoxaprop-p-ethyl

The effects of fenoxaprop-p-ethyl on *ACCase* of different wild oat populations is shown

in table 2. The results indicate that The most unsusceptible ACCase to fenoxaprop-p-ethyl was found in the wild oat population from Yexian2017 (W24), its IC_{50} value was 8172.236 mg/L, the next unsusceptible ACCase to fenoxaprop-p-ethyl were from Fugou (W7), W21(Xihua), Huixian (W3) and Linying (W19), their IC_{50} values were 7656.177, 5111.930, 3966.196, and 2736.872 mg/L, respectively. The most susceptible ACCase to fenoxaprop-p-ethyl was found in the wild oat population from Queshan (W11), its IC_{50} value was 1.134 mg/L. The IC_{50} of the most unsusceptible ACCase to fenoxaprop-p-ethyl of wild oat population from Yexian2017 (W24), which was 7206.557-fold greater than that of the most susceptible ACCase to fenoxaprop-p-ethyl of the wild oat population from Queshan (W11). In addition, the susceptibility of ACCase to fenoxaprop-p-ethyl of the wild oat from Yexian in 2017 (Pingdingshan) is less than that in 2016; the IC_{50} values were 8172.236 and 1339.554 mg/L, respectively, with an 6.101-fold difference.

In vivo ACCase activity of different wild oat populations

The ACCase activity of different wild oat populations is shown in Fig 1. The ACCase activity of the wild oat population from Suiyang (W13) was the highest at 3.278 U/h/mg protein. The next highest ACCase activity were from Zhaoling (W14), Wenxian (W16), Qixian (W12) and Linying (W19), their ACCase activities were 3.245, 3.234, 3.123 and 3.101 U/h/mg protein, respectively. The ACCase activity of the wild oat population from Heshan (W10) was the lowest, which was 0.664 U/h/mg protein. Among them, the highest ACCase activity of wild oat population from Suiyang (W13) was 4.936-fold that of the lowest population from Heshan (W10). In addition, the ACCase activity of wild oat from Yexian (Pingdingshan) in 2017 was higher than that in 2016, which were 2.492 and 1.356 U/h/mg protein, respectively, a 1.838-fold difference.

Mutation point detection

Eight populations were selected for mutation point detection by multiple sequences alignment and a complete ACCase sequence from *Alopecurus myosuroides* (Gene Bank Accession No. AJ310767), which has high homology with wild oat, was used for the alignment stander. There was no amino acid mutation in six possible sites, it indicated the resistance from these populations in the assay to fenoxaprop-p-ethyl wasn't caused

by the six known amino acid mutations. However, the other sites, Glu-1797-Gly, Thr-1805-Ser, Pro-1829-Leu, Thr-1833-lle, Met-1859-Thr, Asp-1904-Gly, Asn-1913-Asp, Phe-1935-Ser, Gln-2009-Arg, Thr-2092-Ala may be possible amino acid mutations (Fig 2).

Differential expressed ACCase unigenes in wild oat suppressed by fenoxaprop-p-ethyl

The differential expression was conducted using RNA-seq, digital gene expression (DGE) profling in wild oat treated by the IC_{50} fenoxaprop-p-ethyl concentration (6.9 mg/L) for 24 hours. The results showed that 8 unigenes were annotated as ACCase, 0 up-regulaed expression and 3 down-regulated expression were observed (Table 3).

The effects of fenoxaprop-p-ethyl on the expression of ACCase gene in some populations

The expression of ACCase gene in the 3 resistant populations from Huixian (W3), W21(Xihua), and Yexian2017 (W24), and 1 relative susceptible population from , Queshan (W11) treated by fenoxaprop-p-ethyl were conducted. The results indicated that the expression of ACCase gene from Huixian (W3), Queshan (W11), W21(Xihua), and Yexian2017 (W24), treated by fenoxaprop-p-ethyl was suppressed by 0.24-, 0.81-, 0.14-, and 0.21-fold (Fig 3).

DISCUSSION

Fenoxaprop-p-ethyl is the main ACCase inhibition herbicide used to manage weeds in wheat fields, some species, i.g. *Alopecurus japonicas*, *Beckmannia syzigachne*, and *Avena fatua* have developed different levels of resistance to fenoxaprop-p-ethyl because of its wide usage (Zhang *et al*. 2009; Guo 2011, 2012; Zhang *et al*. 2013; Bi 2013). ACCase is the only target of fenoxaprop-p-ethyl, so the susceptibility of ACCase in plants to fenoxaprop-p-ethyl *in vitro* or *in vivo* could reflect a relationship between the plant biotype and its susceptibility to the herbicide. In this study, susceptibility of ACCase in 24 different populations of wild oat to fenoxaprop-p-ethyl were conducted *in vitro*. The results indicated that the IC_{50} value of the ACCase of the most unsusceptible to fenoxaprop-p-ethyl in the wild oat population from Yexian2017 (W24) was 7206.557 -fold compared to that of the ACCase of most susceptible to fenoxaprop-

p-ethyl in the wild oat population from Queshan (W11). This indicated that ACCase of wild oat may play a critical role in the resistance to fenoxaprop-p-ethyl.

Point mutations of ACCase may result in resistance of wild oat to the herbicide, thus six point mutations of ACCase loci published (Christoffers & Berg 2002; Liu *et al.* 2007; Yu *et al*. 2007; Hochberg *et al.* 2009; Kaundun 2010) were determined in the 8 wild oat populations from the 7 relative susceptible and the 1 relative unsusceptible populations to fenoxaprop-p-ethyl, these genetic loci mutations can lead to the different levels of resistance. Amino acid mutations in 1999, 2027, 2041 and 2096 loci of the ACCase of weed species has resulted in resistance to one or more AOPP herbicides but not to cyclohexanedione (CHD) or phenylpyrazoline (PPZ) herbicides, and amino acid mutations in 1781, 2078 and 2088 loci of the ACCase of weed species have led to resistance to all ACCase-inhibiting herbicides (Powles & Yu 2010; Collavo 2011). However, our results indicated that six published resistance-related amino acid mutation sites weren't found in the populations texted in the eight wild oat populations. However, the other sites, Glu-1797-Gly, Thr-1805-Ser, Pro-1829-Leu, Thr-1833-lle, Met-1859-Thr, Asp-1904-Gly, Asn-1913-Asp, Phe-1935-Ser, Gln-2009-Arg, Thr-2092-Ala may be possible amino acid mutations, so the resistance of wild oat to the herbicide may be caused by other amino sites mutation, ACCase or detoxification enzymes.

The differential expression was conducted using RNA-seq, digital gene expression (DGE) profling in wild oat treated by the IC_{50} fenoxaprop-p-ethyl concentration (6.9 mg/L) for 24 hours. The results showed that 8 unigenes were annotated as ACCase, 0 up-regulaed expression and 3 down-regulated expression were observed. To further clarify the relationship between ACCase and the wild oat resistance to fenoxaprop-p-ethyl, the gene expresssion of ACCase from the 3 resistant populations W21(Xihua), Huixian (W3), Yexian2017 (W24) and the relative susceptible population Queshan (W11) treated by fenoxaprop-p-ethyl for 24 hours were conducted. This indicated that relative expression level of ACCase gene from 3 relative resistant populations and a relative susceptible population had been suppressed by fenoxaprop-p-ethyl, the resistant populations were significantly less suppressed than the susceptible populations.

390 This proved that the resistance of wild oat to fenoxaprop-p-ethyl is related to the
391 expression level of ACCase gene.

392 **CONCLUSION**

393 Overexpression of ACCase could play a critical role in the resistance of wild oat to
394 fenoxaprop-p-ethyl. The six published resistance-related amino acid mutation sites
395 weren't found in the populations texted in the 8 wild oat populations. Resistance
396 mechanisms of wild oat still need further validation, such as other point mutation
397 detection of ACCase, and detoxication enzymes.

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406 **ADDITIONAL INFORMATION**

407 The sequence information of Accase unigenes is in the Supplementary S1

408 **DISCLOSURE STATEMENT**

409 Competing financial interests: The authors declare no competing financial interests.

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FIGURE LEGEND

Figure 1. In vivo ACCase activity of different wild oat populations.

Figure 2. Alignment of the deduced amino acid sequences of ACCase.

Figure 3. Relative expression of ACCase gene in three resistant populations compared with a susceptible population.

Table 1. Primer design for qPCR

Gene name	Gene ID	Sequence (5′–3′)	Tm (℃)	Efficiency (%)	Product Length (bp)	R^2
18S	Cluster-32023.48114	F: TGCACCACCACCCATAGAAT R: CTGCGGCTTAATTTGACCCA	59.00 58.83	97.2	102	0.998
ACCase	Cluster-32023.49510	F: ACCTGCAACCGTGGATTAAG R: AGCTAGAGCAAGCAGCAAGG	59.99 60.06	99.8	127	0.999

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563 **Table 2. The susceptibility of ACCase to fenoxaprop-p-ethyl on different populations of wild**

564 **oat**

Populations	SLOPE±S.E	IC_{50} (95% Confidence limit) (mg/L)	X^2	Degree of freedom (Df)	R^2
Xiangxian (W1)	0.08±0.016	115.315 (24.337-447.658)	7.039	13	0.971
Huojia (W2)	0.07±0.023	69.285 (43.449-149.531)	4.050	13	0.922
Huixian (W3)	0.05±0.014	3966.196(4511.583-5253.136)	1.573	13	0.844
Wuzhi (W4)	0.12±0.038	2.725 (1.716-6.723)	2.889	13	0.868
Xiuwu (W5)	0.09±0.034	98. 781 (65.103-155.183)	1.282	13	0.925
Xunxian (W6)	0.10±0.040	12.065 (7.904-70.572)	3.527	13	0.965
Fugou (W7)	0.07±0.037	7656.177 (5962.964- 8554.205)	6.218	13	0.932
W8(Wuyang)	0.13±0.039	1.255 (0.986-8.655)	12.939	13	0.909
W9(Lankao)	0.10±0.013	1140.105 (1244.703-5456.843)	7.550	13	0.878
Heshan (W10)	0.30±0.018	13.805 (5.630-29.448)	16.850	13	0.969
Queshan (W11)	0.17±0.037	1.134 (0.525-6.959)	14.761	13	0.943
Qixian (W12)	0.27±0.023	36.828 (11.222-83.391)	11.625	13	0.827
Suiyang (W13)	0.20±0.044	166.126 (31.72 5-209.475)	2.466	13	0.925
Zhaoling (W14)	0.38±0.032	7.140 (2.949-22.873)	31.076	13	0.939
Huaiyang (W15)	0.23±0.065	10. 797 (2.668-17.5 65)	5.102	13	0.946
Wenxian (W16)	0.11±0.015	560. 584 (229.352-883.867)	6.978	13	0.858
Dengfeng (W17)	0.16±0.037	507. 665 (117.969-781.807)	7.065	13	(0.923)
Suiping (W18)	0.16±0.030	130.537 (83.338-476. 601)	5.700	13	0.991
Linying (W19)	0.10±0.024	2736.872 (1454.222-2563.884)	0.819	13	0.927
Sheqi (W20)	0.08±0.032	133.274 (50.615-186.894)	20.564	13	0.937
W21(Xihua)	0.21±0.064	5111.930 (4495.158-5918.343)	4.998	13	0.908
Yanling (W22)	0.13±0.045	166.984 (105.604-372.714)	32.600	13	0.939
Tangyin (W23)	0.05±0.013	400.835 (186.5252-638.301)	3.640	13	0.901
Yexian2017 (W24)	0.06±0.020	8172.236 (6454.222-9591.452)	2.109	13	0.901
Yexian2016 (W25)	0.08±0.019	1339.554 (807.785-1800.296)	1.792	13	0.992

Table 3. Differential expressed ACCase unigenes in wild oat suppressed by fenoxaprop-p-ethyl

Gene ID	FTvsCK.log2. (Fold change).	Similarity organisms	Query cover %	E value	Accession
Cluster-32023.23305	-1.0028	*T. aestivum*	21	1e-16	EU660892.1
Cluster-22064.0	-0.0028251	*X. granatum*	100	4e-155	LC217858.1
Cluster-32023.20930	-2.0028	*T. aestivum*	66	2e-84	EU660895.1
Cluster-32023.63937	-0.56424	*T. aestivum*	63	9e-71	EU660895.1
Cluster-32023.46997	0.5603	*A. tauschii*	77	0.0	XM_020312452.1
Cluster-32023.62340	-0.1636	*A. tauschii*	96	0	EU660897.1
Cluster-32023.48393	0.45485	*A. myosuroides*	89	0	AJ632096.1
Cluster-32023.49510	-1.43894	*A. myosuroides*	97	0	AJ310767.1

Note: Fold Change (FC) = RPKM of neonicotinoid or sulfoxaflor treated samples/RPKM of untreated samples.

RPKM: Reads per kilo bases per million reads.

FDR ≤ 0.001 and the absolute value of $Log_2^{FC} \geq 1$ were used as thresholds to judge the significance of differences in gene expression.

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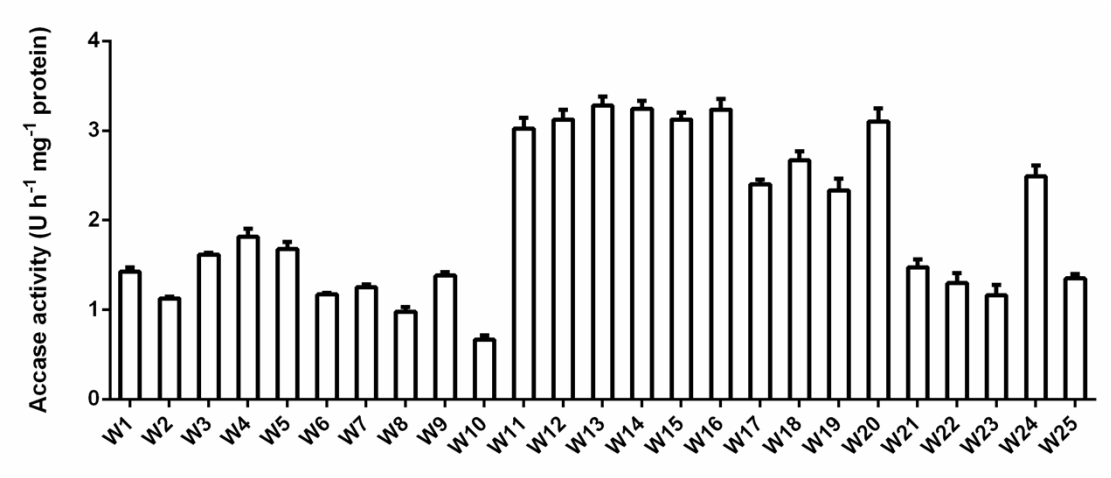

584 **Fig. 1.** ***In vivo*** **ACCase activity of different wild oat populations.**

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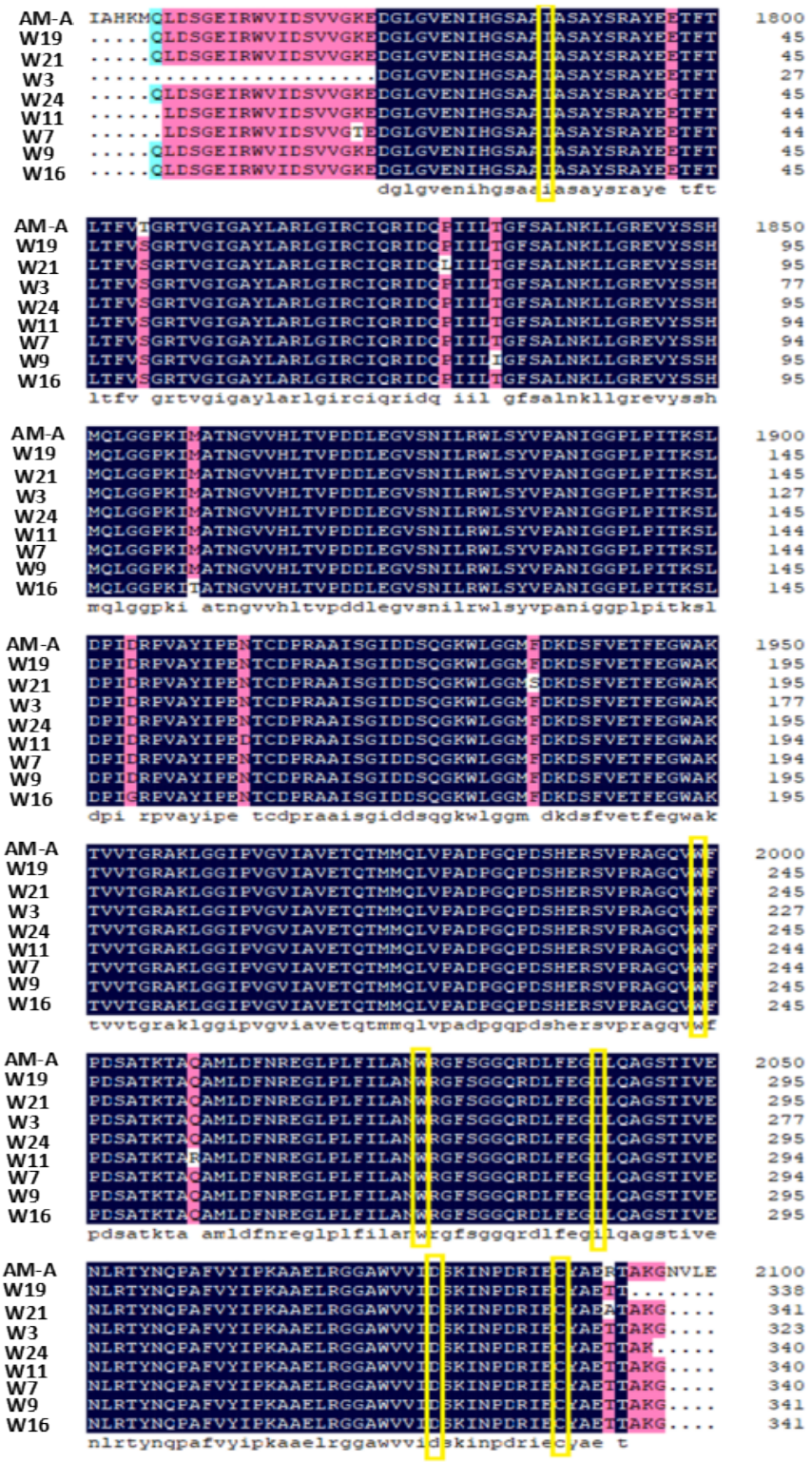

Fig. 2. Alignment of the deduced amino acid sequences of ACCase. Yellow frames represent that six possible amino acid mutation site which had been published. AM-A means model ACCase sequence (*Alopecurus myosuroides*). W11 is most susceptive population, the other 7 are relative resistant.

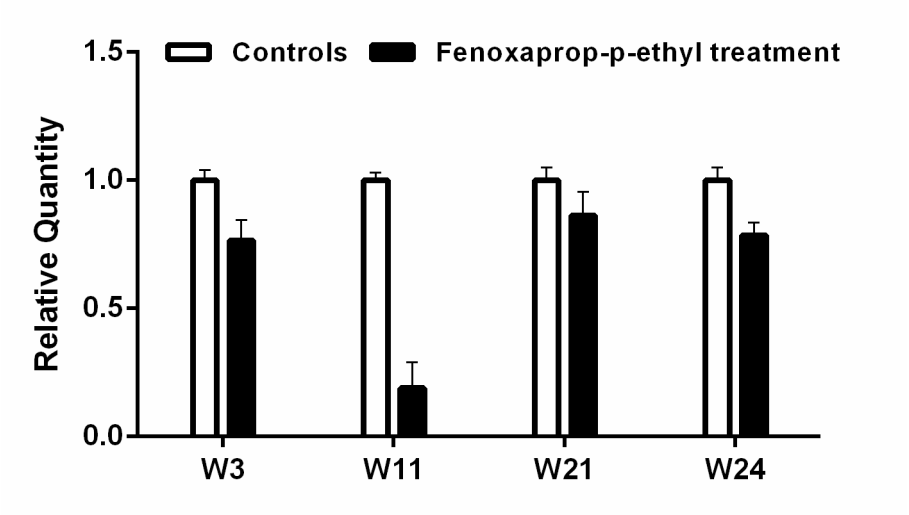

Fig. 3. Relative expression of ACCase gene in three resistant populations and a susceptible population (W11). This four typical populations were treated by fenoxaprop-p-ethyl in 3-leaf stage and compared with the control. Date are means ± tandard error (S.E) of three replicates, and the actin gene was used as a reference gene. The relative expression was calculated using $2^{-\Delta\Delta Ct}$ method base on the value of the control expression, which was ascribed an arbitrary value of 1. W11 is most susceptive population, the other 3 are relative resistant.