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Cross-Resistance of Japanese Foxtail (*Alopecurus japonicus*) to ACCase Inhibitors in China

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The increasing use of ACCase-inhibiting herbicides has resulted in evolved resistance in key grass weeds infesting cereal cropping systems worldwide. Japanese foxtail is one of the most important grass weed species in wheat in China. Most populations have evolved resistance to fenoxaprop-p-ethyl, which is one of the most common ACCase-inhibiting herbicides in wheat. The seeds of two Japanese foxtail populations were collected from wheat fields where farmers complained that control could not be effectively obtained with fenoxaprop-p-ethyl. Seeds from one susceptible population were collected from an area along a roadside where ACCase inhibitors had not been used to be used for validating cross-resistance and elucidating the mechanism of resistance. The experimental results showed that the two populations, Aloja-JS10-R1 and Aloja-JS10-R2, expressed high resistance to fenoxaprop-p-ethyl, with resistance indexes (RIs) of 29.2 and 27.9. These populations also expressed high cross-resistance to clodinafop-propargyl with RIs of 12.8 and 14.7, and moderate cross-resistance to clethodim and pinoxaden with RIs ranging from 2.6 to 11.4. Comparison of the ACCase carboxyl-transferase (CT) domain sequences of the susceptible and resistant populations with blackgrass revealed that tryptophan at position 2027 of the ACCase gene was substituted by cysteine in population Aloja-JS10-R1, and isoleucine at position 1781 of the ACCase gene was substituted by leucine in populations Aloja-JS10-R2. The study confirmed Japanese foxtail resistance to the ACCase inhibitor fenoxafop-p-ethyl, cross-resistance to other ACCase inhibitors, and the resistance mechanism being conferred by specific ACCase point mutations at amino acid position 1781 and 2027.

Nomenclature: Clethodim; clodinafop-propargyl; fenoxaprop-p-ethyl; pinoxaden; blackgrass, *Alopecurus myosuroides*; Japanese foxtail, *Alopecurus japonicus*; wheat, *Triticum aestivum* L.

Key words: ACCase-inhibiting herbicides, ACCase gene, herbicide resistance, mutation detection.

El creciente uso de herbicidas inhibidores de ACCase ha resultado en la evolución de resistencia en especies de malezas gramíneas clave en sistemas de cultivos de cereales en todo el mundo. *Alopecurus japonicus* es una de las malezas gramíneas más importantes en trigo en China. La mayoría de sus poblaciones han evolucionado resistencia a fenoxaprop-p-ethyl, el cual es uno de los herbicidas inhibidores de ACCase más comunes en trigo. Semillas de dos poblaciones de *A. japonicus* fueron colectadas en campos de trigo donde los productores se habían quejado que no se había podido alcanzar un control efectivo con fenoxaprop-p-ethyl. También se colectaron semillas de una población susceptible en un área a la orilla de la carretera donde no se había usado inhibidores de ACCase, para validar la resistencia cruzada y elucidar el mecanismo de resistencia. Los resultados experimentales mostraron que las dos poblaciones, Aloja-JS10-R1 y Aloja-JS10-R2, expresaron un alto nivel de resistencia a fenoxaprop-p-ethyl, con índices de resistencia (RIs) de 29.2 y 27.9. Estas poblaciones también expresaron una alta resistencia cruzada a clodinafop-propargyl con RIs de 12.8 y 14.7, y resistencia cruzada moderada a clethodim y pinoxaden con RIs que variaron entre 2.6 y 11.4. La comparación de la secuencia del dominio carboxyl-transferase (CT) de ACCase de las poblaciones susceptibles y resistentes con *Alopecurus myosuroides* reveló que tryptophan en la posición 2027 del gen ACCase fue sustituido por cysteine en la poblacón Aloja-JS10R1, y isoleucine en la posición 1781 del gene ACCase fue sustituido por leucine en la población Aloja-JS10-R2. Este estudio confirmó la resistencia de *A. japonicus* al inhibidor de ACCase fenoxaprop-p-ethyl, la resistencia cruzada a otros inhibidores de ACCase, y el mecanismo de resistencia, el cual se debe a mutaciones puntuales en las posiciones de los amino ácidos 1781 y 2027 del gen ACCase.

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Japanese foxtail, a member of the Poaceae family, is a strong competitor and a prolific seed producer as one of the most troublesome grass weeds in major wheat-producing areas of China (Yang et al. 2007a,b). Japanese foxtail can substantially reduce crop yield in many crops, such as winter wheat, barley (*Hordeum vulgare* L.), oilseed rape (*Brassica napus* L.), and some vegetables (Li et al. 1996).

Selective, acetyl-coenzyme A carboxylase (ACCase, EC.6.4.1.2) –inhibiting herbicides are widely used to control grass weeds in many crops in China. ACCase is a key enzyme in lipid biosynthesis that catalyzes the formation of malonyl-CoA from the carboxylation of acetyl-CoA (Nikolau et al. 2003). Two types of ACCase have been recognized. The heteromeric prokaryotic ACCase is composed of multiple subunits, whereas the homomeric eukaryotic ACCase is a large multidomain protein. In grasses the plastidic ACCase is homomeric and the target site for the ACCase-inhibiting herbicides (Alban et al. 1994; Gornicki et al. 1997; Incledon and Hall 1997; Price et al. 2003). ACCase-inhibiting herbicides have different chemical classes. Fenoxaprop-p-ethyl and clodinafop-propargyl belong to aryloxyphenoxypropionate (APP), clethodim belongs to cyclohexanedione (CHD), and pinoxaden belongs to phenylpyrazoline (PPZ) classes, respectively (Hofer et al. 2006). Fenoxaprop-p-ethyl inhibits chloroplastic ACCase, causing plant death (Burton et al. 1989), and has been widely used to control grass weed species since its introduction in the 1970s (Délye et al. 2005).

Resistance to ACCase-inhibiting herbicides has been found in some grass weed species, including blackgrass (Cummins et al. 1999; Délye 2005; Délye et al. 2008; Délye and Matéjicek 2002; Menchari et al. 2008; Moss et al. 2003), Italian ryegrass (*Lolium multiflorum*) (Kusk et al. 2008), downy brome (*Bromus tectorum*) (Daniel et al. 2007), wild oat (*Avena fatua*) (Heap 2014), barnyardgrass (*Echinochloa crus-galli*) (Bagavathiannan et al. 2014), and Asia minor bluegrass (*Polypogon fugax*) (Tang et al. 2014). Currently, 46 weed species have been identified with resistance to ACCase inhibitors, mostly in North America and Australia (Délye et al. 2004; Heap 2014; Hochberg et al. 2009; Stephen and Yu 2010).

Fenoxaprop-p-ethyl was registered to control grasses in China in 1992 (Institute for Control of Agrichemicals [ICAMA] 1992). After over 20 yr of use, this herbicide could not control Japanese foxtail as effectively as before in China. Populations from Jurong city of Jiangsu Province were determined to be resistant to haloxyfop, and four nucleotide mutations of Japanese foxtail were detected (Tang et al. 2012). The objectives of this research were to evaluate the resistance of Japanese foxtail to fenoxaprop-p-ethyl and the cross-resistance to other ACCase inhibitors, as well as elucidate the mechanism of resistance in Japanese foxtail by comparing the ACCase gene sequences between resistant and susceptible populations.

Materials and Methods

Seed Source. Seeds of two resistant Japanese foxtail populations, Aloja-JS10-R1 and Aloja-JS10-R2, were collected in 2010 from wheat fields in Hongze county and Yixing city of Jiangsu province, respectively. Fenoxaprop-p-ethyl had been applied to these fields for more than 10 years. The susceptible population, Aloja-JS10-S1, was collected from a roadside in Jiangsu province of China where no ACCase-inhibiting herbicides had been used.

Evaluation of Cross-Resistance to ACCase Inhibitors. Dose–response experiments were performed in the greenhouse to evaluate the cross-resistance of the resistant populations to ACCase inhibitors belonging to different chemical classes. Forty seeds of Japanese foxtail were sown into 12-cm-diam pots containing moist loam soil. Pots were placed in the greenhouse (temperature was maintained at 15 to 25 C, relative humidity maintained at 60 to 85%, in natural sunlight condition), watered and fertilized as required. The greenhouse was located on the site of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, in Beijing. The seedlings were thinned to 10 evenly sized plants per pot before herbicide application.

Four ACCase inhibitors representing three different chemical classes were applied respectively at the three-leaf stage. Fenoxaprop-p-ethyl, 69 g ai L^{-1} emulsion oil in water, supplied by the Bayer (China) Company in Shanghai, was applied at 0, 20, 40, 80, 160, 320, 640, 1,280, and 2,560 g ai ha^{-1}. Clodinafop-propargyl, 15% wettable powder, supplied by the Syngenta (China) Company in Shanghai, was applied at 0, 15, 30, 60, 120, 240, 480, 960, and 1920 g ai ha^{-1}. Clethodim, 240 g ai L^{-1} emulsifiable concentration, supplied by the Xianda Chemical (China) Company in Shandong, was applied at 0, 6.75, 13.5, 27, 54, 108, and 216 g ai ha^{-1}. Pinoxaden, 50 g ai L^{-1} emulsifiable concentration, supplied by the Syngenta (China) Company in Shanghai, was applied at 0, 11.25, 22.5, 45, 90, 180, and 360 g ai ha^{-1}. The herbicides were applied with a compressed air, moving nozzle

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Table 1. Primers utilized for amplification of key regions of the ACCase gene along with necessary polymerase chain reaction conditions and resistance target site.

Primer	Sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (C)	Containing the confirmed amino acids mutations[a]
Forward 1	AAACTCTGGTGCTCGGATTG	615 bp	55	Ile-1781
Reverse 1	GCAACGGGTCTGTCTATTGG			
Forward 2	TGTTGTCCATCTGACTGTTCCA	829 bp	56	Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, Gly-2096
Reverse 2	TAGGCTTCCATTTGCTCCC			

[a] Amino acid positions correspond to the full-length plastidic ACCase in blackgrass.

cabinet sprayer (compressed air cabinet sprayer 3WPSH-500D, Beijing Research Center for Information Technology in Agriculture, Beijing, China) equipped with one TeeJet XR8003 flat-fan nozzle (TeeJet Spraying Systems Co., Springfield, IL) and calibrated to deliver 367.5 L ha^{-1} at 0.3 MPa. Treatments were organized as a completely randomized design with four replications. The experiment was conducted twice to check its reproducibility. Japanese foxtail was harvested from ground level 21 d after treatment, dried at 80 C for 48 h, and dry weights were determined.

The dry weight for each dose was expressed as a percentage of the nontreated control. A four-parameter log-logistic model was fitted to the data by the software Sigmaplot version 10.0:

$$Y = C + (D - C)/\left[1 + (X/\mathrm{GR}_{50})^{b}\right],$$

where Y is the dry weight (percentage of nontreated), X is the herbicide dose (g ai ha^{-1}), C is the lower limit of the response curve at high herbicide doses, D is the upper limit or mean response when herbicide doses are zero, b is the slope of the curve around GR_{50}, and GR_{50} is the dose that reduced dry weight by 50% (Seefeldt et al. 1995). GR_{50} of the resistant population and GR_{50} of the susceptible population were used to calculate the RI , where RI = GR_{50} of the resistant population/GR_{50} of the susceptible population.

DNA Extraction and Plastidic ACCase Carboxyl-Transferase (CT) Domain Cloning. The populations were grown in the greenhouse and treated with fenoxaprop-p-ethyl at 160 g ha^{-1} at the three-leaf growth stage, except for the susceptible population Aloja-JS10-S1. Young shoot tissue from 20 surviving plants from each resistant populations and 5 untreated plants from the susceptible population were harvested and stored at −70 C, respectively. DNA was extracted from 100 mg young shoot tissue of each plant using the Plant Genomic DNA Rapid Extraction kit (Plant Genomic DNA Rapid Extraction kit (Spin-column), Bio Teke Corporation, Beijing, China).

Two pairs of forward and reverse overlapping primers (Table 1) were designed based on the ACCase gene sequences of Japanese foxtail (Accession No. JQ068820) and blackgrass (AJ310767) from the GenBank database to amplify a highly conserved region of the ACCase gene, which is 1,270 bp long and contains seven confirmed point mutations for ACCase-inhibiting herbicides resistance (Kaundun 2010; Kaundun and Windass 2006; Neff et al. 1998; 2002; Scarabel et al. 2011). A thermocycler (DNA engine, Bio-RAD, Hercules, CA) was used to amplify ACCase gene fragments from Japanese foxtail genomic DNA. Each polymerase chain reaction (PCR) contained 1 µl of genomic DNA (about 25 ng $µl^{-1}$), 0.5 µl of each primer (20 µM), 2.5 µl of 10× PCR buffer, 1.5 µl of 2.5 mM deoxynucleotide triphosphates (dNTPs) mixture, and 0.5 µl *Taq* DNA polymerase (5 U $µl^{-1}$) in a final volume of 25 µl. PCR reactions were subjected to a 4-min denaturation at 94 C; 25 cycles of 0.5 min at 94 C, 0.5 min at X C, and 1 min at 72 C, then 3 min at 72 C, where X is the annealing temperature for each primer pair used. Annealing temperatures were 55 and 56 C for primer sets 1 and 2, respectively (Table 1). The desired PCR products were cloned with the competent cell (JM109) and plasmid (pMD19-T) for sequencing. Each desired fragment was sequenced in forward and reverse directions, to minimize sequencing errors, by a commercial sequencing company (Sequencing Service Department, Beijing AuGCT Biotechnology Co., Ltd., Beijing, China). The sequences of five plants of susceptible population were analyzed first and then compared with sequences from resistant populations to determine whether a nucleotide substitution

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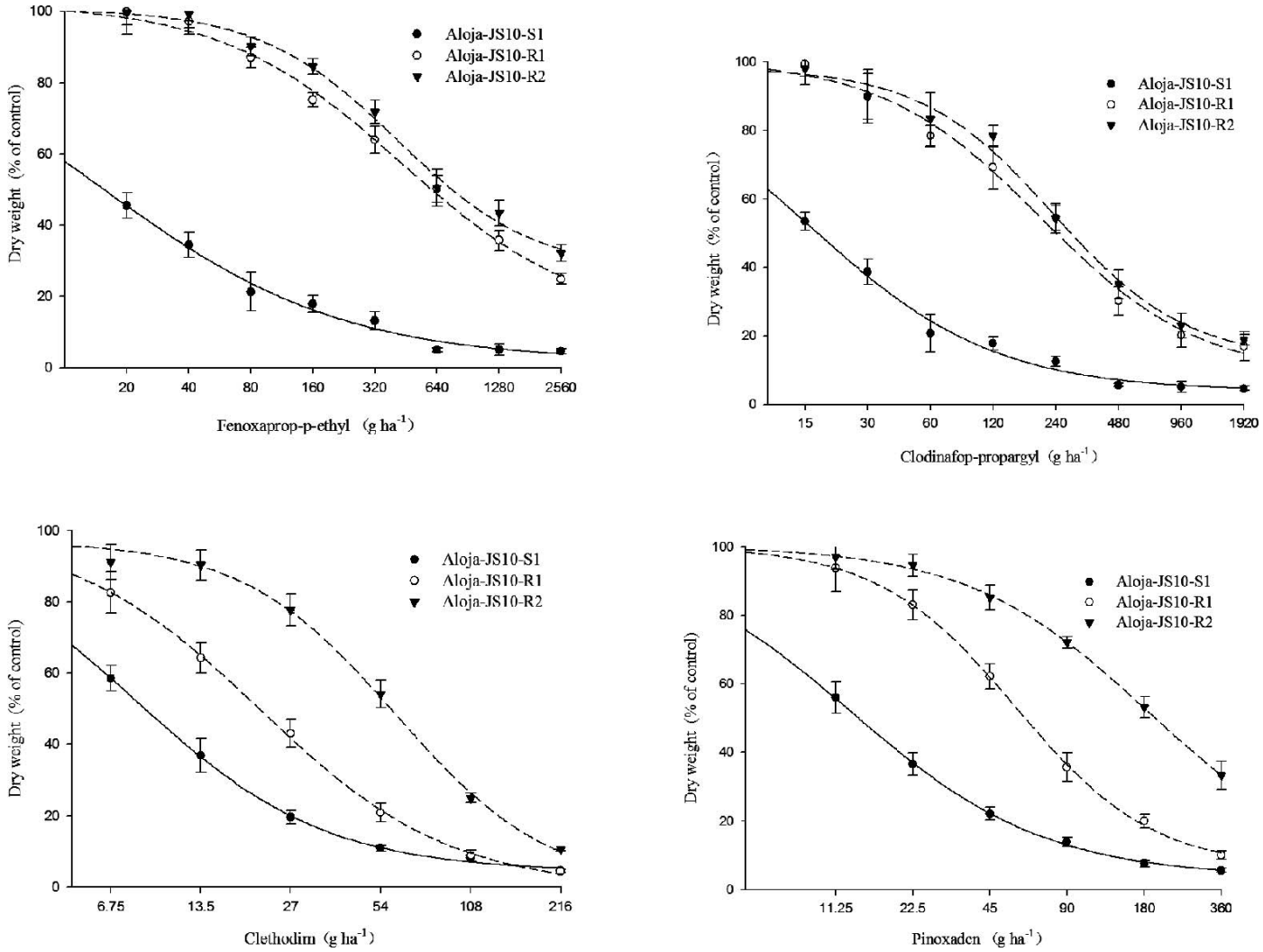

Figure 1. Dry weight above the ground of Japanese foxtail susceptible population and resistant populations with four ACCase inhibiting herbicides.

occurred. A minimum of 20 plants were sequenced for each population examined. DNA Analyzer (ABI 3730xl 96-capillary DNA Analyzer, Applied Biosystems, Los Angeles, CA) with the common primers M13F (–47) (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) was used to obtain the complementary strand of the sequenced ACCase gene fragments. Sequences of Japanese foxtail and blackgrass (*Alopecurus myosuroides*) were assembled and compared with the use of DNAMAN software package (Version 5.2.2, Lynnon Biosoft, Canada).

Results and Discussions

Evaluation of Cross-Resistance to ACCase Inhibitors. Dose–response studies in the greenhouse showed that population Aloja-JS10-S1 was susceptible to fenoxaprop-p-ethyl, and was also susceptible to other ACCase inhibitors. The GR_{50} values of the susceptible population Aloja-JS10-S1 to ACCase inhibitor fenoxaprop-p-ethyl, clodinafop-propargyl, clethodim, and pinoxaden were 14.8, 15.9, 8.2, and 13.0 g ha^{-1}, respectively (Table 2). Populations Aloja-JS10-R1 and Aloja-JS10-R2 expressed high resistance to fenoxaprop-p-ethyl and their RI values were 29.2 and 27.9, respectively. These populations also expressed high cross-resistance to the same chemical class APP herbicide clodinafop-propargyl with a RI of 12.8 and 14.7, moderate cross-resistance to CHD class clethodim (RI of 2.6 and 7.4) and to the PPZ class pinoxaden (RI of 4.4 and 11.4) (Table 2). Aloja-JS10-R1 and Aloja-JS10-R2

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Table 2. Dose–response parameters of three Japanese foxtail populations to select ACCase-inhibiting herbicides.[a]

	APP				CHD		PPZ	
	Fenoxaprop-p-ethyl		Clodinafop-propargyl		Clethodim		Pinoxaden	
Population	GR_{50} (g ha^{-1}) (SE)	RI	GR_{50} (g ha^{-1}) (SE)	RI	GR_{50} (g ha^{-1}) (SE)	RI	GR_{50} (g ha^{-1}) (SE)	RI
Aloja-JS10-S1	14.8 (1.8)	–	15.9 (1.6)	–	8.2 (0.2)	–	13.0 (0.3)	–
Aloja-JS10-R1	431.5 (83.4)	29.2	204.2 (35.7)	12.8	21.3 (0.8)	2.6	56.9 (1.8)	4.4
Aloja-JS10-R2	412.5 (63.1)	27.9	234.0 (32.1)	14.7	60.8 (7.4)	7.4	148.7 (20.3)	11.4

[a] Abbreviations: APP, aryloxyphenoxypropionates; CHD, cyclohexanediones; PPZ, phenylpyrazolines; GR_{50}, herbicide dose that reduces dry weight by 50%; SE, standard error; RI, GR_{50} of the resistant population/GR_{50} of the susceptible population.

were collected from fields where farmers complained about no effective control from the herbicide, correlating with high resistance to this herbicide in our study. In China, farmers would like to increase the dosage of inexpensive fenoxaprop-p-ethyl to provide higher efficacy if the herbicide could not control the weed effectively at lower doses, and do not want to apply other high-priced herbicides. Fenoxaprop-p-ethyl has become a very popular herbicide in wheat fields of China since 1993, and the consumption of the product in wheat in China reached 1,298 tons in 2010.

Clodinafop-propargyl was introduced into China in 2006, and it belongs to the same APP chemical class as fenoxaprop-p-ethyl. The occurrence of APP-resistant Japanese foxtail may be because of continuous application and high dose pressure of ACCase inhibitors in wheat. Even though the populations Aloja-JS10-R1 and Aloja-JS10-R2 had no history of clethodim and pinoxaden being used in these fields, they showed moderate cross-resistance to clethodim and pinoxaden (Figure 1 and Table 2). It is very helpful for weed management to find that the populations Aloja-JS10-R2 with Ile-1781-Leu showed much higher resistance to the APP, CHD, and PPZ chemical classes compared to the Aloja-JS10-R1 with Trp-2027-Cys.

Molecular Basis of Resistance. The gene fragment encompassing the CT domain of the ACCase gene for the two resistant populations and one susceptible population of Japanese foxtail were sequenced. Comparison of the ACCase gene sequences of the susceptible and resistant populations with blackgrass revealed that tryptophan at position 2027 of the ACCase gene was substituted by cysteine in population Aloja-JS10-R1 (GenBank KR061657) and isoleucine at position 1781 of the ACCase gene substituted by leucine in populations Aloja-JS10-R2 (GenBank accession No. KR061656). The results indicated that the resistance of Japanese foxtail to ACCase inhibitors was due to specific ACCase point mutations at amino acid positions 1781 and 2027. These findings provide additional understanding of the molecular basis of resistance to ACCase inhibitor herbicides in Japanese foxtail.

Target site–based ACCase-inhibitor resistance is conferred by single amino acid substitutions of the ACCase gene, which occur at multiple sites within the ACCase gene such as Ile-1781-Leu in blackgrass (Petit et al. 2010), wild oat (Christopher and Holtum 2000), green foxtail (Yu et al. 2007; Zhang and Powles 2006), ryegrass sp.; Trp-2027-Cys in blackgrass; Ile-2041-Asn in blackgrass and ryegrass species; and Ile-2041-Val in ryegrass sp. (Kotoula-Syka et al. 2000; Prado et al. 2005; Preston et al. 1996; Tal and Rubin 2004; White et al. 2005). Seven different mutant ACCase alleles (Ile-1781-Leu, Trp-1999-Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala) were detected in the pinoxaden-resistant ryegrass sp. (Scarabel et al. 2011), Asp-2078-Gly in blackgrass and Gly-2096-Ala in blackgrass (Kaundun and Windass 2006; Kaundun 2010). Scarabel et al. (2011) reported that the Ile-2041-Asn and Ile-2041-Val alleles in ryegrass spp. were associated with dominant or partially dominant resistance to APP, no substantial resistance to CHD and a moderate resistance to pinoxaden. The Cys-2088-Arg allele endowed a partially dominant resistance to clodinafop, sethoxydim and most likely to pinoxaden. Délye et al. (2008) found that Ile-1781-Leu, Trp-2027-Cys, Ile-2041-Asn, Gly-2096-Ala conferred the resistance to fenoxaprop, clodinafop and haloxyfop at field rates, and Trp-2027-Cys could not confer the resistance to clethodim and cycloxydim. Four nucleotide mutations in

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Jurong city of Japanese foxtail were detected, and the point mutations were replaced by Arg-1734 with Gly, Met-1738 with Leu, Thr-1739 with Ser and Ile-2041 with Asn in the resistant population respectively (Tang et al. 2012). For the Japanese foxtail, the mutation at position 2041 was reported first (Tang et al. 2012), and the mutation at position 1781 was first reported in this research.

From this research, the Trp-2027-Cys in population Aloja-JS10-R1 and Ile-1781-Leu in populations Aloja-JS10-R2 might be also associated with dominant resistance to APP fenoxaprop-p-ethyl. These two populations showed high cross-resistance to APP clodinafop-propargyl and moderate cross-resistance to CHD clethodim and PPZ pinoxadem. The populations Aloja-JS10-R2 with Ile-1781-Leu showed much higher resistance to APP, CHD, and PPZ chemical class herbicides, comparing to the Aloja-JS10-R1 with Trp-2027-Cys. The mechanisms of cross-resistance will be the subject of future studies, and will be very important for the understanding of appropriate long-term weed management in wheat.

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