

Research Article

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Corresponding authors:

Haiqun Cao; Email: caohq@ahau.edu.cn;

Ning Zhao; Email: zhaon@ahau.edu.cn

*These authors contributed equally to this work.

Detection of two common ACCase mutations associated with high levels of fenoxaprop-*P*-ethyl resistance in shortawn foxtail (*Alopecurus aequalis*) using loop-mediated isothermal amplification

Fan Yin^{1,*}, Mali Wang^{1,*}, Min Liao², Haiqun Cao³ and Ning Zhao⁴ 

¹Graduate Student, Anhui Province Key Laboratory of Crop Integrated Pest Management and Anhui Province Engineering Laboratory for Green Pesticide Development and Application, School of Plant Protection, Anhui Agricultural University, Hefei, China; ²Associate Professor, Anhui Province Key Laboratory of Crop Integrated Pest Management and Anhui Province Engineering Laboratory for Green Pesticide Development and Application, School of Plant Protection, Anhui Agricultural University, Hefei, China; ³Professor, Anhui Province Key Laboratory of Crop Integrated Pest Management and Anhui Province Engineering Laboratory for Green Pesticide Development and Application, School of Plant Protection, Anhui Agricultural University, Hefei, China and ⁴Associate Professor, Anhui Province Key Laboratory of Crop Integrated Pest Management and Anhui Province Engineering Laboratory for Green Pesticide Development and Application, School of Plant Protection, Anhui Agricultural University, Hefei, China

Abstract

The resistance to fenoxaprop-*P*-ethyl, a herbicide that inhibits acetyl-CoA carboxylase (ACCcase), has emerged in shortawn foxtail (*Alopecurus aequalis* Sobol.) since the 1990s, presenting a considerable challenge to wheat (*Triticum aestivum* L.) production in China. One of the primary mechanisms responsible for this high-level resistance is the presence of mutations at codons 1781, 2041, and 2078 in the ACCcase gene. However, the conventional methods used to detect these mutations, such as polymerase chain reaction (PCR) and gene sequencing, are time-consuming and labor-intensive. To address this issue and enable the prompt and effective detection of these common ACCcase mutations in *A. aequalis*, a loop-mediated isothermal amplification (LAMP) strategy was developed. The LAMP assay specifically targets the Ile-1781-Leu and Asp-2078-Gly mutations within the ACCcase gene of *A. aequalis*. Through the optimization of primers, systems, and conditions, the LAMP assay enables rapid differentiation between wild-type individuals and mutants of *A. aequalis* carrying either of these two mutations. Including SYBR Green I dye in the final reaction mixtures enables detection of the target mutation through a noticeable color change that can be observed with the naked eye. It is noteworthy that the sensitivity of the LAMP assay was approximately 10⁴-fold greater than that of conventional PCR methods. Additionally, a derived cleaved amplified polymorphic sequence (dCAPS) assay was established for each mutation to distinguish between homozygous and heterozygous mutants. Overall, the developed LAMP assay could efficiently detect the Ile-1781-Leu and Asp-2078-Gly mutations in the ACCcase gene of *A. aequalis*, offering significant advantages for the monitoring and management of fenoxaprop-*P*-ethyl resistance.

Introduction

Shortawn foxtail (*Alopecurus aequalis* Sobol.) stands as a prominent and pervasive weed of global concern, extensively distributed across North America, Europe, and temperate Asia (Cope 1982). This species, which may be annual or biennial, is capable of producing up to 18 tillers and an average of more than 7,300 seeds per plant, thereby exhibiting notable competitive capabilities (Li et al. 1990; Zhao et al. 2018). In China, *A. aequalis* has substantially encroached upon some overwintering crops, especially wheat (*Triticum aestivum* L.) fields in rotation with rice (*Oryza sativa* L.), resulting in substantial yield losses of up to 50% (Zhu and Tu 1997). Over the past two decades, the management of *A. aequalis* has heavily relied on herbicides, particularly fenoxaprop-*P*-ethyl and mesosulfuron-methyl. However, their recurrent application over time has selected individuals resistant to multiple herbicides with distinct modes of action (MOAs) (Zhao et al. 2019), posing a significant threat to sustainable crop production.

Among the common agricultural chemicals, fenoxaprop-*P*-ethyl stands out as a prominent aryloxyphenoxypropionate herbicide exhibiting high efficiency by specifically targeting the plastidic acetyl-CoA carboxylase (ACCcase) in plants (Zhang et al. 2003). ACCcase plays a pivotal role in the initial stage of fatty acid biosynthesis (Harwood 1988), with its isoforms typically comprising three catalytic domains: the carboxyl transferase (CT), the biotin carboxylase, and the

Table 1. Information on the *Alopecurus aequalis* seeds for the three populations tested.^a

Population	Biotype	ACCcase mutation	GPS coordinate	GR ₅₀ (SE)	RI
AH FY-3	Susceptible	Wild type	117.64°E, 32.75°N	g ai ha ⁻¹ 3.34 (0.41)	1.00
AH TC-2	Resistant	Ile-1781-Leu (ATA to TTA)	118.99°E, 32.70°N	415.52 (49.67)	124.41
SZ SX-2	Resistant	Asp-2078-Gly (GAT to GGT)	117.89°E, 33.49°N	224.74 (33.33)	67.29

^aAbbreviations: ACCcase, acetyl-CoA carboxylase; GR₅₀, the herbicide dose causing a 50% growth reduction; RI, resistance index.

biotin carboxyl carrier (Nikolau *et al.* 2003). Among these, the CT domain of the plastid form of ACCase stands as the primary binding site for ACCase-inhibiting herbicides (Zhang *et al.* 2004). In weed species, amino acid substitutions (AASs) within the genes encoding these target enzymes are classified as target site-based resistance (TSR), now recognized as a major mechanism of herbicide resistance (Powles and Yu 2010). To date, more than 16 AASs at codons 1781, 1999, 2027, 2041, 2078, 2088, 2096, and 2097 of ACCase have been identified, conferring ACCase resistance in grass weed species (Murphy and Tranel 2019). Concerning *A. aequalis*, four AASs—specifically Ile-1781-Leu, Ile-2041-Asn/Thr, and Asp-2078-Gly—have been recognized in its ACCase, conferring ACCase resistance, with Ile-2041-Thr being a relatively uncommon occurrence (Guo *et al.* 2018). Resistance patterns in weed plants, induced by target-gene mutations, can vary significantly depending on the specific mutation positions and types within the target gene (Powles and Yu 2010). This underscores the importance of the prompt and accurate detection of these mutations in guiding the judicious use of herbicides.

Over the past two decades, diverse molecular detection tools have emerged for identifying resistance mutations in weeds. Traditional approaches, including polymerase chain reaction (PCR)-restriction fragment-length polymorphism (PCR-RFLP), PCR amplification of specific alleles, and (derived) cleaved amplified polymorphic sequence (dCAPS) analysis, rely on DNA sequencing and/or PCR methodologies, often necessitating expensive equipment and complex procedures (Boutsalis 2001; Corbett and Tardif 2006; Kaundun *et al.* 2019; Kaundun and Windass 2006). In a move to address these constraints, a pioneering technique for nucleic acid amplification, known as loop-mediated isothermal amplification (LAMP), was thus devised in 2000 (Notomi *et al.* 2000). LAMP exhibits the ability to amplify target-gene sequences under constant thermal conditions, yielding positive results discernible through a color change (Tomita *et al.* 2008). This feature positions LAMP as a promising tool for field-based detection. At present, LAMP-based methodologies have been extensively adopted for detecting of a range of agricultural challenges including plant-parasitic nematodes (Ahuja and Somvanshi 2021) and diverse pathogens (Garg *et al.* 2022; Trippa *et al.* 2024), as well as the identification of genetically modified crops (Ahuja and Somvanshi 2021). However, its application in detecting herbicide-resistance mutations in arable weeds remains relatively scarce, with limited instances reported in the literature (Pan *et al.* 2015a, 2015b; Panozzo *et al.* 2023; Wang *et al.* 2023).

In a prior investigation, an efficient LAMP assay was successfully established for the rapid identification of the predominant Ile-to-Asn mutation at codon position 2041 within the ACCase genes of *A. aequalis* species (Wang *et al.* 2023). In this ongoing study, sequential advances were made in LAMP-based detection for the remaining two prevalent resistance mutations—specifically, Ile-to-Leu and Asp-to-Gly—occurring at codon positions 1781 and 2078 within the ACCase genes of *A. aequalis*

(Guo *et al.* 2018). Furthermore, dCAPS assays were concurrently developed for the two mutations to discern their heterozygosity among plants exhibiting resistance. These tools may facilitate the prompt diagnosis of fenoxaprop-*P*-ethyl resistance in *A. aequalis*, thereby contributing to the efficacious management of this weed.

Materials and Methods

Plant Material and Growth Conditions

In May 2021, mature seeds were collected from a susceptible (S, AH FY-3) and two putative resistant (R) populations (AH TC-2 and SZ SX-2) of *A. aequalis* at distinct locations in Anhui Province, China (Table 1). AH FY-3 was sourced from uncultivated land and had been characterized as susceptible previously (Wang *et al.* 2023), while AH TC-2 and SZ SX-2 were separately obtained from an individual wheat field that had been exposed to fenoxaprop-*P*-ethyl for more than one decade. Mature seeds from each population, collected from more than 200 individuals, were meticulously blended, air-dried, and subsequently stored in paper bags at 4 C until being used.

The *A. aequalis* seeds from each population were germinated in petri dishes containing deionized water. When their coleoptiles reached about 1-cm length, eight seeds were planted in each of the separate plastic pots (10 cm by 10 cm by 8.5 cm) filled with loam soil. The pots were placed in a controlled glasshouse environment with natural light at temperatures of 25/15 C and approximately 75% relative humidity. To ensure uniform growth conditions across all plants, their pots were subjected to regular watering and rearrangement. At the 2- to 3-leaf stage, weed plants were selectively thinned to retain five plants of similar size in each pot and continuously cultivated.

Single-Dose Herbicide-Resistance Testing

Thirty seedlings were randomly chosen from each population and grown to the 3- to 4-leaf stage under the specified conditions. In each population, 15 plants underwent application of fenoxaprop-*P*-ethyl (69 g L⁻¹ emulsion in water; Puma®, Bayer Crop Science, Hangzhou, China) at the recommended field rate (62.1 g ai ha⁻¹), while the remaining 15 plants received water as a negative control. These liquids were sprayed using a compressed-air cabinet sprayer fit with a mobilizable flat-fan nozzle (9503EVS, Spraying Systems, Wheaton, IL, USA) following established methods (Zhao *et al.* 2019). Subsequently, all plants were returned to the same glasshouse and allowed to grow for an additional 3 wk. At 21 d after treatment (DAT), growth status was visually assessed according to established protocols (Kumar and Jha 2017). Fresh leaves were then harvested from control individuals in each population and utilized for genomic DNA extraction.

Table 2. Primers designed for detecting the Ile-1781-Leu and Asp-2078-Gly mutations in *Alopecurus aequalis*^a

Mutation	Usage	Primer	Primer type	Primer sequence (5'→3')
Ile-1781-Leu	dCAPS	d1781-F d1781-R	PCR forward PCR reverse	TGTGGTGGGAAAGGAGGATGGACTAGGTGTGGAGt ^b AC TGGGCTGGTCATTACGCTGT
Asp-2078-Gly	dCAPS	d2078-F d2078-R	PCR forward PCR reverse	TGCAGAGCTACGTGGAGGGCCCTGGGTCGTGAc ^b Ta ^b CCCTGGAGTCTTGCTTTTCAG
Ile-1781-Leu	LAMP	L1781-F3 L1781-B3 L1781-FIP L1781-BIP	Forward outer Backward outer Forward inner Backward inner	T ^c -TAG ^b ATGGAAGTGTCTGCTAT TGGGAGCTGTACACCTCC GGCTCCGATTCCAACAGTTCGT-GTGCCTATTCTAGGGCGCTAC GGCATAACGGTGATACAGCGT-CGCCAAGAAGCTTGTTC
Asp-2078-Gly	LAMP	L2078-F3 L2078-B3 L2078-FIP L2078-BIP	Forward outer Backward outer Forward inner Backward inner	GGAGCTGGGTCGTGAaa ^b GG ^c ATCAGATAGGCTTCCATTTC CCTTGAGTTTCGAGAACATT-AACCCAGATCGCATCGAGTG GGTCAGAGGAACCTCAAAGAA-TCTTGCTTTTCAGATCTATCA

^aAbbreviations: dCAPS, derived cleaved amplified polymorphic sequence; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

^bThe lowercase nucleotides are mismatches manually added to distinguish different amino acid substitutions specifically.

^cThe nucleotide in bold is the mutant site in the sequence of the *ACC*ase gene in the resistant (R) biotype compared with the susceptible (S) biotype.

Whole-Plant Dose–Response Experiment

The S and R seedlings at the 3- and 4-leaf stage were used for testing. Based on the single-dose testing results, a range of concentrations of fenoxaprop-*P*-ethyl was determined and applied to the three populations. For the S population, fenoxaprop-*P*-ethyl was applied at the doses of 0, 0.8, 2.3, 6.9, 20.7, 62.1, and 186.3 g ai ha⁻¹. For the two R populations, doses of 0, 20.7, 62.1, 186.3, 558.9, 1,676.7, and 5,030.1 g ai ha⁻¹ were used. Herbicide application was conducted using a laboratory cabinet sprayer, as detailed previously. At 21 DAT, the aboveground biomass for the plants in each treatment was measured and calculated as a percentage relative to the untreated control. This testing was arranged in a randomized complete block design with three biological replicates, and the whole experiment was replicated twice (runs).

An ANOVA analysis using SPSS v. 19.0 software (IBM, Armonk, NY, USA) revealed no significant interactions ($P > 0.05$) between treatment and runs. The data from the repeated experiments were thus pooled and fit to a four-parameter nonlinear logistic model (Equation 1) using SigmaPlot v. 14.0 (Systat Software, San Jose, CA, USA).

$$y = C + \frac{D - C}{1 + \left(\frac{x}{GR_{50}}\right)^b} \quad [1]$$

where C and D represent the lower and upper limits, b represents the slope at which the herbicide dose causes a 50% growth reduction (GR_{50}), and y represents the growth response (i.e., the percentage of biomass residue compared with the untreated control) at the herbicide application dose x .

Based on the GR_{50} values, a resistance index (RI) was computed to assess the extent of resistance in the R population relative to the S population (Beckie and Tardif 2012).

PCR and Gene Sequencing for Detection of Different *ACC*ase Mutations

Genomic DNA extraction followed the classical cetyltrimethylammonium bromide (CTAB)-based method (Porebski et al. 1997). PCR amplification of a 1,437-bp *ACC*ase gene fragment was conducted using 2× Super Pfx MasterMix (CoWin Biosciences, Beijing, China) with the primer pair as reported in Bi et al. (2016). The amplified products were subjected to Sanger sequencing (TsingKe Biotech, Nanjing, China), and the resulting nucleotide

and deduced amino acid sequences were analyzed and compared between S and R populations using DNAMAN v. 6.0 (Lynnon Biosoft, Quebec, Canada).

LAMP Methods for Detection of Different *ACC*ase Mutations

To specifically detect the Ile-1781-Leu and Asp-2078-Gly mutations in the *ACC*ase gene of *A. aequalis*, two sets of specific LAMP primers were designed (Table 2). This design utilized the PrimerExplorer v. 4 software (available at <http://primerexplorer.jp/elamp4.0.0/index.html>) and targeted the highly conserved regions surrounding codons 1781 and 2078 of *ACC*ase. The primer sets included outer primers that initiated the reaction and inner primers that completed it. To prevent occasional nonspecific gene amplification from susceptible plants—a phenomenon attributed to the high sensitivity of the LAMP method—one mismatch was deliberately introduced into the forward outer primer L1781-F3, and two mismatches were introduced into L2078-F3 (Table 2).

A total volume of 25 µl was utilized in the preparation of the LAMP reaction system. The optimization of this system involved evaluating a series of concentrations for several key components: MgSO₄ (Sigma-Aldrich, St Louis, MO, USA) ranging from 4 to 8 mM, dNTP (Sangon Biotech, Shanghai, China) from 0.4 to 1.6 mM, betaine (Sigma-Aldrich) from 0.32 to 0.96 M, Bst DNA Polymerase (NEB, Ipswich, MA, USA) from 0.16 to 0.64 U µl⁻¹, F3/B3 from 0.2 to 0.6 µM, and Forward Inner Primer (FIP)/Backward Inner Primer (BIP) from 1.6 to 6.4 µM. To determine the optimal reaction condition, the mixture was incubated at a temperature range of 60 to 65 C for a duration of 40 to 50 min. The reaction was terminated by heating at 80 C for 10 min, followed by the addition of SYBR Green I dye at a proportion of 1:100 (dye: reaction system). The emergence of a yellow color in the reaction systems indicated the presence of a specific mutation.

Comparison of Sensitivity between LAMP and Conventional PCR

The primer pairs used in the dCAPS analysis of the Ile-1781-Leu and Asp-2078-Gly mutations underwent modification to eliminate introduced mismatches. They were subsequently used in conventional PCR targeting a 185-bp region for the Ile-1781-Leu mutation and a 206-bp region for the Asp-2078-Gly mutation in *ACC*ase. Genomic DNA extraction followed the previously described method, and the original DNA samples isolated from populations AHTC-2 and SZSX-2 served as standard DNA stock solutions.

DNA concentrations for the two populations were determined as $3.2 \times 10^2 \text{ ng } \mu\text{l}^{-1}$ and $5.1 \times 10^2 \text{ ng } \mu\text{l}^{-1}$, respectively. After an accurate 10-fold serial dilution from $10^2 \text{ ng } \mu\text{l}^{-1}$ to $10^{-6} \text{ ng } \mu\text{l}^{-1}$, the resulting solutions were acquired for use as templates in both LAMP and PCR. Optimal reaction conditions were employed for LAMP, and the PCR system and condition were prepared using 2× Super Pfx MasterMix (Covin Bio.) following the manufacturer's recommendations.

dCAPS Markers for Detection of Different ACCase Mutations

To specifically identify the Ile-1781-Leu and Asp-2078-Gly mutations in *ACCase* genes of *A. aequalis* at the molecular level, two dCAPS markers were also developed. Using the wild-type (WT) and mutant nucleotide sequences of *ACCase* as a basis, two primer pairs were designed with the assistance of dCAPS Finder v. 2.0 software (Neff et al. 2002; Table 2). For the Ile-1781-Leu mutation, a 185-bp DNA fragment covering codon 1781 of *ACCase* was amplified with the primer pair d1781-F and d1781-R. For the Asp-2078-Gly mutation, a 206-bp DNA fragment spanning codon 2078 of *ACCase* was amplified using the primer pair d2078-F and d2078-R. One (A to T) and two mismatches (T to C and G to A) were artificially introduced into d1781-R and d2078-F to create restriction sites AGT[^]ACT and A[^]CTAGT for the *ScaI* and *SpeI* enzymes (TaKaRa, Dalian, China), respectively. PCR was conducted using 2× Super Pfx MasterMix (Covin Bio.) following the manufacturer's recommendations, and the resulting products were directly digested with the respective restriction enzymes. In instances where the PCR products comprised solely WT sequences, they remained undigested by any of the restriction enzymes, manifesting as a single band on the gel. Conversely, PCR products containing mutant sequences were cleaved by the respective enzymes, resulting in the formation of two distinct bands.

Results and Discussion

As documented, 272 weed species have developed resistance to 21 of the 31 known herbicide sites of action and to 168 different herbicides (Heap 2024), and diverse mutations related to herbicide resistance have been identified occurring in the target genes (Murphy and Tranel 2019). As an invasive grass species, *A. aequalis* has been found to have evolved resistance to multiple herbicides with different MOAs, including both *ACCase*- and acetolactate synthase (ALS)-inhibiting herbicides. Among these, Ile-1781-Leu, Ile-2041-Asn, and Asp-2078-Gly of *ACCase* were the three common mutations responsible for the *ACCase* resistance determined in *A. aequalis* (Guo et al. 2018). Given that the cross-resistance patterns have been meticulously characterized for the three *ACCase* mutations (Guo et al. 2015a, 2015b, 2016), the prompt and dependable detection of these mutations will significantly contribute to precision in selection of alternative herbicides for resistance management. Although we have successfully established a reliable LAMP reaction for detecting the Ile-to-Asn mutation at codon 2041 of *ACCase* in *A. aequalis* (Wang et al. 2023), LAMP is incapable of detecting the other two critical mutations linked to high levels of *ACCase* resistance. Therefore, the main aim of this current study was to develop LAMP-based reactions for the quick identification of these remaining mutations.

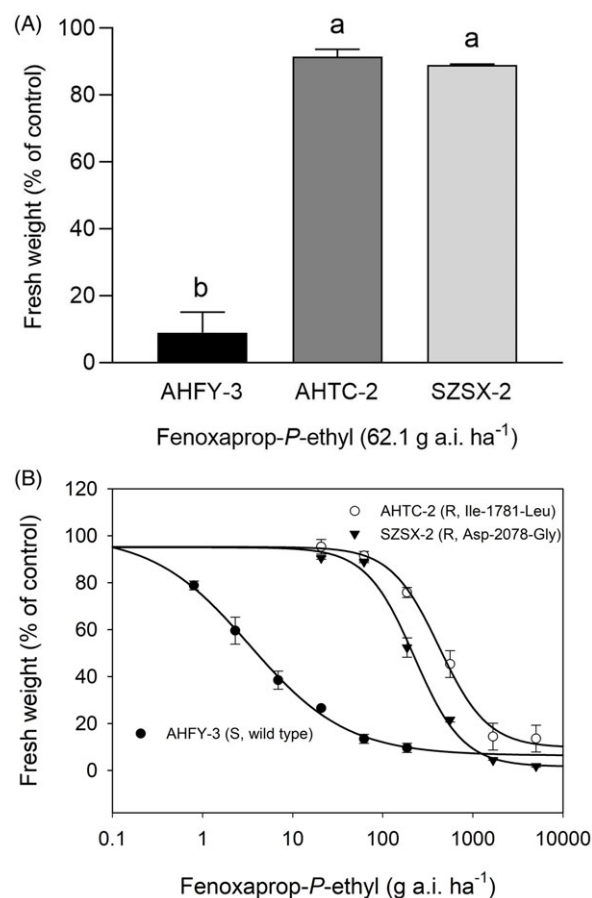


Figure 1. Effects of fenoxaprop-*P*-ethyl treatments on the growth of the susceptible (S, AHFY-3) and two resistant (R, AHTC-2 and SZSX-2) populations of *Alopecurus aequalis*. (A) Weed plants from all three populations, at the 3- to 4-leaf stage, received fenoxaprop-*P*-ethyl at the recommended field rate (62.1 g a.i. ha⁻¹) or water as an untreated control. Fresh weights for plants under each treatment were assessed 21 d after treatment and expressed as a percentage (%) of the untreated control. Distinct letters denote statistically significant differences at $P < 0.05$. (B) Dose-response curves of the aboveground biomass (% of control) of the S (AHFY-3) and two R (AHTC-2 and SZSX-2) populations of *A. aequalis* for a series of application rates of fenoxaprop-*P*-ethyl. The data for AHFY-3, AHTC-2, and SZSX-2 were fit with four-parameter nonlinear logistic models: $y = 6.37 + 93.92/[1 + (x/3.34)]^{0.81}$ ($R^2 = 0.9983$), $y = 9.72 + 85.54/[1 + (x/415.52)]^{1.62}$ ($R^2 = 0.9961$), and $y = 1.65 + 93.41/[1 + (x/224.74)]^{1.62}$ ($R^2 = 0.9950$), respectively. The curves and data for the S population AHFY-3 were reused with permission from Wang et al. (2023), ©2024 John Wiley and Sons. Vertical bars represent the standard errors of the mean.

High Levels of Fenoxaprop-*P*-Ethyl Resistance in the Two R Populations

The single-dose testing and whole-plant dose-response experiments were conducted concurrently between this study and our prior research, employing identical parameters (Wang et al. 2023). The data for the S population, including its GR₅₀ value to fenoxaprop-*P*-ethyl, were identical and were used in this study for comparative analysis.

Single-dose testing revealed that at 21 DAT, approximately 80% of the tested plants from the two R populations survived, exhibiting reductions in aboveground biomass below 15% (Figure 1A). In contrast, all plants from the S population had died, displaying biomass reductions exceeding 90% (Figure 1A). This observation indicates that the two R populations had developed resistance to fenoxaprop-*P*-ethyl.

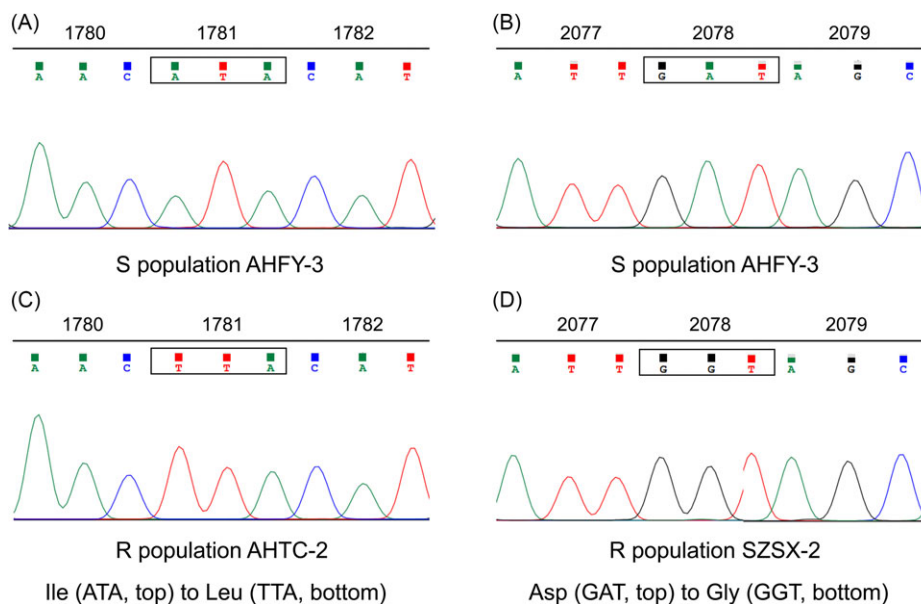


Figure 2. DNA sequencing results showing the wild-type codons (A) Ile-1781 and (B) Asp-2078 in the *ACCase* genes of the susceptible (S) plants (AHFY-3, top) of *Alopecurus aequalis* and the mutant codons (C) Ile-1781-Leu and (D) Asp-2078-Gly in those of the resistant (R) plants (AHTC-2 and SZSX-2, bottom). The *ACCase* codons at positions 1781 and 2078 are highlighted in black boxes.

To assess the resistance levels of the two R populations to fenoxaprop-*P*-ethyl, whole-plant dose–response experiments were carried out (Figure 1B). The GR₅₀ values of AHTC-2 and SZSX-2 to fenoxaprop-*P*-ethyl were 415.52 and 224.74 g ai ha⁻¹, respectively, significantly higher than that of AHFY-3 (3.34 g ai ha⁻¹) (Table 1). Based on the RI values, AHTC-2 and SZSX-2 were confirmed to be about 124- and 67-fold more resistant to fenoxaprop-*P*-ethyl, respectively, characterizing them as highly resistant (RI > 10).

Gene Sequencing of *ACCase* Revealing Two Common Resistance Mutations

Following PCR, *ACCase* gene fragments of 1,437-bp length were successfully amplified from different individuals within each population ($n = 15$). These gene fragments were subsequently aligned with the documented *ACCase* sequence (GenBank: AJ310767) of black-grass (*Alopecurus myosuroides* Huds.), revealing a remarkable >99% similarity. Comparison of the *ACCase* fragments between the S and R samples identified single-nucleotide changes, specifically A-to-T and A-to-G in the *ACCase* of AHTC-2 and SZSX-2, respectively. These changes led to Ile-to-Leu and Asp-to-Gly substitutions at codons 1781 and 2078 of the *ACCase*, as illustrated in Figure 2. Furthermore, the frequency of the resistance mutation was found to be 87% and 73% in the *ACCase* genes of AHTC-2 and SZSX-2 plants, respectively.

LAMP Detection of Different *ACCase* Mutations in *Alopecurus aequalis*

Previous reports have highlighted the challenge in selecting the optimal set of primers and determining the ideal reaction conditions for LAMP detection, despite the relative ease of primer design (Badolo et al. 2015; Tomita et al. 2008). In this study, artificially introduced mismatches were employed to enable the designed primers to specifically and efficiently amplify mutant *ACCase* sequences from *A. aequalis* (Table 2; Figure 3). The final

optimal LAMP reaction systems for detecting each mutation were also determined and found to be identical (Table 3). Regarding the reaction conditions, LAMP reactions were incubated at 63.0 C for 50 min for Ile-1781-Leu and at 63.5 C for 45 min for Asp-2078-Gly. To aid in visual identification, SYBR Green I was added into the final system, allowing for direct observation with the naked eye.

DNA extracted from the S and two R populations was employed to evaluate the efficacy of the LAMP primer sets in detecting each specific mutation. The final LAMP products from the DNA samples of the two R populations, AHTC-2 (Ile-1781-Leu) and SZSX-2 (Asp-2078-Gly), displayed ladder-like banding patterns, while that from the DNA of the S population, AHFY-3 (WT), and the negative control (CK) with no templates showed no bands on agarose gels (Figure 4A and 4B). Upon addition of dye, the color of the R systems turned yellow, whereas that of the S system and the negative controls maintained orange red (Figure 4C and 4D). Through optimal LAMP reactions, both primer sets functioned effectively in detecting specific *ACCase* mutations in *A. aequalis*.

To date, a total of four distinct *ACCase* mutations have been identified to confer resistance to *ACCase*-inhibiting herbicides in the *ACCase* genes of *A. aequalis* (Guo et al. 2018). Employing optimized primers, systems, and conditions, the LAMP assays developed in this study can rapidly distinguish between WT individuals and mutants of *A. aequalis* harboring Ile-1781-Leu or Asp-2078-Gly in the *ACCase* enzyme. In conjunction with our previous research, LAMP-based methodologies have been successfully devised for the detection of the three most prevalent mutations, whereas that for identifying the infrequent Ile-2041-Thr mutation in the *ACCase* of *A. aequalis* has yet to be developed. In addition, in *A. aequalis*, six amino acid substitutions at codons 197 and 574 of *ALS* have been identified to influence the efficacies of *ALS* inhibitors, including mesosulfuron-methyl, which is commonly used to control grass species such as *A. aequalis* (Tang et al. 2023). The development of LAMP-based assays targeting these mutations will enhance their rapid identification

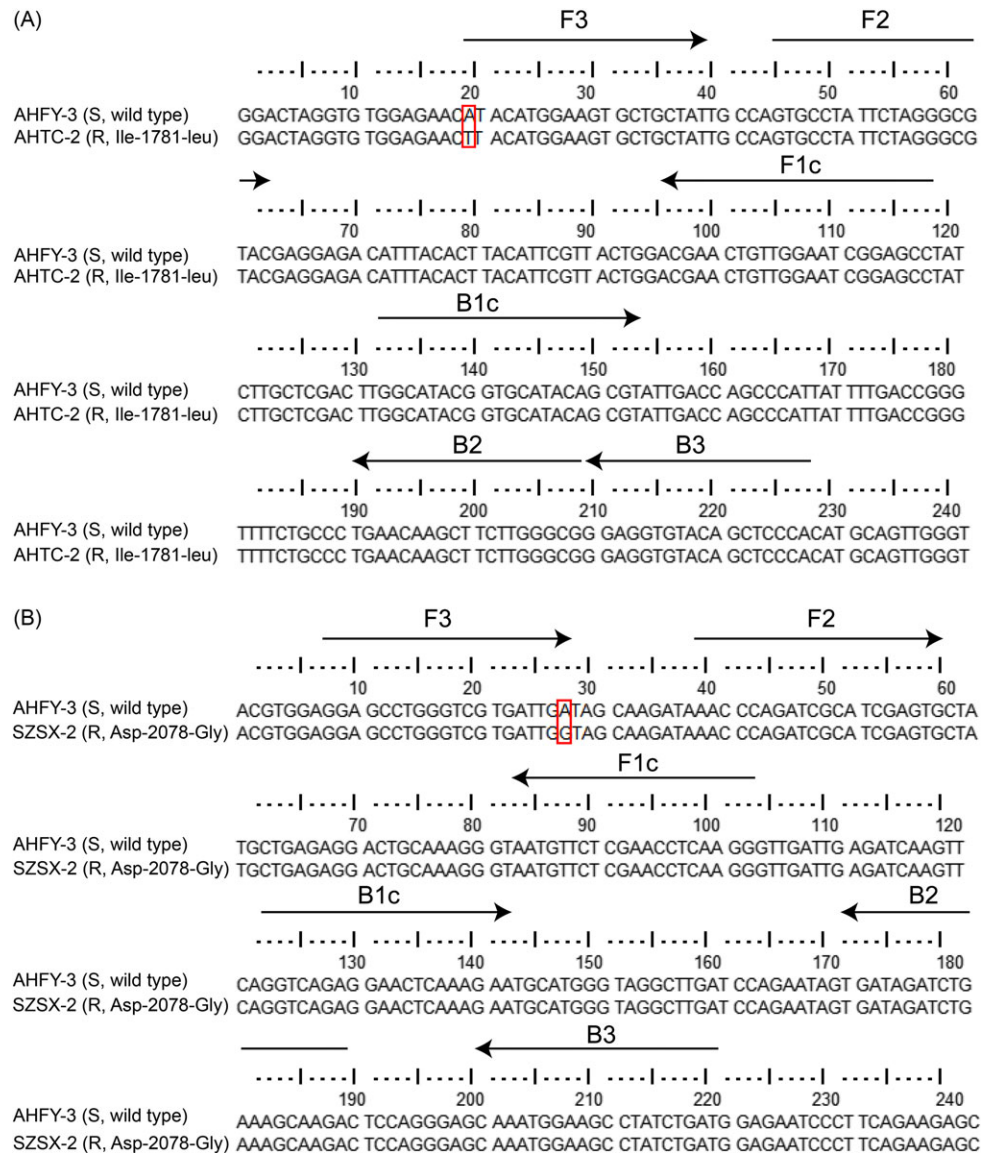


Figure 3. Alignment of partial nucleotide sequences of *ACCase* from the susceptible (S) AHFY-3 and two resistant (R) populations, (A) AHTC-2 and (B) SZSX-2, of *Alopecurus aequalis*. The sequences employed as loop-mediated isothermal amplification (LAMP) primers are indicated by arrows, with F3 and B3 serving as the outer primers, and Forward Inner Primer (FIP) comprising F2, the complementary sequence of F1 (F1c), and backward inner primer (BIP) comprising B2 and the complementary sequence of B1 (B1c). The point mutations in the *ACCase* genes of resistant plants are highlighted in red boxes.

and enable the precise selection of alternative herbicides for effective resistance management. However, the presence of copy number variation in the *ALS* gene of *A. aequalis* may reduce the efficacy and accuracy of detection (Iwakami *et al.* 2017; Panozzo *et al.* 2023). Alternative techniques, such as the Cas12a-based one-pot single-nucleotide polymorphism detection method, may offer a more reliable solution for accurately identifying these known *ALS* mutations in *A. aequalis* (Zhang *et al.* 2023).

Difference in Sensitivity between LAMP and Conventional PCR

Detection limits of the developed LAMP reactions and conventional PCR were determined and compared by employing DNA dilutions of different resistant plants as templates. LAMP products were analyzed through agarose gel electrophoresis and visualized with SYBR Green I, whereas PCR products were exclusively

separated on agarose gels. For both the Ile-1781-Leu and Asp-2078-Gly mutations, LAMP successfully amplified products with dilutions as low as 10^{-6} ng μl^{-1} (Figure 5). The agarose gels displayed ladder-like patterns (Figure 5A and 5D), and the reaction systems turned yellow upon dye addition (Figure 5B and 5E). In contrast, PCR only amplified products with dilutions as low as 10^{-2} ng μl^{-1} for the two mutations (Figure 5C and 5F). The developed LAMP reaction exhibited a sensitivity about 10^4 -fold higher than conventional PCR.

Genotyping of *ACCase* Mutant Plants Using dCAPS Markers

Two dCAPS markers were subsequently developed to rapidly identify the Ile-1781-Leu and Asp-2078-Gly mutations in fenoxaprop-*P*-ethyl-resistant plants of *A. aequalis*. Regarding Ile-1781-Leu, after *ScaI* digestion, individuals carrying

Table 3. Optimized reaction systems for the developed LAMP detections

Mutation	Component (concentration)	Volume per reaction
Ile-1781-Leu or Asp-2078-Gly	10× isothermal amplification buffer (1× final)	2.5
	100 mM MgSO ₄ (6.00 mM final)	1.5
	10 mM dNTP (0.80 mM final)	2.0
	40 μM FIP (3.20 μM final)	2.0
	40 μM BIP (3.20 μM final)	2.0
	5 μM F3 (0.20 μM final)	1.0
	5 μM B3 (0.20 μM final)	1.0
	4 M Betaine (0.80 M final)	5.0
	8 U μl ⁻¹ Bst DNA polymerase (0.32 U μl ⁻¹ final)	1.0
	DNA	1.0
	ddH ₂ O	6.0
	Total	25.0

Note: BIP, backward inner primer; FIP, forward inner primer.

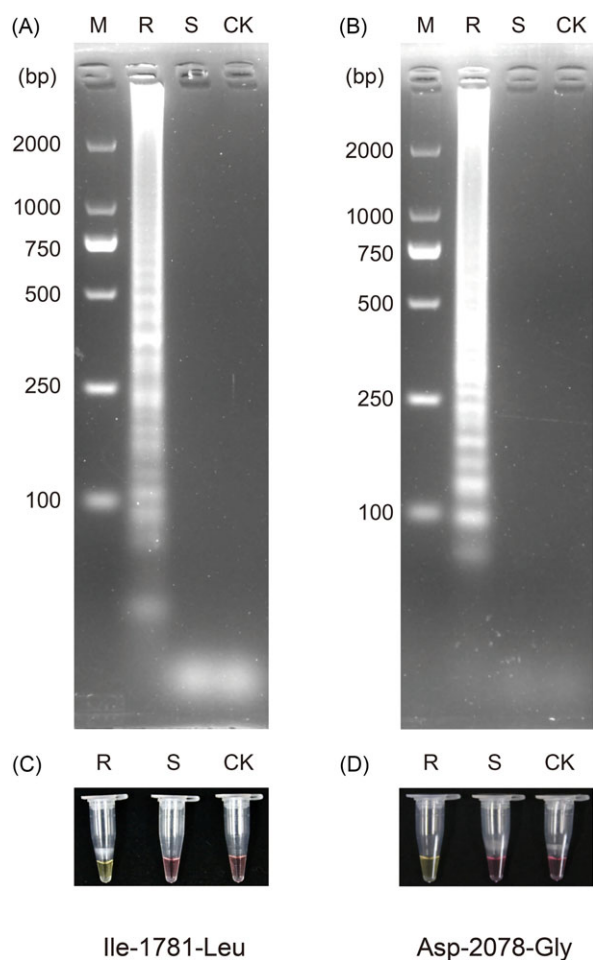


Figure 4. Specificity of loop-mediated isothermal amplification (LAMP) in the detection of the (A, C) Ile-1781-Leu and (B, D) Asp-2078-Gly mutations of *ACCase* in the fenoxaprop-*P*-ethyl-resistant *Alopecurus aequalis* populations AHTC-2 and SZSX-2, respectively. The visualization of LAMP products is achieved through the addition of dye and agarose gel electrophoresis. R, AHTC-2 (A, C) or SZSX-2 (B, D) population; S, AHFY-3 population; CK, double-distilled water; and M, 2k DNA marker.

heterozygous mutant alleles (RS) exhibited both an undigested 185-bp DNA band and two digested bands at 150 bp and 35 bp. Meanwhile, those with only WT alleles (SS) displayed an undigested 185-bp DNA band (Figure 6A). For Asp-2078-Gly, following *SpeI* digestion, individuals carrying homozygous mutant alleles (RR) exhibited two digested bands at 174 bp and 32 bp. Those with heterozygous mutant alleles (RS) exhibited both an undigested 206-bp DNA band and two digested bands at 174 bp and 32 bp. Finally, those with only WT alleles (SS) presented an undigested 206-bp DNA band (Figure 6B). Both dCAPS markers reliably differentiate between homozygous and heterozygous mutant plants, and the dCAPS analysis results were consistent with the sequencing data for each individual.

In the detection of various *ACCase* resistance mutations, the established LAMP reactions offer some advantages compared with conventional methods. For instance, PCR detection typically requires much higher DNA concentrations, involves tedious and technical procedures, and utilizes more expensive reagents and equipment. In contrast, the LAMP reaction enables the detection of specific gene mutations in DNA samples at a relatively low concentration and can be conducted using a regular water bath, providing a constant isothermal condition (Notomi et al. 2000). Furthermore, the resulting products are easily visualized through color change, facilitating the rapid recognition of known mutations in target weeds under field conditions. Nevertheless, certain limitations were noted for the LAMP method, especially its inability to distinguish heterozygosity in *ACCase* mutations. Considering that another simple molecular detection technology, the dCAPS method, can easily distinguish individuals carrying homozygous and heterozygous resistant alleles using agarose gel electrophoresis (Délye et al. 2011), a combination of dCAPS and LAMP would be more powerful in determining *ACCase* mutation type and homozygosity. In this current study, a dCAPS assay was also developed for the detection of each *ACCase* mutation, and restriction digestion patterns could clearly indicate the mutant status of target codons by counting the number of bands displayed in gels (Figure 6). Similar methods have been established for the detection of different *ACCase* mutations in *ACCase*-resistant American sloughgrass [*Beckmannia syzigachne* (Steud.) Fernald] and demonstrated feasibility (Pan et al. 2015a). Of course, it is anticipated that future advancements in the LAMP assay will facilitate the precise differentiation between homozygous and heterozygous mutation genotypes.

In summary, this study has successfully developed LAMP-based tools for the efficient detection of two common *ACCase* mutations, namely Ile-1781-Leu and Asp-2078-Gly, which have been found to be associated with high levels of fenoxaprop-*P*-ethyl resistance in *A. aequalis* species. Moreover, dCAPS assays have been established for each of these mutations, enabling the differentiation of heterozygosity in resistant plants. These tools hold promise for effectively controlling *A. aequalis* plants carrying such mutations through the timely adoption of alternative chemical agents.

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Competing interests. The authors declare no conflicts of interest.

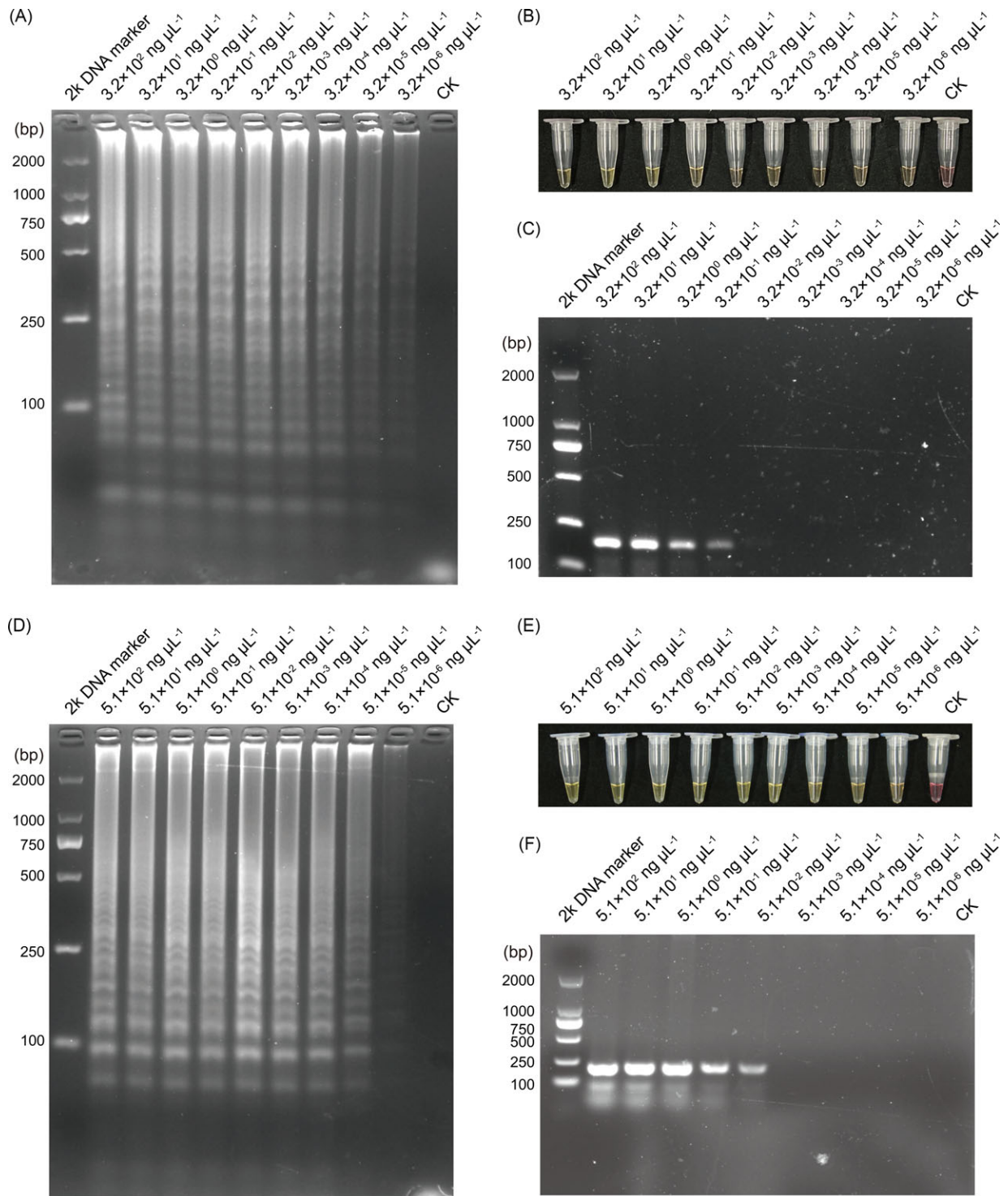


Figure 5. Comparison of the sensitivity between loop-mediated isothermal amplification (LAMP) and conventional polymerase chain reaction (PCR) in the detection of the (A–C) Ile-1781-Leu and (D–F) Asp-2078-Gly mutations of *ACCase* in *Alopecurus aequalis*, respectively. (A, D) LAMP assessment through agarose gel electrophoresis. (B, E) LAMP assessment based on color change. (C, F) Conventional PCR assessment via agarose gel electrophoresis. DNA dilutions with concentrations ranging from 10^2 ng μL^{-1} to 10^{-5} ng μL^{-1} , and double-distilled water (CK) were used as templates, respectively.

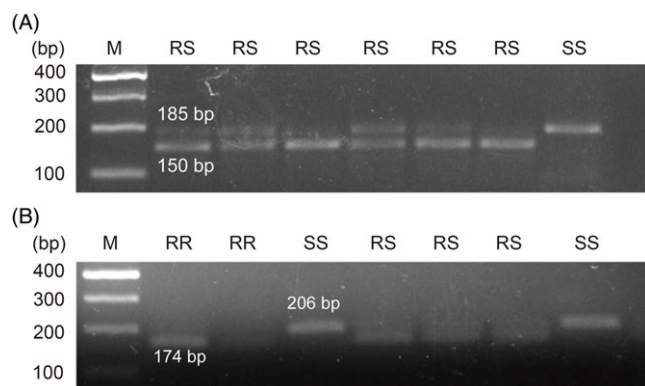


Figure 6. Derived cleaved amplified polymorphic sequence (dCAPS) markers for the detection of (A) Ile-1781-Leu and (B) Asp-2078-Gly mutations in the *ACCase* genes of *Alopecurus aequalis*. In (A), the *ScaI* digestion pattern reveals a single undigested 185-bp band corresponding to susceptible Ile-1781 alleles, while the two digested bands of 150 bp and 35 bp correspond to resistant Ile-1781-Leu alleles (with the 35-bp band invisible on agarose gel). In (B), the *SpeI* digestion pattern shows a single undigested 206-bp band for susceptible Asp-2078 alleles, and the two digested bands of 174 bp and 32 bp correspond to resistant Asp-2078-Gly alleles (with the 32-bp band invisible on agarose gel). M, 100-bp DNA ladder; RR, homozygous mutant alleles; RS, heterozygous mutant alleles; and SS, susceptible (wild-type) alleles.

References

- Ahuja A, Somvanshi VS (2021) Diagnosis of plant-parasitic nematodes using loop-mediated isothermal amplification (LAMP): a review. *Crop Prot* 147:105459
- Badolo A, Bando H, Traoré A, Ko-Ketsu M, Guelbeogo WM, Kanuka H, Ranson H, Sagnon NF, Fukumoto S (2015) Detection of G119S *ace-1^R* mutation in field-collected *Anopheles gambiae* mosquitoes using allele-specific loop-mediated isothermal amplification (AS-LAMP) method. *Malar J* 14:1–8
- Beckie HJ, Tardif FJ (2012) Herbicide cross resistance in weeds. *Crop Prot* 35:15–28
- Bi YL, Liu WT, Guo WL, Li LX, Yuan GH, Du L, Wang JX (2016) Molecular basis of multiple resistance to ACCase- and ALS-inhibiting herbicides in *Alopecurus japonicus* from China. *Pestic Biochem Physiol* 126:22–27
- Boutsalis P (2001) Syngenta quick-test: a rapid whole-plant test for herbicide resistance. *Weed Technol* 15:257–263
- Cope TA (1982) Poaceae. Page 678 in Nasir E, Ali SI, eds. *Flora of Pakistan*. Fascicle No. 143. Islamabad, Pakistan: National Herbarium
- Corbett CAL, Tardif FJ (2006) Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: a review. *Pest Manag Sci* 62:584–597
- Délye C, Pernin F, Michel S (2011) “Universal” PCR assays detecting mutations in acetyl-coenzyme A carboxylase or acetolactate synthase that endow herbicide resistance in grass weeds. *Weed Res* 51:353–362
- Garg N, Ahmad FJ, Kar S (2022) Recent advances in loop-mediated isothermal amplification (LAMP) for rapid and efficient detection of pathogens. *Curr Res Microb Sci* 3:100120
- Guo W, Chi Y, Feng L, Tian X, Liu W, Wang J (2018) Fenoxaprop-*P*-ethyl and mesosulfuron-methyl resistance status of shortawn foxtail (*Alopecurus aequalis* Sobol.) in eastern China. *Pestic Biochem Physiol* 148:126–132
- Guo W, Liu W, Li L, Yuan G, Du L, Wang J (2015a) Molecular basis for resistance to fenoxaprop in shortawn foxtail (*Alopecurus aequalis*) from China. *Weed Sci* 63:416–424
- Guo W, Lv L, Zhang L, Li Q, Wu C, Lu X, Liu W, Wang J (2016) Herbicides cross resistance of a multiple resistant short-awn foxtail (*Alopecurus aequalis* Sobol.) population in wheat field. *Chil J Agric Res* 76:163–169
- Guo W, Yuan G, Liu W, Bi Y, Du L, Zhang C, Li Q, Wang J (2015b) Multiple resistance to ACCase and AHAS-inhibiting herbicides in shortawn foxtail (*Alopecurus aequalis* Sobol.) from China. *Pestic Biochem Physiol* 124:66–72
- Harwood JL (1988) Fatty acid metabolism. *Annu Rev Plant Biol* 39:101–138
- Heap IM (2024) The International Herbicide-Resistant Weed Database. <http://www.weedscience.org>. Accessed: January 24, 2024
- Iwakami S, Shimono Y, Manabe Y, Endo M, Shibaie H, Uchino A, Tominaga T (2017) Copy number variation in acetolactate synthase genes of thifensulfuron-methyl resistant *Alopecurus aequalis* (shortawn foxtail) accessions in Japan. *Front Plant Sci* 8:254
- Kaundun SS, Marchegiani E, Hutchings SJ, Baker K (2019) Derived polymorphic amplified cleaved sequence (dPACS): a novel PCR-RFLP procedure for detecting known single nucleotide and deletion–insertion polymorphisms. *Int J Mol Sci* 20:3193
- Kaundun SS, Windass JD (2006) Derived cleaved amplified polymorphic sequence, a simple method to detect a key point mutation conferring acetyl CoA carboxylase inhibitor herbicide resistance in grass weeds. *Weed Res* 46:34–39
- Kumar V, Jha P (2017) First report of Ser653Asn mutation endowing high-level resistance to imazamox in downy brome (*Bromus tectorum* L.). *Pest Manag Sci* 73:2585–2591
- Li G, Li Y, Zhang M, Zou J (1990) Study on the habit of *Alopecurus aequalis* and its control methods. *Hubei Agric Sci* 9:29–30. Chinese
- Murphy BP, Tranel PJ (2019) Target-site mutations conferring herbicide resistance. *Plants* 8:382
- Neff MM, Turk E, Kalishman M (2002) Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet* 18:613–615
- Nikolau BJ, Ohlrogge JB, Wurtele ES (2003) Plant biotin-containing carboxylases. *Arch Biochem Biophys* 414:211–222
- Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28:e63
- Pan L, Li J, Xia W, Zhang D, Dong L (2015a) An effective method, composed of LAMP and dCAPS, to detect different mutations in fenoxaprop-*P*-ethyl-resistant American sloughgrass (*Beckmannia syzigachne* Steud.) populations. *Pestic Biochem Physiol* 117:1–8
- Pan L, Li J, Zhang WN, Dong L (2015b) Detection of the I1781L mutation in fenoxaprop-*P*-ethyl-resistant American sloughgrass (*Beckmannia syzigachne* Steud.), based on the loop-mediated isothermal amplification method. *Pest Manag Sci* 71:123–130
- Panozzo S, Farinati S, Sattin M, Scarabel L (2023) Can allele-specific loop-mediated isothermal amplification be used for rapid detection of target-site herbicide resistance in *Lolium* spp.? *Plant Methods* 19:14
- Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol Biol Rep* 15:8–15
- Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* 61:317–347
- Tang Z, Wang Z, Wang M, Yin F, Liao M, Cao H, Zhao N (2023) Molecular mechanism of resistance to mesosulfuron-methyl in shortawn foxtail (*Alopecurus aequalis*) from China. *Weed Sci* 71:224–232
- Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 3:877–882
- Trippa D, Scalenghe R, Basso MF, Panno S, Davino S, Morone C, Giovino A, Oufensou S, Luchi N, Yousefi S, Martinelli F (2024) Next-generation methods for early disease detection in crops. *Pest Manag Sci* 80:245–261
- Wang M, Tang Z, Liao M, Cao H, Zhao N (2023) Loop-mediated isothermal amplification for detecting the Ile-2041-Asn mutation in fenoxaprop-*P*-ethyl-resistant *Alopecurus aequalis*. *Pest Manag Sci* 79:711–718

- Zhang H, Tweel B, Tong L (2004) Molecular basis for the inhibition of the carboxyltransferase domain of acetyl-coenzyme-A carboxylase by haloxyfop and diclofop. *Proc Natl Acad Sci USA* 101:5910–5915
- Zhang H, Yang Z, Shen Y, Tong L (2003) Crystal structure of the carboxyltransferase domain of acetyl-coenzyme A carboxylase. *Science* 299:2064–2067
- Zhang HX, Zhang C, Lu S, Tong X, Zhang K, Yin H, Zhang Y (2023) Cas12a-based one-pot SNP detection with high accuracy. *Cell Insight* 2:100080
- Zhao N, Li Q, Guo W, Zhang L, Wang J (2018) Effect of environmental factors on germination and emergence of shortawn foxtail (*Alopecurus aequalis*). *Weed Sci* 66:47–56
- Zhao N, Yan Y, Ge L, Zhu B, Liu W, Wang J (2019) Target site mutations and cytochrome P450s confer resistance to fenoxaprop-*P*-ethyl and mesosulfuron-methyl in *Alopecurus aequalis*. *Pest Manag Sci* 75:204–214
- Zhu W, Tu S (1997) Study on damage from *Alopecurus aequalis* Sobol and its economical threshold in wheat fields of Hubei province. *J Huazhong Agric Univ* 16:268–271