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Short title: Rapid mutation detection

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

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Abstract

The resistance to fenoxaprop-*P*-ethyl, a herbicide that inhibits acetyl-CoA carboxylase (ACCase), 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 *ACCase* 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 *ACCase* 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 *ACCase* 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. By including SYBR Green I dye in the final reaction mixtures, the target mutation can be visually detected 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 10^4-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 *ACCase* gene of *A. aequalis*, offering significant advantages for the monitoring and management of fenoxaprop-*P*-ethyl resistance.

Keywords: ACCase; resistance diagnostics; target-site resistance; monocot

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 over 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 (APP) herbicide exhibiting high efficiency by specifically targeting the plastidic acetyl-CoA carboxylase (ACCase) in plants (Zhang et al. 2003). ACCase 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 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 is classified as target-site based resistance (TSR), now recognized as a major mechanism of herbicide resistance (Powles and Yu 2010). To date, over 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 (PASA), and (derived) cleaved amplified polymorphic sequence ((d)CAPS) analysis, rely on DNA sequencing and/or PCR methodologies, often necessitating expensive equipment and intricate 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. Presently, LAMP-based methodologies have been extensively adopted for detecting of a range of agricultural challenges including plant-parasitic nematodes (Ahuja & Somvanshi, 2021) and diverse pathogens (Garg et al. 2022; Trippa et al. 2023), 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 advancements were made in LAMP-based detection for the remaining two prevalent resistance mutations—specifically, Ile-to-Leu and Asp-to-

Gly—occurred 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, AHFY-3) and two putative resistant (R) populations (AHTC-2 and SZSX-2) of *A. aequalis* at distinct locations in Anhui Province, China (Table 1). AHFY-3 was sourced from an uncultivated land and had been characterized as susceptible previously (Wang et al. 2023), while AHTC-2 and SZSX-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 over 200 individuals, were meticulously blended, air-dried, and subsequently stored in paper bags at 4 °C until 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 into distinct plastic pots (10 cm × 10 cm × 8.5 cm), each filled with loam soil. The pots were placed in a controlled glasshouse environment with natural light at temperatures of 25 °C/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 (Puma, 69 g L^{-1} emulsion in water; Bayer Crop Science, Hangzhou, China) at the recommended field rate (RFR, 62 g active ingredient a.i. ha^{-1}), while the remaining 15 plants received water as a negative control. These liquids were sprayed using a compressed-air cabinet sprayer fitted with a mobilizable flat-fan nozzle (9503EVS, Spraying Systems Co., 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 three weeks. At 21 d after treatment (DAT), the 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.

Whole-Plant Dose-Response Experiment

The S and R seedlings at the 3- and 4-leaf stage were used for the 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 a.i. ha^{-1}. For the two R populations, doses of 0, 20.7, 62.1, 186.3, 558.9, 1676.7, and 5030.1 g a.i. 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).

Using SPSS v19.0 software (IBM, Armonk, NY, USA), ANOVA analysis was performed, revealing no remarkable interactions ($P > 0.05$) between treatment and runs. The data from the repeated experiments were thus pooled and fitted to a four-parameter nonlinear logistic model (Equation 1) using SIGMAPLOT v14.0 (Systat Software, San Jose, CA, USA).

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

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 to 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 ACCase Mutations

Genomic DNA extraction followed the classical cetyltrimethylammonium bromide (CTAB)-based method (Porebski et al. 1997). PCR amplification of a 1437-bp *ACCase* gene fragment was conducted using 2 × Super Pfx MasterMix (Cowin Bio., Beijing, China) with the primer pair as reported in Bi et al. (2016). The amplified products were subjected to Sanger sequencing by TsingKe Biotech Co., Ltd. (Nanjing, China), and the resulting nucleotide and deduced amino acid sequences were analyzed and compared between S and R populations using DNAMAN v6.0 (Lynnon Biosoft, Quebec, Canada).

LAMP Methods for Detection of Different ACCase Mutations

To specifically detect the Ile-1781-Leu and Asp-2078-Gly mutations in the *ACCase* gene of *A. aequalis*, two sets of specific LAMP primers were respectively designed (Table 2). This design utilized the PRIMER EXPLORER V4 software, available at http://primerexplorer.jp/elamp4.0.0/index.html, and targeted the highly conserved regions surrounding codons 1781 and 2078 of *ACCase*. The primer sets included outer primers that initiated the reaction and inner primers that completed it. To prevent occasional non-specific gene amplification from susceptible plants—a phenomenon attributed to the high sensitivity of the LAMP method—two mismatches were deliberately introduced into the forward outer primer L1781-F3, and one mismatch was introduced into L2078-F3 (Table 2).

In the preparation of the LAMP reaction system, a total volume of 25 μL was utilized. The optimization of this system involved evaluating a series of concentrations for several key components: $MgSO_4$ (Sigma-Aldrich, St Louis, MO, USA) ranging from 4 to 8 mM, dNTP (Sangon Biotech Co., Ltd., 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 FIP/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 *ACCase*. 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 3.2×10^{2} ng μL^{-1} and 5.1×10^{2} ng μL^{-1}, respectively. After an accurate 10-fold serial dilution from 10^{2} ng μL^{-1} to 10^{-6} ng μL^{-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 (Cowin 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 v2.0 software (Table 2) (Neff et al. 2002). For the Ile-1781-Leu mutation, a 185-bp DNA fragment covering codon 1781 of *ACCase* would be 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* would be 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 *Sca*I and *Spe*I enzymes (TaKaRa, Dalian, China), respectively. PCR was conducted using 2 × Super Pfx MasterMix (Cowin 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, should the products contain mutant sequences, they 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 the precision 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), it 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.

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 GR_{50} value to fenoxaprop-*P*-ethyl, were identical and utilized 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% (Fig. 1A). In contrast, all plants from the S population had died, displaying biomass reductions exceeding 90% (Fig. 1A). This observation indicates that the two R populations had developed resistance to fenoxaprop-*P*-ethyl.

To assess the resistance levels of the two R populations to fenoxaprop-*P*-ethyl, whole-plant dose-response experiments were carried out (Fig. 1B). The GR_{50} 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 1437-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 Fig. 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 A. 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, Fig. 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 (Fig. 4 A-B). Upon adding dye, the color of the R systems turned yellow, whereas that of the S systems and the negative controls maintained orange red (Fig. 4 C-D). 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 the optimized primers, systems, and conditions, the LAMP assays developed in this study can rapidly distinguish between wild-type 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 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 (SNP) 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} (Fig. 5). The agarose gels displayed ladder-like patterns (Fig. 5 A, D), and the reaction systems turned yellow upon dye addition (Fig. 5 B, E). In contrast, PCR amplified products only with dilutions as low as 10^{-2} ng μL^{-1} for the two mutations (Fig. 5 C, F). 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 *Sca*I digestion, individuals carrying 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 (Fig. 6A). For Asp-2078-Gly, following *Spe*I 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. Lastly, those with only WT alleles (SS) presented an undigested 206-bp DNA band (Fig. 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 to 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 incapability to distinguish heterozygosity in *ACCase* mutations. Considering that another simple molecular detection technology, the (d)CAPS method, can easily distinguish individuals carrying homozygous and heterozygous resistant alleles using agarose gel electrophoresis (Délye et al. 2011), a combination of (d)CAPS 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 (Fig. 6). Similar methods have been established for the detection of different *ACCase* mutations in ACCase-resistant *B. syzigachne* 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 author(s) declare none.

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Tables

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

Population	Biotype	*ACCase* mutation	GPS coordinate	GR_{50} (SE)	RI
				g a.i. ha^{-1}	
AHFY-3	Susceptible	Wild type	117.64°E, 32.75°N	3.34 (0.41)	1.00
AHTC-2	Resistant	Ile-1781-Leu ($\underline{\text{A}}$TA to $\underline{\text{T}}$TA)	118.99°E, 32.70°N	415.52 (49.67)	124.41
SZSX-2	Resistant	Asp-2078-Gly (G$\underline{\text{A}}$T to G$\underline{\text{G}}$T)	117.89°E, 33.49°N	224.74 (33.33)	67.29

Abbreviations: ACCase, acetyl-CoA carboxylase; GR_{50}, the herbicide dose causing a 50% growth reduction; RI, resistance index.

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

Mutation	Usage	Primer	Primer type	Primer sequence (5′–3′)
Ile-1781-Leu	dCAPS	d1781-F	PCR forward	TGTGGTGGGAAAGGAGGAT
		d1781-R	PCR reverse	TGTGGTGGGAAAGGAGGATGGACTAGGTGTGGAGt[a]AC
Asp-2078-Gly	dCAPS	d2078-F	PCR forward	TGCAGAGCTACGTGGAGGGGCCTGGGTCGTGAc[a]Ta[a]
		d2078-R	PCR reverse	CCCTGGAGTCTTGCTTTCAG
Ile-1781-Leu	LAMP	L1781-F3	Forward outer	T**T**[b]ag[a]ACATGGAAGTGCTGCTAT
		L1781-B3	Backward outer	TGGGAGCTGTACACCTCCC
		L1781-FIP	Forward inner	GGCTCCGATTCCAACAGTTCGT-GTGCCTATTCTAGGGCGTAC
		L1781-BIP	Backward inner	GGCATACGGTGCATACAGCGT-CGCCCAAGAAGCTTGTTCA
Asp-2078-Gly	LAMP	L2078-F3	Forward outer	GGAGCCTGGGTCGTGAAa[a]G**G**[b]
		L2078-B3	Backward outer	ATCAGATAGGCTTCCATTTG
		L2078-FIP	Forward inner	CCTTGAGGTTCGAGAACATT-AACCCAGATCGCATCGAGTG
		L2078-BIP	Backward inner	GGTCAGAGGAACTCAAAGAA-TCTTGCTTTCAGATCTATCA

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

[a] The lowercase nucleotides are mismatches manually added to distinguish different amino acid substitutions specifically.

[b] The nucleotide in bold is the mutant site in the sequence of the *ACCase* gene in the resistant (R) biotype compared with the susceptible (S) biotype.

Table 3. Optimized reaction systems for the developed LAMP detections.

Mutation	Component (concentration)	Volume per reaction
		µL
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_2O	6.0
	Total	25.0

Figure legends

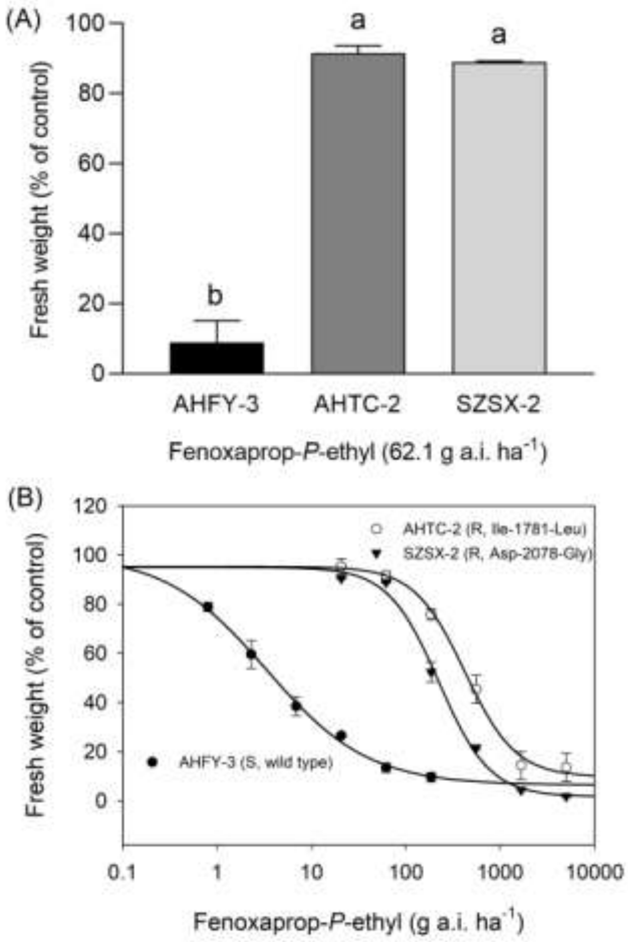

Fig. 1. Effects of fenoxaprop-*P*-ethyl treatments on the growth of the susceptible (S, AHFY-3) and two resistant (R, AHTC-2 and SZSX-2) populations of *Alopecurus aequalis*. (A) Weed plants from all three populations, at the 3- to 4-leaf stage, received fenoxaprop-*P*-ethyl at the recommended field 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 fitted 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 re-used with permission from Wang et al. (2023), Copyright 2024 John Wiley and Sons. Vertical bars represent the SE of the mean.

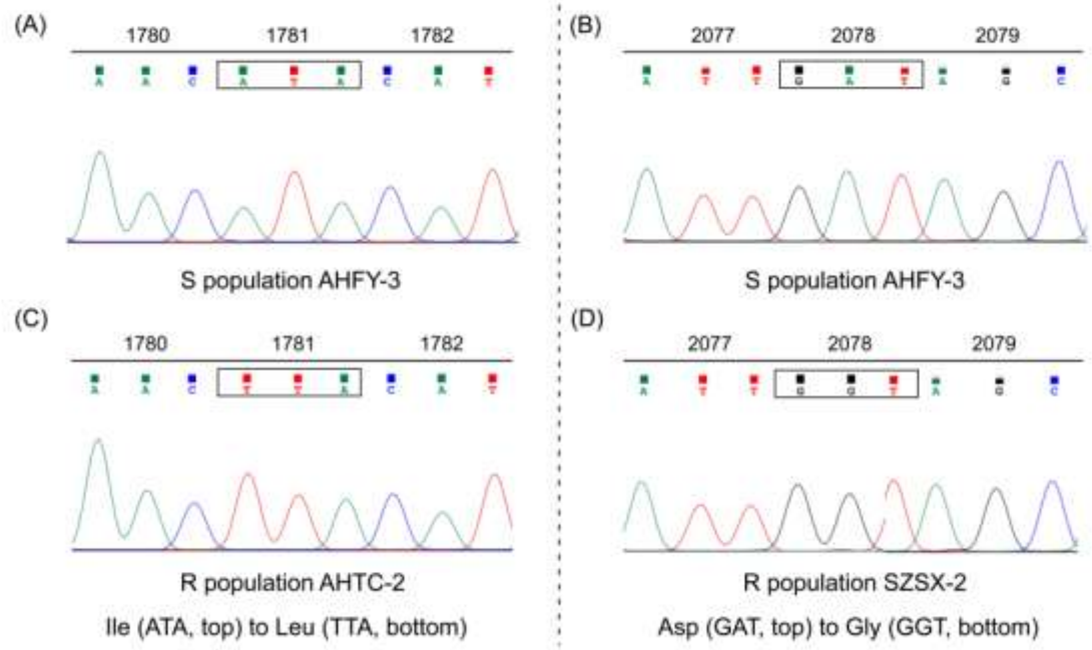

Fig. 2. DNA sequencing results showing the wile-type codons (A) Ile-1781 and (B) Asp-2078 in the *ACCase* genes of the susceptible (S) plants (AHFY-3, top) of *Alopecurus aequalis* and the mutant codons (C) Ile-1781-Leu and (D) Asp-2078-Gly in those of the resistant (R) plants (AHTC-2 and SZSX-2, bottom).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

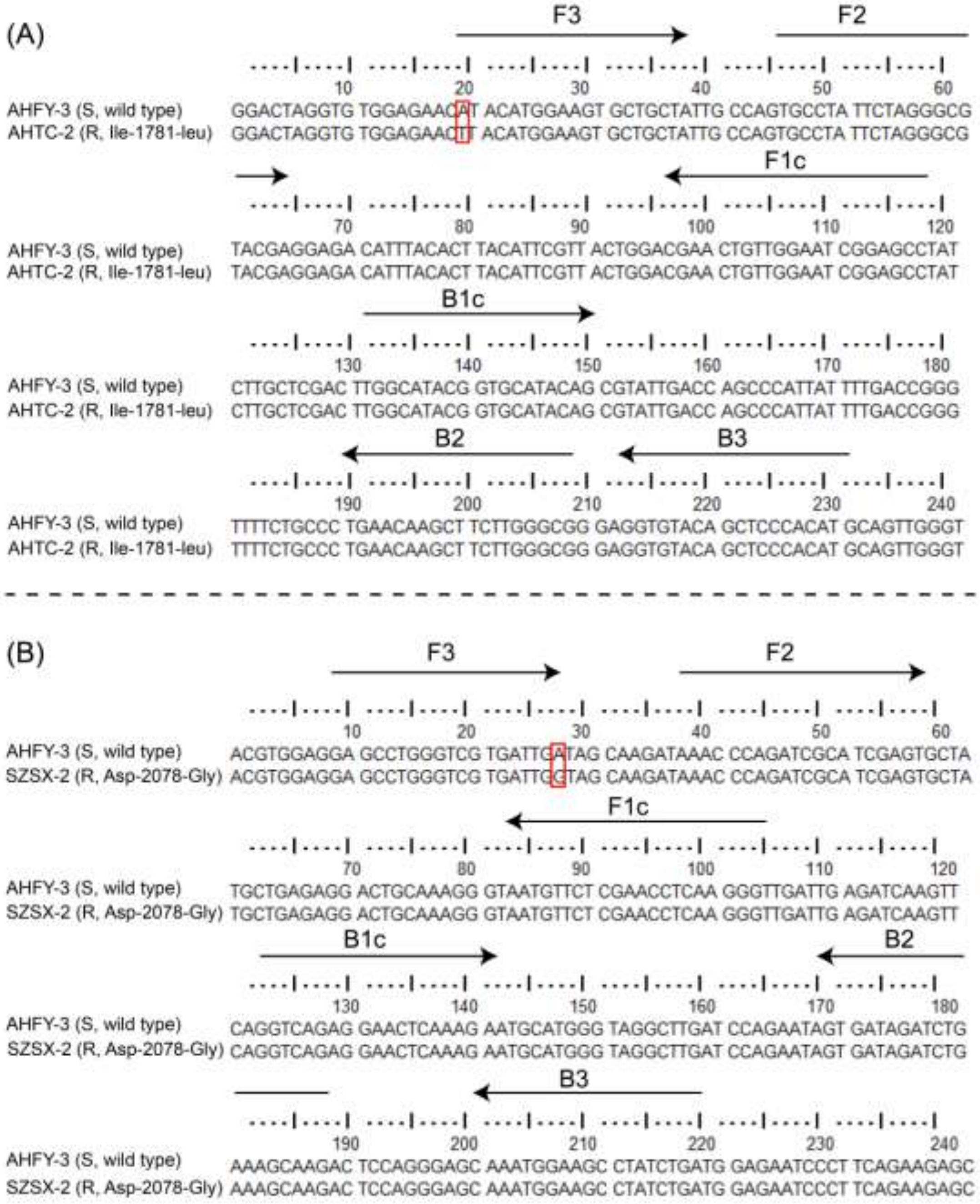

Fig. 3. Alignment of partial nucleotide sequences of *ACCase* from the susceptible (S) AHFY-3 and two resistant (R) populations, (A) AHTC-2 and (B) SZSX-2, of *Alopecurus aequalis*. The sequences employed as loop-mediated isothermal amplification (LAMP) primers are indicated by arrows, with F3 and B3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

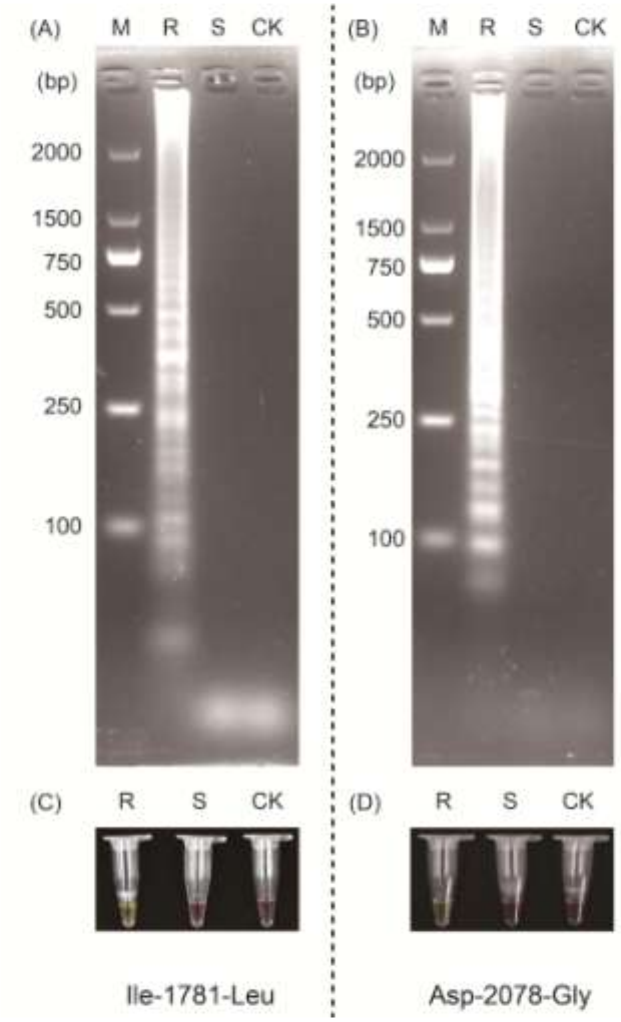

Fig. 4. Specificity of loop-mediated isothermal amplification (LAMP) in the detection of the (A, C) Ile-1781-Leu and (B, D) Asp-2078-Gly mutations of *ACCase* in the fenoxaprop-*P*-ethyl-resistant *Alopecurus aequalis* populations AHTC-2 and SZSX-2, respectively. The visualization of LAMP products is 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.

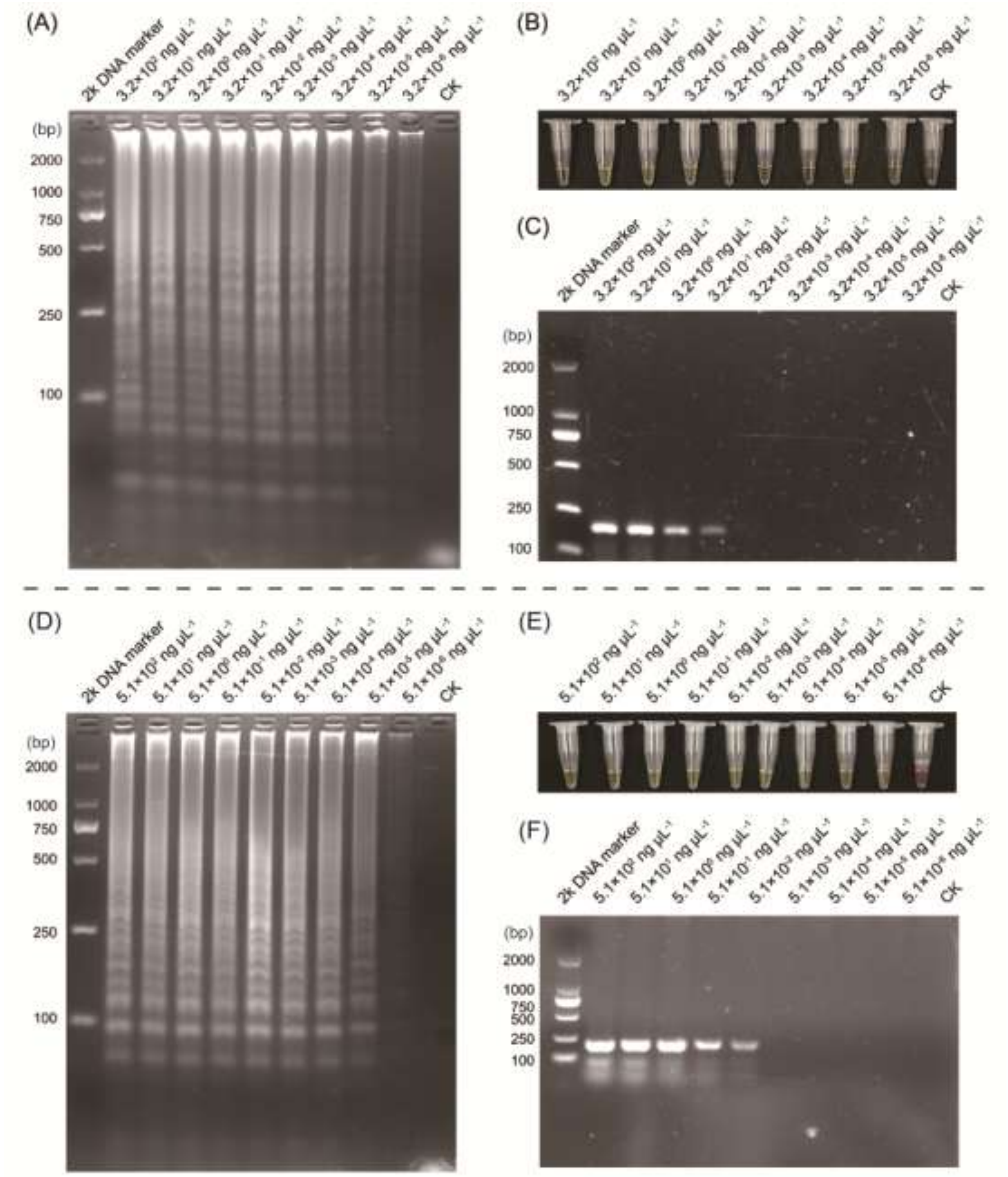

Fig. 5. Comparison of the sensitivity between loop-mediated isothermal amplification (LAMP) and conventional PCR in the detection of the (A-C) Ile-1781-Leu and (D-F) Asp-2078-Gly mutations of *ACCase* in *Alopecurus aequalis*, respectively. (A, D) LAMP assessment through agarose gel electrophoresis. (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.

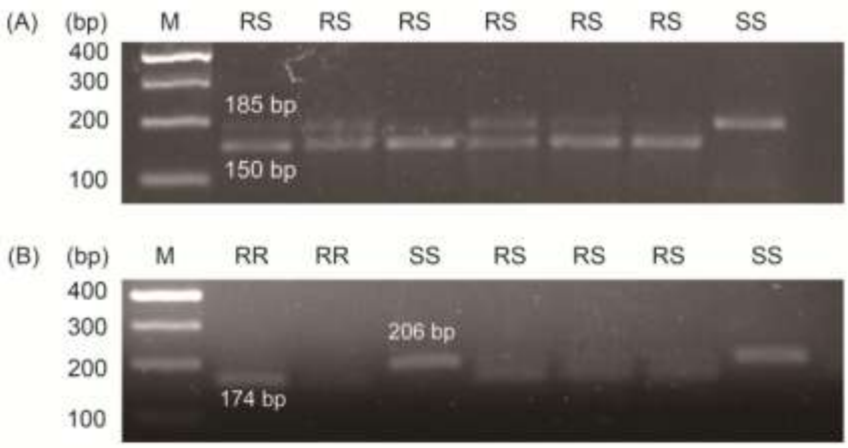

Fig. 6. Derived cleaved amplified polymorphic sequence (dCAPS) markers for the detection of (A) Ile-1781-Leu and (B) Asp-2078-Gly mutations in the *ACCase* genes of *Alopecurus aequalis*. In (A), the *Sca*I digestion pattern reveals a single undigested 185-bp band corresponding to susceptible Ile-1781 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 *Spe*I 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). In both dCAPS markers, the 32- and 35-bp bands were invisible on the gels. M, 100-bp DNA ladder; RR, homozygous mutant alleles; RS, heterozygous mutant alleles; and SS, susceptible (wild-type) alleles.