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Short Title: Pro-197-Thr mutation in ALS

Pro-197-Thr mutation in *ALS* gene confers higher pyroxsulam resistance than Pro-197-Gln in Italian ryegrass (*Lolium perenne* ssp. *multiflorum*)

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Abstract:

Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], a pernicious weed in wheat fields, has evolved severe resistance to the acetolactate synthase (ALS)-inhibiting herbicides, like pyroxsulam. Here, the derived cleaved amplified polymorphic sequence (dCAPS) markers were developed to detect two distinct mutations at the 197th position of the *ALS* gene. The method was used to examine and purify the resistant population. Homozygous populations with different 197 site mutations from the same population were obtained, and the target-site resistance mechanisms were investigated. Whole-plant dose-response bioassays show that the resistance index (RI) of the Pro-197-Thr mutant population to pyroxsulam was 508.92, whereas that of the Pro-197-Gln population was 9.75. Similar trends were observed for different herbicides within same mode of action. *In vitro* ALS assays demonstrated that the Pro-197-Thr population exhibited lower sensitivity to pyroxsulam than the Pro-197-Gln population, consistent with plant bioassays. Furthermore, *ALS* gene expression of the Pro-197-Thr population analysis is significantly higher than that in the Pro-197-Gln population which may also explain why the Pro-197-Thr population exhibits higher resistance level than the Pro-197-Gln population. Our findings suggest that different amino acid substitutions at one *ALS* gene locus can confer herbicide resistance with different levels in *L. perenne* ssp. *multiflorum*. This study provides valuable insights into the mechanisms of herbicide resistance in *L. perenne* ssp. *multiflorum*.

Keywords: pyroxsulam, ALS, target-site resistance, dCAPS

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Introduction

Acetolactate synthase (ALS) is a key enzyme that catalyzes the first step in the branched-chain amino acid biosynthesis of valine (Val), leucine (Leu), and isoleucine (Ile) (Dimou et al. 2022; Zhao et al. 2021). ALS inhibiting herbicides are commonly used in the fields to control noxious weeds and mainly consist of five different families: imidazolinones (IMI), sulfonylureas (SU), pyrimidinyl thiobenzoates (PTB), sulfonylamino-carbonyl-triazolinones (SCT), and triazolopyrimidines (TP) (Zhao et al. 2019).

ALS-inhibiting herbicides are widely used due to their high efficacy, broad-spectrum weed control, high selectivity, and exceptional safety for humans and livestock (Turra et al. 2023). However, their extensive and prolonged use has led to the emergence of increasing resistance to ALS inhibitors in weeds. Globally, resistance to ALS inhibiting herbicides has been documented in 176 weed species, comprising 108 dicotyledons / 68 monocotyledons (Heap 2025). Herbicide resistance mechanisms are primarily categorized into two types: target-site resistance (TSR) and non-target-site resistance (NTSR). TSR includes target-site mutations, as well as changes in the expression of target genes (Délye C et al. 2013). In most cases, ALS target-site mutations are the key cause of herbicide resistance (Wang et al. 2024). Mutations in the *ALS* gene of weed species have been extensively documented, with numerous mutation sites identified across five conserved domains (A-E) (Chtourou et al. 2024; Kaya et al. 2022). To date, more than 30 distinct mutation sites have been reported. These mutations primarily include Pro-197 in domain A, Trp-574 in domain B, Ala-122 in domain C, Ala-205 in domain D, and Ser-653 in domain E (Xu et al. 2022; Yu and Powles 2014). The first documented case was the Trp-574-Leu mutation discovered in 1993 (Heap 2025). These mutations confer varying resistance patterns to different types of ALS inhibitors. The Pro-197 mutation typically confers resistance to SU and IMI, while maintaining susceptibility to TP in common lambsquarters (*Chenopodium album* L.) (Cao et al. 2022). In contrast, the Trp-574 mutation in barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] generally confers broad-spectrum resistance to all ALS inhibiting herbicides (Panozzo et al. 2013). The Ala-122 mutation primarily confers resistance to IMI, while the Ser-653 mutation

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shows specific resistance to IMI and TP (Massa et al. 2011). Notably, mutation patterns and resistance spectrum vary significantly among different weed species and geographical locations (Menchari et al. 2006).

Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] is an annual, biennial or short-term perennial herb of the grass family, which has developed into a persistent weed in wheat fields in China (Wu et al. 2022). It exhibits widespread distribution across numerous regions and has demonstrated a significant capacity to develop resistance to various herbicides (Bobadilla et al. 2021). Resistance to glyphosate was initially documented in the United States in 1998, and herbicide-resistant populations of *L. perenne* ssp. *multiflorum* have been identified in at least 13 countries to date (Heap 2025). The first report of ALS herbicide resistance in *L. perenne* ssp. *multiflorum* occurred in Australia in 1982, followed by subsequent reports in Tunisia, Morocco, and other African countries (Heap 2025). The mechanism of resistance of *L. perenne* ssp. *multiflorum* to ALS inhibiting herbicides includes TSR and NTSR. Many studies focused on TSR, mainly due to resistance caused by target-site mutations. Tehranchian et al. (2019) reported the substitution of Trp-574-Leu and Asp-376-Glu in the *ALS* gene in *L. perenne* ssp. *multiflorum* populations resistant to an ALS-inhibiting herbicide. Emine et al. (2022) found that the mechanism of target resistance of *L. perenne* ssp. *multiflorum* to ALS inhibitors of sulfonylureas was related to the mutation of proline at position 197 of the *ALS* gene (Wu et al. 2022). Zhu et al. (2023) found that the mutation of proline at position 197 to threonine or glutamine and the mutation of tryptophan to leucine at position 574 in *ALS* of *L. perenne* ssp. *multiflorum* would make it resistant to pyroxsulam. However, it remains unclear whether different mutation types in the *ALS* gene of *L. perenne* ssp. *multiflorum* confer varying levels of herbicide resistance and what the nature of these differences might be.

The rapid and accurate detection of resistant weeds is crucial for implementing timely and effective weed management strategies. Currently, several rapid detection methods have been developed, including allele-specific PCR (AS-PCR) (Kadaru et al. 2008; Zhang et al. 2025), quantitative PCR (qPCR) (Dongo et al. 2012), and clustered regularly interspaced short

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palindromic repeats (CRISPR)-based detection system (Ban et al. 2025). The derived Cleaved amplified polymorphic sequences (dCAPS) method, first developed by Neff et al. (1998), has emerged as a particularly valuable tool. The dCAPS method creates a restriction enzyme recognition site by introducing one or more mismatched bases into specific primers. Based on differences in fragment lengths after enzymatic digestion, it allows for accurate genotyping of samples. This method is characterized by its simple operation, intuitive results, short processing time, and low cost, making it suitable for detecting high-throughput samples (Yin et al. 2024). Currently, dCAPS can be used to analyze the genetic diversity of plant populations, study evolutionary relationships, and investigate gene functions, including identifying single nucleotide polymorphisms (SNPs) associated with specific traits (Huang et al. 2020; Wang et al. 2018). Using the dCAPS method to detect mutations conferring target-site-mediated herbicide resistance in weeds can significantly be used to recommend alternative herbicides and thus to reduce the occurrence of resistant weeds in the field and mitigate their impact on agricultural production. However, there have been no reports on rapid detection methods for *ALS* gene mutations in *L. perenne* ssp. *multiflorum* to date.

Therefore, the objectives of this study were (1) to develop a rapid detection method for *ALS* gene mutations in *L. perenne* ssp. *multiflorum*, utilizing this method to purify and isolate resistant populations; (2) to evaluate the resistance differences between populations with different mutation types at one *ALS* locus.

Materials and Methods

Plants, herbicides and chemicals

In 2020, the herbicide-resistant population of *L. perenne* ssp. *multiflorum* (designated as R) was collected from a wheat field in Zhumadian, Henan, China (32.97°N, 114.03°E). The field has a long-term history of using ALS-inhibiting herbicides, Acetyl-CoA carboxylase (ACCase)-inhibiting herbicides and photosystem II (PSII)-inhibiting herbicides, and the *L. perenne* ssp. *multiflorum* population had evolved multiple resistance to herbicides from different modes of action (Zhu et al. 2023). In contrast, seeds of the herbicide-sensitive population

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(designated as S) were collected from a recreational site in Jiangsu, China (32.05°N, 118.80°E), which had no history for herbicide use. Of each population, seeds were manually harvested from more than 50 mature plants, subsequently dried for 7 days, and stored at 4 C for preservation.

Pyroxsulam (7.5%, WG) was provided by Corteva Agrisciences, Beijing, China; Mesosulfuron-methyl was provided by Bayer Crop Sciences (30 g L^{-1}, OF), Hangzhou, China; Fenoxaprop-P-ethyl (69 g L^{-1}, EW) was provided by Bayer Crop Sciences, Hangzhou, China; Isoproturon (50%, WP), Jiangsu Futian Agrochemical Co., Ltd., China; Cypyrafluone (6%, SC), KingAgroot, Qingdao, China. The CYP450 inhibitors piperonyl butoxide (PBO, 95%) was purchased from Aladdin (Shanghai, China, CAS#51-03-6).

Characterization and purification of individual *L. perenne* ssp. *multiflorum* **with distinct mutations**

Seeds from the herbicide-resistant *L. perenne* ssp. *multiflorum* population with confirmed *ALS* gene mutations (Zhu et al. 2023) were cultivated in 7 cm × 7 cm × 7 cm plastic pots filled with a standardized soil mixture (organic substrate and sandy soil with a 1:2 w/w ratio, pH=6.2). Each pot, with drainage holes at the bottom, was sown with 20 seeds, were later thinned to 10 uniform seedlings, 4 replicate pots in total. The plants were maintained in controlled greenhouse conditions with a 12-hour photoperiod, a diurnal temperature regime of 20/15 C (day/night), a light intensity of 120 μmol m^{-2} s^{-1}, and a relative humidity of 65%. At the three- to four-leaf stage, leaf tissue samples of resistant *L. perenne* ssp. *multiflorum* population were collected for genomic DNA extraction. Mutant genotypes were identified using the dCAPS method, and homozygous mutant individuals were selected and labeled for subsequent analysis. Upon reaching the five- to six-leaf stage, individual plants were transplanted into larger pots (20 cm diameter × 20 cm height) for isolated outdoor cultivation. The growth cycle extended from October to June of the following year, with physical isolation measures implemented to prevent pollen contamination. Homozygous Pro-197-Gln (RR) plants underwent two rounds of purification (Fig. 1). The susceptible S population did not require further purification.

Derived cleaved amplified polymorphic sequences (dCAPS) markers

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DNA isolation

The plant growth conditions are as described above, 100 mg young shoot tissues at the 3-4 leaf stage of individual plants of S and R populations were collected and ground with liquid nitrogen. Genomic DNA was isolated using a commercial plant genomic DNA extraction kit (Tiangen Biotechnology, Beijing, China) in accordance with the manufacturer's instructions.

PCR amplification and mutation detection

The dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) was employed to design specific primers for detecting mutations at the Pro-197 position. Initial screening primers were designed to identify potential mutations, followed by the development of mutation-specific primers based on the screening results. For the detection of the Pro-197-Thr mutation, a modified reverse primer incorporating a mismatched base (A) was designed to create a *Mlu*I restriction site. This design enabled differentiation of genotypes through restriction fragment analysis: (1) samples with the sensitive Pro-197 genotype produced an uncut 211 bp fragment; (2) homozygous resistant Pro-197-Thr genotypes yielded 181 bp and 30 bp fragments after *Mlu*I digestion; and (3) heterozygous samples displayed all three fragments (211 bp, 181 bp, and 30 bp). The same dCAPS approach was applied to design primers for Pro-197-Gln mutation and Pro-197 genotype, with detailed primer information provided in Table 1 and Table 2.

PCR was performed in a final volume of 25 µL containing 300 ng DNA, 1 µL of each primer (10 µM), 12.5 µL 2× PCR TaqMix (Vazyme Biotech, Nanjing, China) and 9.5 µL ddH_2O. The cycling program consisted of 95 C for 3 min, followed by 35 cycles of 30 s at 95 C, 30 s at annealing temperature (shown in Table 2) and 15 s at 72 C, with a final extension step of 5 min at 72 C. The PCR products were purified according to the manufacturer's instructions (EasyPure® PCR Purification Kit, TransGen Biotech, Beijing, China). The restriction digestion reaction was performed in a final volume of 20 µL, containing 0.4 µL [2 U] of restriction enzyme (New England Biolabs), 2 µL of 10×NEB buffer, and 0.4 µg of PCR product, with the remaining volume adjusted to 20 µL using ddH_2O. The reaction mixture was gently mixed by slow pipetting followed by centrifugation. The digestion was then carried out at 37 C for 30 minutes.

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After the digestion reaction was completed, 10 µL of the digestion product was loaded onto a 3% agarose gel stained with ethidium bromide and electrophoresed at 120 V for 40 minutes. A 2000 bp DNA marker was used, and the resulting bands were visualized using a gel imaging system. The whole experiment was repeated twice.

Whole-plant dose-response assay

Resistance of different** L. perenne* ssp. *multiflorum **populations to pyroxsulam

To evaluate the pyroxsulam resistance in the populations, whole-plant dose-response assays were conducted. Square plastic pots (7 cm×7 cm×7 cm, with drainage holes) were prepared with a growth medium composed of sandy soil and organic substrate (2:1 ratio, pH 6.2). Each pot was sown with 20 seeds, which were later thinned to 10 uniform seedlings at the 2-3 leaf stage to ensure consistent competition. The plants were maintained in a greenhouse under a 12/12 h photoperiod, with daytime and nighttime temperatures regulated at 20 C and 15 C, respectively. Photosynthetically active radiation (PAR) was maintained at 120 µmol m^{-2} s^{-1}, and relative humidity was stabilized at 65%. At the 3-4 leaf stage, herbicide application was performed using a precision spray system (3WP-2000, Nanjing Institute of Agricultural Mechanization) calibrated to deliver 280 L ha^{-1} at 230 kPa. The S population was treated with pyroxsulam (recommended dose = 14 g a.i. ha^{-1}) at eight concentrations (0.11, 0.22, 0.44, 0.88, 1.75, 3.5, 7, 14 g a.i. ha^{-1}), while the resistant R population received seven higher doses (3.5, 7, 14, 28, 56, 112, 224 g a.i. ha^{-1}). Following treatment, plants were returned to the greenhouse and irrigated as needed. After 21 days, aboveground biomass was harvested, and fresh weight was recorded. Growth reduction (GR_{50}) values were calculated using non-linear regression analysis. The experiment followed a randomized complete block design with three replicates. Once the experiment was completed, one more repeat was conducted.

Cross resistance and multiple resistance patterns of different** L. perenne* ssp. *multiflorum **populations to herbicides

The resistance of S and R populations to herbicides from different mechanisms of action was evaluated through whole plant bioassays. Plants were grown under controlled conditions as

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described above, and herbicide treatments were applied at the 3-4 leaf stage of *L. perenne* ssp. *multiflorum*. Four classes of herbicides were tested, including an ALS inhibitor (mesosulfuron-methyl), an ACCase inhibitor (fenoxaprop-P-ethyl), a PSII inhibitor (isoproturon), and an HPPD (4-hydroxyphenylpyruvate dioxygenase) inhibitor (cypyrafluone). The treatment dosages of herbicides are shown in Table 3. Herbicide application was performed according to the protocol outlined above. At 21 days after treatment, the aboveground fresh weight was measured to calculate the fresh weight inhibition rate and GR_{50} values. The experiment was conducted in duplicate, with each treatment including three biological replicates.

The effects of PBO on the resistance of different *L. perenne* ssp. *multiflorum* ***populations to pyroxsulam***

The plant growth conditions followed the protocol detailed above. Upon reaching the target growth stage (3-4 leaves), *L. perenne* ssp. *multiflorum* populations received an initial treatment with the P450 inhibitor PBO at 4200 g a.i. ha^{-1} (Liu et al. 2024). The PBO application was performed using a spray system (3WP-2000, Nanjing, China). Following a one hour interval after PBO application, pyroxsulam was applied at predetermined concentrations. All treated plants were then maintained under controlled greenhouse conditions. Plant response to treatments was evaluated 21 days after application by measuring shoot fresh biomass. To verify experimental consistency, the complete trial was repeated under identical growing conditions.

ALS enzymatic activity in different *L. perenne* ssp. *multiflorum* **populations**

ALS enzymatic activity was detected according to the previously described methods (Fang et al. 2022; Liu et al. 2024), with some modifications. *Lolium perenne* ssp. *multiflorum* populations were cultivated in a growth chamber under controlled conditions (20/15 C, 12/12 h, 65% humidity) until they reached the 3-4 leaf stage. 3 g of fresh tissue was collected from each sample. The leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powdered sample was immediately transferred to a pre-chilled beaker, and pre-cooled ALS extraction buffer (0.5 mM $MgCl_2$, 0.5 mM Thiamine pyrophosphate [TPP], 10 μM Flavin adenine dinucleotide [FAD], 10 mM Sodium pyruvate, 1 mM DL-Dithiothreitol [DTT], 1 mM

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Phenyl methane sulfonyl fluoride [PMSF], 0.5% Polyvinyl pyrrolidone [PVP] (m/v), 10% Glycerol (v/v), 0.1 M K_2HPO_4-KH_2PO_4 phosphate buffer with pH = 7.5) was added. The mixture was allowed to stand on ice for 10 min. The plant residue was removed by filtration, and the filtrate was transferred to a pre-chilled 50 mL Beckman centrifuge tube. The sample was centrifuged at 27000 ×g for 15 min at 4 C. A saturated ammonium sulfate solution was slowly added to the supernatant to achieve a final concentration of 50%, and the mixture was gently stirred until protein precipitation was observed. The solution was centrifuged again at 27000 ×g for 12 min at 4 C. The pellet was resuspended in ALS assay buffer and stored at -20 C until further analysis.

The reaction mixture consisted of 100 µL of enzyme extract, 200 µL of assay buffer (containing 100 mM Potassium phosphate buffer [pH 7.5], 200 mM Sodium pyruvate, 20 mM $MgCl_2$, 2 mM TPP, 20 µM FAD, and 1 mM DTT), and 100 µL of pyroxsulam at final concentrations of 0, 0.005, 0.05, 0.5, 5, or 50 µM. The mixture was incubated at 37 C in the dark for 60 minutes to produce acetate, 8 µL 6 N H_2SO_4 was added to terminate the reaction. The mixture was subsequently incubated at 60 C for 30 min to convert acetolactate to acetoin. Subsequently, 100 µL of 0.55% (w/w) creatine solution and 100 µL of 5.5% (v/v) α-naphthol in 5 N NaOH were added. ALS enzyme activity was determined by measuring the absorbance of the acetoin-creatine-naphthol complex at 530 nm. Two independent enzyme extractions were performed, and each herbicide concentration was assayed in triplicate.

ALS gene expression in different populations

ALS gene expression in R and S populations was determined by real-time quantitative PCR (RT-qPCR). As described above, *L. perenne* ssp. *multiflorum* plants were grown until the 3-4 leaf stage, followed by foliar application of pyroxsulam at the recommended field rate (14 g a.i. ha^{-1}). Leaf tissues were harvested at 0 h (without pyroxsulam treatment), 12 h, 24 h, 3 d, and 5 d after treatment, flash-frozen in liquid nitrogen, and stored at -80 C until RNA extraction. Total RNA was isolated using the RNA extraction reagent (Pudi Biotech, Shanghai, China), and its concentration and purity were assessed with a spectrophotometer (ND-100C). Genomic DNA

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was removed, and first-strand cDNA was synthesized from 1000 ng of total RNA using HiScript® II Q RT SuperMix (+gDNA wiper, Vazyme, China).

The *ALS* gene was amplified using specific primers (ALS/F: GCGATCAAGAAGATGCTTGAGAC; ALS/R: TCCTGCCATCACCTTCCATGAT), designed with Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Real-time qPCR was conducted on a QuantStudio 1 system (Thermo Fisher Scientific, USA), with the Ras family GTPase (RGTP) gene as the internal control (Gaines et al. 2014).

Data analysis

Statistical analysis of all dose-response measurements was performed using one-way ANOVA in SPSS version 21 (IBM Corp., Chicago, IL). No significant trial-by-treatment interactions were observed in whole plant bioassay ($P > 0.05$). The dose-response relationships for whole-plant experiments were analyzed using a four-parameter nonlinear log-logistic model (Equation 1) through SigmaPlot v. 15.0 (SigmaPlot Software, Chicago, IL, USA). This analysis enabled the quantification of herbicide concentrations required for 50% growth reduction (GR_{50}), based on fresh weight measurements, as well as the concentrations needed to suppress 50% of ALS enzyme activity (IC_{50}) in the biochemical assays (Fang et al. 2022).

$$y = c + (d - c)/[1 + \left(\frac{x}{g^b}\right)] \quad (1)$$

where y represents fresh weight, expressed as % of the untreated control at herbicide dose x, b is the slope, c is the lower limit, d is the upper limit, and g corresponds to GR_{50} or IC_{50}. The RI was calculated by dividing the GR_{50} (or IC_{50}) of the resistant (R) population by that of the susceptible (S) population (RI > 2 means herbicide resistance.) (Wang et al. 2024). Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001), with three biological replicates per treatment and two independent experimental runs.

Results and Discussion

dCAPS markers for mutation purification in *L. perenne* ssp. *multiflorum*

Two novel dCAPS markers were developed to detect specific mutations in the *ALS* gene (Pro-197-Thr and Pro-197-Gln) (Table 2). The Pro-197-Thr mutation was specifically identified

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using the restriction endonuclease *Mlu*I. In the absence of mutation, the amplified DNA fragment remains intact, resulting in a single band of 211 bp. Homozygous mutations result in two fragments of 181 bp and 30 bp, whereas heterozygous mutations produce three bands corresponding to 211 bp, 181 bp, and 30 bp, as shown in Fig. 2A. The Pro-197-Gln mutation was detected using the restriction enzyme *Bsg*I. Wild-type (WT) alleles produced a single undigested band of 175 bp, whereas mutant alleles yielded a detectable 127 bp fragment and an undetectable 48 bp band. In the case of heterozygous *ALS* loci, three distinct bands of 175 bp, 127 bp, and 48 bp were observed (Fig. 2B).

Currently, dCAPS detection methods for mutations in the target-site resistance genes of ALS-inhibiting herbicides have been developed for many weed species, such as Chinese sprangletop [*Leptochloa chinensis* (L.) Nees] and shortawn foxtail (*Alopecurus aequalis* Sobol.) (Délye and Boucansaud 2008; Giacomini et al. 2017; Jiang et al. 2025; Yin et al. 2024). However, such methods have not yet been fully established for *L. perenne* ssp. *multiflorum*. In this study, for the first time, a dCAPS-based rapid detection method was developed for target-site mutations, the method enables rapid and accurate detection of single-target mutations at position 197 in the *ALS* gene of *L. perenne* ssp. *multiflorum*.

Whole-plant dose-response assays

Sensitivity to pyroxsulam

As shown in Table 4, the Pro-197-Thr population and Pro-197-Gln population isolated from the same R population had significantly higher resistance to pyroxsulam than the S population. At the same time, the GR_{50} (the herbicide concentration required for 50% growth reduction) value of the Pro-197-Thr population was significantly higher than that of the Pro-197-Gln population. The resistance index (RI) of the Pro-197-Thr population was 508.92, while the RI of the Pro-197-Gln population was 9.75. This indicates that the Pro-197-Thr population exhibited a much higher resistance to pyroxsulam, being 51.20 times more resistant than the Pro-197-Gln population.

Mutations in the *ALS* gene confer resistance to weeds, and different mutations can result in

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varying degrees of resistance (Yu et al. 2008), Deng et al. (2017) found that flixweed [*Descurainia sophia* (L.) Webb ex Prantl] populations with Pro-197-Thr and Trp-574-Leu mutations in the *ALS* gene exhibited resistance to SU herbicides, as well as to IMI and PTB herbicides. Massa et al. (2011) reported that silky windgrass [*Apera spica-venti* (L.) P. Beauv.] populations with Trp-574-Leu and Arg-377-His mutations in the *ALS* gene showed higher resistance to SU and TP herbicides compared to populations with Pro-197-Thr and Pro-197-Asn mutations. However, these studies did not clarify which type of mutation confers a higher degree of resistance, because they did not compare the differences in resistance levels caused by different amino acid mutations at the same site. In our study, the same population of *L. perenne* ssp. *multiflorum* was found to have two different amino acid substitutions at position 197, both of which confer resistance to pyroxsulam (Table 4). However, the Pro-197-Thr population was more resistant to pyroxsulam than that of the Pro-197-Gln population. This suggests that the substitution of proline by threonine at site 197 has a greater effect on the resistance level of *L. perenne* ssp. *multiflorum* than that by glutamine.

Sensitivity to other herbicides

We further investigated the resistance levels of different mutant types of *L. perenne* ssp. *multiflorum* populations to herbicides with different modes of action. The results showed that the Pro-197-Thr and Pro-197-Gln populations exhibited varying degrees of resistance to ALS inhibitors (Table 5). The Pro-197-Thr population showed a higher resistance level to mesosulfuron-methyl, with an RI value of 35.0, compared to the Pro-197-Gln population, which had an RI value of 7.9. However, there was little difference in resistance levels between the Pro-197-Thr and Pro-197-Gln populations to herbicides with other modes of action (Table 5). For the ACCase inhibitor fenoxaprop-P-ethyl, the Pro-197-Thr population had an RI value of 4.6, while the Pro-197-Gln population had an RI value of 3.9, both indicating a certain level of resistance, but the difference between the two populations was minimal. For the PSII inhibitor isoproturon and HPPD inhibitor cypyrafluone, both the Pro-197-Thr and Pro-197-Gln populations remained sensitive (Table 5).

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Amino acid substitution at different sites of *ALS* gene can cause weeds to evolve different degrees of resistance, cross-resistance to different herbicides of the same mode of action (Powles and Yu 2010; Yu and Powles 2014). For example, Merriam et al. (2023) found that different amino acid substitution populations at *ALS* gene 197 from different regions have been shown to confer cross-resistance to SU and IMI herbicides in prickly lettuce (*Lactuca serriola* L.). In the cross-resistance experiments of our study, the Pro-197-Thr and Pro-197-Gln populations showed significant resistance to mesosulfuron-methyl, with the Pro-197-Thr population displaying a higher level of resistance (Table 5).

***In vitro* ALS enzymatic activity between *L. perenne* ssp. *multiflorum* populations with different mutations**

Through *in vitro* determination of the sensitivity of ALS enzyme to pyroxsulam, it was found that the Pro-197-Thr and Pro-197-Gln population exhibited significantly lower sensitivity to pyroxsulam compared to the S population (Fig. 3). The Pro-197-Thr population showed significantly lower sensitivity to pyroxsulam than the Pro-197-Gln population. The IC_{50} (the herbicide concentration required for 50% inhibition of enzymatic activity) value for the S population was 7.06 nM, while the IC_{50} values for the Pro-197-Thr and Pro-197-Gln populations were 1172.17 nM and 757.12 nM, respectively (Table 6). The IC_{50} value for the Pro-197-Thr population was 415.05 nM higher than that of the Pro-197-Gln population. This result is consistent with the whole-plant bioassay (Table 4), indicating that the Pro-197-Thr population has lower sensitivity to pyroxsulam.

The change in *in vitro* ALS enzymatic activity is a key factor in the development of herbicide resistance in weeds. Cao et al. (2022) found that the ALS enzyme activity in two resistant populations of *C. album* was 17.1-fold and 19.3-fold higher than that in the sensitive population. Sun et al. (2024) demonstrated that the IC_{50} value of ALS enzyme activity in resistant *E. crus-galli* population was 4.12 times higher than that in the sensitive population, which is the reason for the resistance of these populations to penoxsulam. Our study yielded analogous results, the heightened resistance level of the Pro-197-Thr population to pyroxsulam,

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compared to the Pro-197-Gln population, may be attributed to a reduced sensitivity of the ALS enzyme to pyroxsulam.

ALS gene expression in populations with different mutations

The expression levels of the *ALS* gene in different populations were monitored at four time points after treatment with pyroxsulam (the untreated samples of 0 h were used to normalize gene expression) (Fig. 4). The results indicated that the expression levels of *ALS* gene in the S population or the Pro-197-Gln population did not show significant changes after herbicide treatment, and no significant differences were observed among the different time points. However, the expression of the *ALS* gene in the Pro-197-Thr population was upregulated following pyroxsulam treatment, peaking at 5 days and showing significantly higher levels compared to the Pro-197-Gln subpopulation, which was 2.68 times that of the Pro-197-Gln subpopulation. Under pyroxsulam treatment, the *ALS* gene expression level in the Pro-197-Thr population was higher than that in the S population and the Pro-197-Gln population. However, there was no significant difference in *ALS* gene expression between the Pro-197-Gln population and the S population (Fig 4). The increased expression of target genes may also contribute to herbicide resistance (Gaines et al. 2010; Singh et al. 2018). In this study, the *ALS* gene exhibited higher expression levels in the Pro-197-Thr population compared to the Pro-197-Gln population following herbicide treatment. This upregulation could lead to increased accumulation of ALS enzyme, potentially influencing resistance levels (Tanigaki et al. 2021). This mechanism aligns with previous findings, suggesting that elevated target gene expression is a common strategy for herbicide resistance in weeds. For instance, Yu et al. (2020) found that in annual sedge (*Cyperus compressus* L.), the expression levels of the *ALS* gene in two resistant populations were 5.2 times and 3.6 times higher, respectively, compared to the susceptible population. Elevated gene expression may therefore be an important factor in enhancing herbicide tolerance.

Sensitivity of populations to pyroxsulam upon treatment with PBO

The RI value of the S population to pyroxsulam did not change significantly after the application of cytochrome P450 inhibitor PBO (Table 4). However, the RI value of the R

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population showed significant changes, the RI value of the Pro-197-Thr population decreased from 508.92 to 159.84, indicating that the resistance to pyroxsulam remained high. The RI decrease (~3.2-fold in Thr and ~4.7-fold in Gln) suggests a P450-based resistance in both populations. However, the Pro-197-Thr population still has stronger resistance after PBO treatment. Therefore, the mutation of the *ALS* gene at position 197 in *L. perenne* ssp. *multiflorum*, where proline is replaced by threonine, confers a higher resistance to pyroxsulam compared to the mutation where proline is replaced by glutamine. At the same time, it also indicates that the resistance mechanism of *L. perenne* ssp. *multiflorum* to pyroxsulam is not only the TSR but also the NTSR.

In summary, we developed a new rapid detection method that can efficiently and rapidly detect the 197 site mutations (Pro-197-Thr and Pro-197-Gln) in the *ALS* gene of *L. perenne* ssp. *multiflorum*, and acquired the homozygous populations with Pro-197-Thr and Pro-197-Gln mutations from one resistant population. Through the whole-plant bioassays, we found that the Pro-197-Thr population had a higher degree of resistance to ALS-inhibiting herbicides pyroxsulam and mesosulfuron-methyl than the Pro-197-Gln population, indicating that the resistance levels caused by the different mutations in the same target site was quite different. The reason for this difference may result from the differences of ALS enzyme activity and/or *ALS* gene expression. Particularly, when PBO and pyroxsulam were applied, the resistance level of the resistant population to pyroxsulam significantly decreased. This suggests that PBO may reduce weed resistance to herbicides by inhibiting P450 enzyme activity, offering a novel strategy for managing herbicide resistance in weeds.

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Competing interests

The authors declare no conflicts of interest.

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References

Ban Y, Zhang Z, Wei J, Xu G, Ma Y, Yin S, Dong L, Feng Z (2025) Establishment of a CRISPR-based system for rapidly detecting the target-site resistance of American sloughgrass (*Beckmannia syzigachne*) to pinoxaden. Pest Manag Sci 81:5649-5658

Bobadilla LK, Hulting AG, Berry PA, Moretti ML, Mallory-Smith C (2021) Frequency, distribution, and ploidy diversity of herbicide-resistant Italian ryegrass (*Lolium perenne* spp. *multiflorum*) populations of western Oregon. Weed Sci 69:177-185

Cao Y, Zhou X, Wei S, Huang H, Lan Y, Li W, Sun Y, Huang Z (2022) Multiple resistance to ALS-inhibiting and PPO-inhibiting herbicides in *Chenopodium album* L. from China. Pestic Biochem Physiol 186:105155

Chtourou M, Osuna MD, Vázquez-García JG, Lozano-Juste J, Prado RD, Torra J, Souissi T (2024) Pro197Ser and the new Trp574Leu mutations together with enhanced metabolism contribute to cross-resistance to ALS inhibiting herbicides in *Sinapis alba*. Pestic Biochem Physiol 201:105882

Délye C, Boucansaud K (2008) A molecular assay for the proactive detection of target site-based resistance to herbicides inhibiting acetolactate synthase in *Alopecurus myosuroides*. Weed Res 48:97-101

Délye C, Jasieniuk M, Corre V (2013) Deciphering the evolution of herbicide resistance in weeds. Trends Genet 29:649-658

Deng W, Yang Q, Zhang Y, Jiao H, Mei Y, Li X, Zheng M (2017) Cross-resistance patterns to acetolactate synthase (ALS)-inhibiting herbicides of flixweed (*Descurainia sophia* L.) conferred by different combinations of ALS isozymes with a Pro-197-Thr mutation or a novel Trp-574-Leu mutation. Pestic Biochem Physiol 136:41-45

Dimou A, Tsimihodimos V, Bairaktari E (2022) The critical role of the branched chain amino acids (BCAAs) catabolism-regulating enzymes, branched-chain aminotransferase (BCAT) and branched-chain α-keto acid dehydrogenase (BCKD), in human pathophysiology. Int J Mol Sci 23:4022

https://doi.org/10.1017/wsc.2025.10054 Published online by Cambridge University Press

Dongo A, Leflon M, Simier P, Delavault P (2012) Development of a high-throughput real-time quantitative PCR method to detect and quantify contaminating seeds of *Phelipanche ramosa* and *Orobanche cumana* in crop seed lots. Weed Res 52:34-41

Fang J, Yang D, Zhao Z, Chen J, Dong L (2022) A novel Phe-206-Leu mutation in acetolactate synthase confers resistance to penoxsulam in barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv). Pest Manag Sci 78:2560-2570

Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL, Westra P (2010) Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. Proc Natl Acad Sci USA 107:1029-1034

Gaines TA, Lorentz L, Figge A, Herrmann J, Maiwald F, Ott M, Han H, Busi R, Yu Q, Powles SB, Beffa R (2014) RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. Plant J 78:865-876

Giacomini DA, Umphres AM, Nie H, Mueller TC, Steckel LE, Young BG, Scott RC, Tranel PJ (2017) Two new PPX2 mutations associated with resistance to PPO-inhibiting herbicides in *Amaranthus palmeri*. Pest Manag Sci 73:1559-1563

Huang J, Long L, Mao X, Wang J, Liu H, Li C, Jing R (2020) dCAPS markers developed for nitrate transporter genes TaNRT2L12s associating with 1 000-grain weight in wheat. J Integr Agr 19:1543-1553

Jiang M, Chen X, He T, Hu W, Zhao N, Liao M, Cao H (2025) A novel ACCase Trp-1999-Gly mutation confers resistance to ACCase-inhibiting herbicides in Chinese sprangletop (*Leptochloa chinensis*). Plant Physiol Biochem 221:109596

Kadaru S, Zhang W, Yadav A, Oard JH (2008) Development and application of allele-specific PCR assays for imazethapyr resistance in rice (*Oryza sativa*). Euphytica 160:431-438

Kaya AE, Erken MS, Zandstra BH, Menna H (2022) Target-site point mutation conferring resistance to ALS herbicides in Italian ryegrass (*Lolium multiflorum* L.). Phytoparasitica 50:1133-1142

Liu Y, Shi K, Gao H, Yin S, Dong L, Feng Z (2024) A Trp-574-Leu mutation in acetolactate

https://doi.org/10.1017/wsc.2025.10054 Published online by Cambridge University Press

synthase confers imazamox resistance in barnyardgrass (*Echinochloa crus-galli*) from China. Weed Sci 72:683-692

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402-408

Massa D, Krenz B, Gerhards R (2011) Target-site resistance to ALS-inhibiting herbicides in *Apera spica-venti* populations is conferred by documented and previously unknown mutations. Weed Res 51: 294-303

Menchari Y, Camilleri C, Michel S, Brunel D, Dessaint F, Le Corre V, Délye C (2006) Weed response to herbicides: regional-scale distribution of herbicide resistance alleles in the grass weed *Alopecurus myosuroides*. New Phytol 171:861-873

Merriam AB, Malone JM, Hereward JP, Gill G, Preston C (2023) Point mutations including a novel Pro-197-Phe mutation confer cross-resistance to acetolactate synthase (ALS) inhibiting herbicides in *Lactuca serriola* in Australia. Pest Manag Sci 79:5333-5340

Pan Y, Wang Z, Zhao S, Wang X, Li Y, Liu J, Wang S, Xi J (2022) The herbivore-induced plant volatile tetradecane enhances plant resistance to *Holotrichia parallela* larvae in maize roots. Pest Manag Sci 78:550-560

Panozzo S, Scarabel L, Tranel PJ, Sattin M (2013) Target-site resistance to ALS inhibitors in the polyploid species *Echinochloa crus-galli*. Pestic Biochem Physiol 105:93-101

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317-347

Singh S, Singh V, Lawton-Rauh A, Bagavathiannan MV, Roma-Burgos N (2018) EPSPS gene amplification primarily confers glyphosate resistance among Arkansas Palmer amaranth (*Amaranthus palmeri*) populations. Weed Sci 66:293-300

Sun P, Niu L, He P, Yu H, Chen J, Cui H, Li X (2024) Trp-574-Leu and the novel Pro-197-His/Leu mutations contribute to penoxsulam resistance in *Echinochloa crus-galli* (L.) P. Beauv. Front Plant Sci 15:1488976

Tanigaki S, Uchino A, Okawa S, Miura C, Hamamura K, Matsuo M, Yoshino N, Ueno N,

https://doi.org/10.1017/wsc.2025.10054 Published online by Cambridge University Press

Toyama Y, Fukumi N, Kijima E, Masuda T, Shimono Y, Tominaga T, Iwakami S (2021) Gene expression shapes the patterns of parallel evolution of herbicide resistance in the agricultural weed *Monochoria vaginalis*. New Phytol 232:928-940

Tehranchian P, Nandula VK, Matzrafi M, Jasieniuk M (2019) Multiple herbicide resistance in California Italian ryegrass (*Lolium perenne* ssp. *multiflorum*): characterization of ALS-inhibiting herbicide resistance. Weed Sci 67:273-280

Turra GM, Cutti L, Machado, FM, Dias GM, Andres A, Markus C, Merotto JA (2023) Application of ALS inhibitors at pre-emergence is effective in controlling resistant barnyardgrass biotypes depending on the mechanism of resistance. Crop Prot 172:106325

Wang H, Fang J, Li X, Sun P, Gao H, Ren Y, Liu Y, Feng Z, Dong L (2024) Epigenetic regulation of CYP72A385-mediated metabolic resistance to novel auxin herbicide florpyrauxifen-benzyl in *Echinochloa crus-galli* (L.) P. Beauv. J Agric Food Chem 72:9199-9214

Wang J, Qi J, Ouyang Y, Zhou S, Qin L, Zhang B, Bai L, Pan L (2024) The mutation Asp-376-Glu in the ALS gene confers resistance to mesosulfuron-methyl in *Beckmannia syzigachne*. Plant Physiol Biochem 215:109083

Wang Y, Xu Q, Chang X, Hao C, Li R, Jing R (2018) A dCAPS marker developed from a stress associated protein gene TaSAP7-B governing grain size and plant height in wheat. J Integr Agr 17:276-284

Wu C, Song M, Zhang T, Zhou C, Liu W, Jin T, Zhao N (2022) Target-site mutation and cytochrome P450s confer resistance to multiple herbicides in Italian ryegrass (*Lolium multiflorum* Lam.) from China. Crop Prot 161:106068

Xu H, Pan X, Wang C, Chen Y, Chen K, Zhu S, Klinken RD (2020) Species identification, phylogenetic analysis and detection of herbicide-resistant biotypes of Amaranthus based on ALS and ITS. Sci Rep 10:11735

Yin F, Wang M, Liao M, Cao H, Zhao N (2024) 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. Weed Sci 72:693-702

https://doi.org/10.1017/wsc.2025.10054 Published online by Cambridge University Press

Yu J, McCullough PE, McElroy JS, Jespersen D, Shilling DG (2020) Gene expression and target-site mutations are associated with resistance to ALS inhibitors in annual sedge (*Cyperus compressus*) biotypes from Georgia. Weed Sci 68:460-466

Yu Q, Han H, Powles SB (2008) Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. Pest Manag Sci 64:1229-1236

Yu Q, Powles SB (2014) Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag Sci 70:1340-1350

Zhang Z, Ban Y, Wei J, Wu Q, Dong L, Feng Z (2025) Rapid resistance detection of *Amaranthus retroflexus* to fomesafen via Kompetitive allele-specific PCR (KASP). Plants 14:515

Zhao J, Meng Z, Ma X, Zhao S, An Y, Xiao Z (2021) Characterization and regulation of the acetolactate synthase genes involved in acetoin biosynthesis in *Acetobacter pasteurianus*. Foods 10:1013

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 G, Wang H, Gao H, Liu Y, Li J, Feng Z, Dong L (2023) Multiple resistance to three modes of action of herbicides in a single Italian ryegrass (*Lolium multiflorum* L.) population in China. Agronomy 13:216

https://doi.org/10.1017/wsc.2025.10054 Published online by Cambridge University Press

Figure Legends

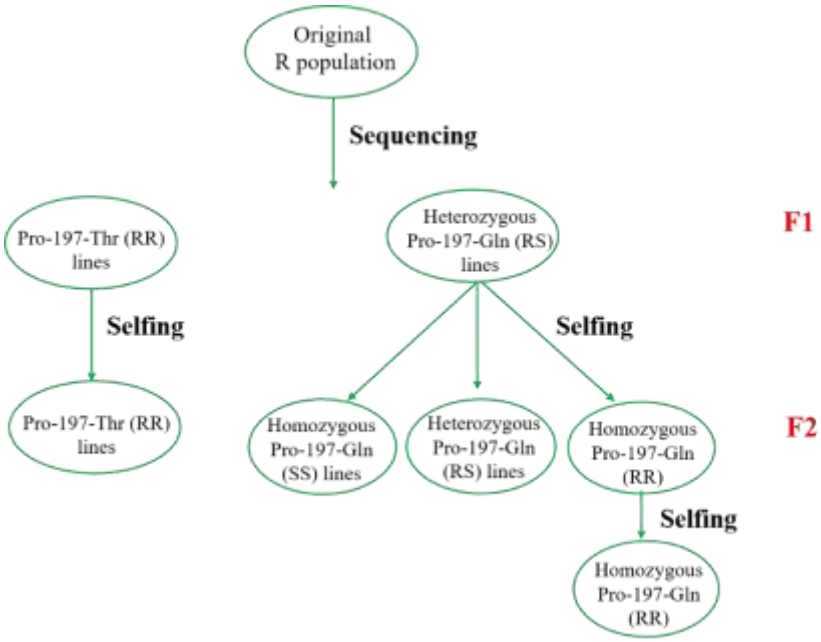

Figure 1. Separation and purification of different resistant populations.

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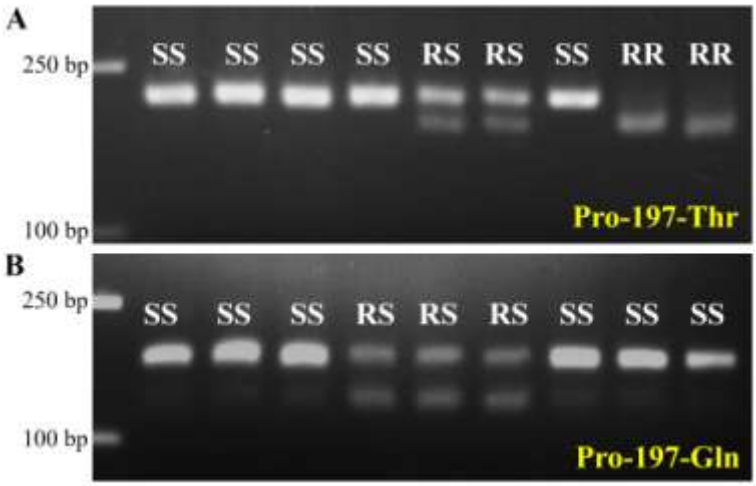

Figure 2. dCAPS markers for detecting the ALS mutations at position 197. (A) *Mlu*I digestion pattern of the dCAPS marker for the Pro-197-Thr mutation. (B) *Bsg*I digestion pattern of the dCAPS marker for the Pro-197-Gln mutation.

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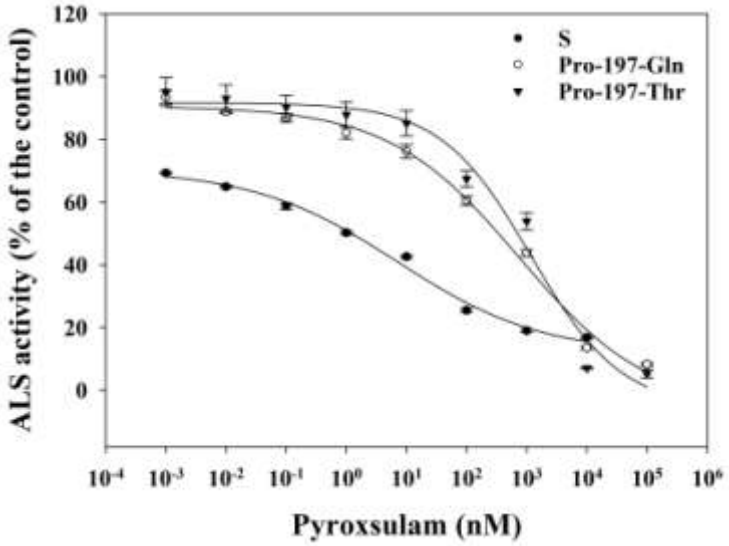

Figure 3. Inhibition of *in vitro* ALS enzyme activities of different *L. perenne* ssp. *multiflorum* populations upon treatment with pyroxsulam.

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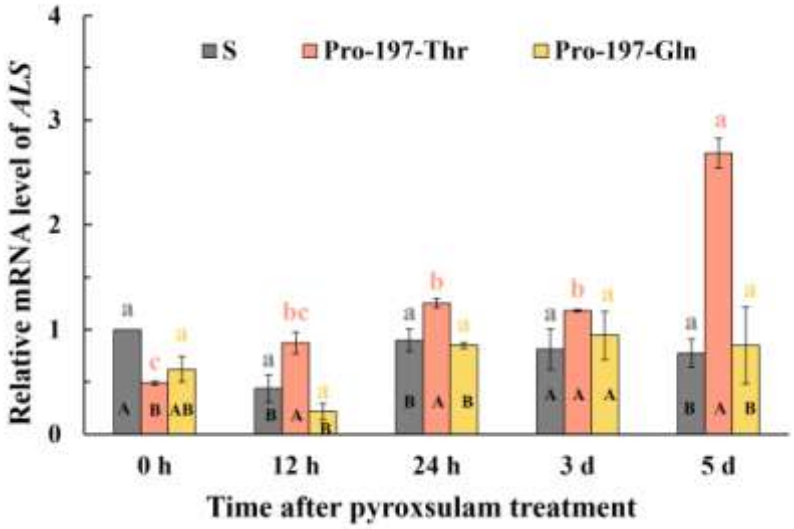

Figure 4. Relative mRNA level of *ALS* gene in different *L. perenne* ssp. *multiflorum* populations with or without pyroxsulam treatment. Lowercase letters indicate comparisons between different time points within the same population, while uppercase letters denote comparisons between different populations at the same time point. ANOVA significance is shown with different letters, $P < 0.05$. Data is derived from at least three biological replicates.

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Table 1. Restriction enzymes for dCAPS assay.

Target-site mutation	Restriction enzyme	Cutting site	Product size	
			Wild	Mutant
P197	*Apa*I	GGGCC^C	30, 145	175
P197T	*Mlu*I	A^CGCGT	211	30, 181
P197Q	*Bsg*I	GTGCAG(N)16^	175	48, 127

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Table 2. dCAPS primers.

Primer	Sequence (5'-3') [a]	Annealing temperature	Restriction enzyme	Target-site mutation
197-F	TCCCCATGGTGGCCATCACGGGGCAGGC	68.2	*Apa*I	P197
197-R	CCAGAGGAGGCGAGGAAGAAGG			
P197T-F	ACCAACCACCTCTTCCGCCACG	66.4	*Mlu*I	P197T
P197T-R	GGAAGGCGTCCGTGCCGATCATGCGACGCG			
P197Q-F	TCCCCATGGTGGCCATCACGGGGCAGGTGC	68.4	*Bsg*I	P197Q
P197Q-R	CCAGAGGAGGCGAGGAAGAA			

[a]The introduced mismatched bases are underlined.

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Table 3. Herbicide dosage for cross resistance and multiple resistance.

Herbicide	Field dose	Dose setting	
		S	R
	g ai/ha	g ai/ha	g ai/ha
Mesosulfuron-methyl	13.5	1.69, 3.38, 6.75, 13.5, 27	27, 54, 108, 216, 432
Fenoxaprop-P-ethyl	90	90, 180, 360, 720, 1440	360, 720, 1440, 2880, 5760
Isoproturon	937.5	14.65, 29.30, 58.59, 117.19, 234.38	117.19, 234.38, 468.75, 937.5, 1875
Cypyrafluone	157.5	39.38, 78.75, 157.5, 315, 630	157.5, 315, 630, 1260, 2520, 5040

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Table 4. Sensitivities of different *L. perenne* ssp. *multiflorum* **populations to pyroxsulam with/without P450 metabolic inhibitors.**

Treatment	S		Pro-197-Thr			Pro-197-Gln		
	GR_{50}[a] (±SE)	RI[b]	GR_{50}[a] (±SE)	RI[b]	ri[c]	GR_{50}[a] (±SE)	RI[b]	ri[c]
	g ai/ha		g ai/ha			g ai/ha		
Pyroxsulam	0.19±0.06 a	1	96.64±0.14 a	508.92		1.85±0.03 a	9.75	
Pyroxsulam+PBO[d]	0.12±0.07 a	0.83	30.35±0.51 b	159.84	192.58	0.39±0.02 b	2.06	3.25

[a] GR_{50} is the effective dose of herbicide causing 50% inhibition of fresh weight and is expressed as grams of active ingredient per hectare (g a.i. ha^{-1}). Different letter in the column indicates significant differences ($P < 0.05$).

[b] RI is the relative tolerance index, ratio of GR_{50} values relative to the susceptible *L. perenne* ssp. *multiflorum* population (S)+PBO at the same treatment.

[c] ri is the relative tolerance index, ratio of R+PBO populations GR_{50} values relative to the S+PBO treatment at the same treatment.

[d] PBO: piperonyl butoxide; 4200 g a.i. ha^{-1}, applied 1 h before herbicide application.

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Table 5. Sensitivities of different *L. perenne* ssp. *multiflorum* populations to herbicides.

Population	Mesosulfuron-methyl (ALS)		Fenoxaprop-P-ethyl (ACCase)		Isoproturon (PSII)		Cypyrafluone (HPPD)	
	GR_{50}[a] (±SE)	RI[b]	GR_{50}[a] (±SE)	RI[b]	GR_{50}[a] (±SE)	RI[b]	GR_{50}[a] (±SE)	RI[b]
	g ai/ha		g ai/ha		g ai/ha		g ai/ha	
S	2.7±0.3 c	1.0	2849.5±307.9 b	1.0	174.7±54.5 c	1.0	616.0±27.3 c	1.0
Pro-197-Thr	95.5±7.9 a	35.0	13075.7±773.8 a	4.6	263.4±21.3 a	1.5	1144.5±85.1 a	1.9
Pro-197-Gln	21.5±1.1 b	7.9	11115.2±902.2 a	3.9	232.0±12.4 b	1.3	770.7±47.1 b	1.3

[a] GR_{50} is the effective dose of herbicide causing 50% inhibition of fresh weight and is expressed as grams of active ingredient per hectare (g a.i. ha^{-1}). Different letter in the column indicates significant differences ($P < 0.05$).

[b] RI is the relative tolerance index, ratio of GR_{50} values relative to the susceptible *L. perenne* ssp. *multiflorum* population (S) at the same treatment.

Abbreviations: ALS, Acetolactate synthase; ACCase: Acetyl-coA carboxylase; PSII: Photosystem II complex; HPPD: 4-Hydroxyphenylpyruvate dioxygenase.

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Table 6. *In vitro* ALS enzymatic activities of different *L. perenne* ssp. *multiflorum* populations upon treatment with pyroxsulam.

Population	IC_{50}[a] (±SE)
	nM
S	7.06±0.71 c
Pro-197-Gln	757.12±1.42 b
Pro-197-Thr	1172.17±1.44 a

[a]IC_{50} is the effective dose of herbicide causing 50% inhibition of ALS enzymatic activity.

Different letters indicate significant differences ($P < 0.05$).

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