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Primary metabolism in an *Amaranthus palmeri* population with multiple resistance to glyphosate and pyrithiobac herbicides

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ABSTRACT

The objective of this work was to characterize the resistance mechanisms and the primary metabolism of a multiple resistant (MR) population of *Amaranthus palmeri* to glyphosate and to the acetolactate synthase (ALS) inhibitor pyrithiobac. All MR plants analysed were glyphosate-resistant due to 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene amplification. Resistance to pyrithiobac was more variable among individuals and was related to point mutations at five positions in the *ALS* gene sequence: A122, A205, W574, S653 and G654. All MR plants were heterozygous for W574, the most abundant mutation. In nontreated plants, the presence of mutations did not affect ALS functionality, and plants with the W574L mutation showed the highest ALS resistance level to pyrithiobac. The accumulation of the transcripts corresponding to several genes of the aromatic amino acid (AAA) and branched-chain amino acid (BCAA) pathways detected in nontreated MR plants indicated additional effects of *EPSPS* gene amplification and *ALS* mutations. The physiological performance of the MR population after treatment with glyphosate and/or pyrithiobac was compared with that of a sensitive (S) population. The increase induced in total soluble sugars, AAA or BCAA content by both herbicides was higher in the S population than in the MR population. Physiological effects were not exacerbated after the mixture of both herbicides in S or in MR populations. This study provides new insights into the physiology of a multiple resistant *A. palmeri*, which could be very useful for achieving effective management of this weed.

1. Introduction

Two enzymes of amino acid biosynthesis are important targets for herbicide action in different biosynthetic pathways: 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) in the aromatic amino acid (AAA) biosynthetic pathway and acetolactate synthase (ALS, EC 2.2.1.6; also termed acetohydroxyacid synthase) in the branched-chain amino acid (BCAA) biosynthetic pathway [1] (Suppl. Fig. S1).

EPSPS is a key enzyme in the biosynthesis of AAAs (tyrosine, phenylalanine and tryptophan) and is the target of the herbicide glyphosate [2]. Glyphosate is a once-in-a century herbicide; it was developed in the early 1970s and is currently the most widely used herbicide in the world [3]. It is a wide-spectrum, nonselective post-emergence herbicide that is widely used in nonagricultural areas, but biotechnology and the use of glyphosate-tolerant crops (maize, soybean, cotton and canola) have exacerbated the importance of glyphosate in weed management [4] (Suppl. Fig. S1).

ALS is the first common enzyme in the biosynthesis of BCAAs (valine, leucine and isoleucine) (Suppl. Fig. S1). ALS-inhibiting herbicides were commercialized in 1982, and this was a milestone in weed control history [5]. They have become one of the most important herbicide groups because of their wide-spectrum weed-control activity, high crop selectivity, low application rates and low mammalian toxicity [6]. Several chemical families have ALS as their site of action, including sulfonylureas, imidazolinones and pyrimidinyl benzoates. Pyrithiobac is one of these active ingredients that belongs to the pyrimidinyl-benzoate chemical family.

Both EPSPS and ALS activities are well-established herbicide target sites, but it is not fully understood how plants actually die after their inhibition, and the sequence of events from herbicide application to plant death is still being debated. Previous findings showed common physiological effects in response to both types of herbicides [7,8], which suggests that although they target different enzymes located in different pathways, both types of herbicides kill plants by similar mechanisms. Both types of herbicides provoke similar effects on the primary metabolism of the treated plants, such as carbohydrate and amino acid accumulation [9–11]. Additionally, the effects of glyphosate on the gene expression of the shikimate pathway have been reported [12,13].

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Amaranthus palmeri is a dioecious species belonging to the family Amaranthaceae and one of the most competitive troublesome and economically damaging weeds due to its high genetic diversity, high seed production and high vigour [14]. Management of *A. palmeri* is possible using several active ingredients; however, repeated and intensive use of herbicides resulted in the evolution of resistance to multiple herbicides with various mechanisms of action, such as glyphosate and ALS inhibitors [15].

Mechanisms of resistance to herbicides can be 1) target site modifications (target site resistance, TSR) involving amino acid substitution or the increased expression of the target gene and 2) processes external to the target site that result in a reduced amount of the herbicide reaching the site of action or protection from phytotoxic effects, which are collectively known as nontarget site resistance (NTSR) [16–18]. Although NTSR mechanisms to glyphosate have been described in *A. palmeri* [19], the most common resistance mechanism is target site amplification of the *EPSPS* gene [20,21]. When this gene is overexpressed, the EPSPS enzyme accumulates so that the recommended field dose of glyphosate is not sufficient to kill the plant. Concerning ALS, the most commonly reported mechanism conferring resistance to ALS inhibitors in weed species, including *A. palmeri*, is the TSR mechanism of point mutations in the *ALS* gene [22,23]. To date, amino acid substitutions endowing ALS resistance at eight positions (Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654) of the *ALS* gene have been identified in weed species [24].

Multiple herbicide-resistant weeds are of greater concern today, as they are an increasing problem that reduces options for herbicide rotation and increases weed control costs. Evolution of resistance to multiple herbicides in a single population is also widespread in *A. palmeri*, with resistance to glyphosate and ALS inhibitors being the most common multiple resistance [15,25]. A multiresistant (MR) *A. palmeri* population to glyphosate and pyrithiobac was first documented in Arizona in 2012 [15], and the mechanisms of resistance remain unknown. The availability of a population with multiple resistance to glyphosate and ALS inhibitors provides an opportunity to analyse how resistance mechanisms affect physiological factors by comparison with a sensitive population.

The objectives of this study were to unravel the resistance mechanisms of an MR *A. palmeri* population to glyphosate and ALS inhibitors and to monitor the primary metabolism and the expression of the targeted amino acid pathways. First, to analyse resistance mechanisms at molecular level, the gene copy number of *ALS* and *EPSPS* and the *ALS* gene sequence were analyzed. Second, the effects of glyphosate and/or pyrithiobac on known physiological markers of these herbicides (shikimate content, amino acid content, carbohydrate content and expression level of the amino acid biosynthetic pathways) were compared between the MR and sensitive populations.

2. Material and methods

2.1. Plant material and treatment application

The seeds of the *Amaranthus palmeri* biotypes were kindly provided by Dr. Gaines (Colorado State University, Fort Collins, CO, USA). Seeds of the MR population to glyphosate and pyrithiobac were originally collected from Arizona. To minimize plant variability, all experiments were performed with seeds obtained from a single female plant that previously survived consecutive treatments of glyphosate and pyrithiobac. Seeds of sensitive (S) standard [26] were originally collected from North Carolina and kept under cold storage (4 °C) until use.

Germination and plant growth were performed according to procedures described earlier [26]. After germination, the seeds were briefly transferred to aerated 2.7 L hydroponic tanks in a phytotron (day/night, 16 h/8 h; light intensity, 500 μmol s^{-1} m^{-2} PAR; temperature, 22/18 °C; relative humidity of the air, 60/70%). Plants remained in the vegetative phenological stage throughout the course of the experiment.

All treatments were applied to three-week-old plants after selecting individuals of similar size and vigour. Glyphosate (commercial formula, FORTIN Green, Key, Lleida, Spain) was applied at the recommended field rate (0.84 kg ha^{-1}) [27]. The ALS inhibitor pyrithiobac was applied at 89 g ha^{-1} (recommended field rate) [28]. Analytical grade pyrithiobac (Dr. Ehrenstorfer LGC, Augsburg, Germany) was applied to leaves containing 5.4% sodium lauryl sulfate adjuvant (commercial formula Biopower 27.65% (p/v)) (Bayer Crop Science, Madrid, Spain). Treatments were performed using an aerograph (Junior Start model; Definik; Sagola, Vitoria-Gasteiz, Spain) connected to a compressor (Werther one, Breverrato) with the following settings: 60 W; 10 L m^{-1}; 2.5 bar at a rate of 500 L ha^{-1}. Plants of the combined treatment were sprayed first with glyphosate and then with pyrithiobac. Control plants were only sprayed with adjuvant.

Leaves of both populations of *A. palmeri* were collected 3 days after treatment and ground to a fine powder under liquid N_2 using a mixer mill as previously described [26]. The experiment was repeated twice.

2.2. DNA analysis

2.2.1. DNA extraction

Genomic DNA was extracted from approximately 0.1 g of previously ground *A. palmeri* leaves, as described previously [26]. DNA was quantified and analysed using a Synergy HT microplate reader (Biotek Instruments, Vermont, USA). The DNA quality was checked using 1% agarose gel electrophoresis. Extracted gDNA was used to measure genomic *EPSPS* and *ALS* copy number, for *ALS* gene sequencing and to perform the dCAPS assay for resistant mutations in several ALS positions.

2.2.2. EPSPS and ALS gene copy number

Quantitative real-time PCR (qRT–PCR) was performed to determine the genomic copy number of *EPSPS* and *ALS* relative to the endogenous single-copy control gene *CPS*, which encodes the large subunit of carbamoylphosphate synthetase (EC 6.3.5.5) [29]. *EPSPS* and *ALS* primers were developed by [20], and *CPS* primers were obtained from [29]. Reactions were performed using a Bio–Rad CFX connected to a thermal cycler (Bio–Rad Inc., Hercules, CA, USA) and the following amplification profile: 50 °C for 2 min, 95 °C for 1 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min, and a melting curve program ranging from 52 °C to 95 °C with a heating rate of 0.1 °C per second. PCRs were set up with 10 ng gDNA in 20 μL of SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan) following the manufacturer's instructions. The cycle was recorded for each sample at which the fluorescence reading crossed a threshold (C_t), indicating an exponential increase, and the relative *EPSPS* or *ALS* gene copy number was calculated using the comparative C_t method as $2^{\Delta Ct}$ ($\Delta C_t = C_t^{EPSPS/ALS} - C_t^{CPS}$) [30]. Controls containing water were included to check for contamination in the qPCR reactions. Standard curves were generated for each primer pair to confirm appropriate efficiency.

2.2.3. ALS gene sequencing

All mutations conferring resistance to ALS inhibitors in *A. palmeri* at positions Ala122, Pro197, Trp574 and Ser653 of the CAD and BE domains [31] were analysed in the gDNA of all plants of the MR population and in some plants of the S population to serve as nonmutated wild-type plants. Amplifications were performed as described in [32]. Sequencing of the purified amplified DNA fragments was carried out by STAB (Service of Applied Techniques to Bioscience) at the University of Extremadura (Spain).

2.2.4. dCAPS assay for the resistant mutation

Derived cleaved amplified polymorphic sequence (dCAPS) analyses were conducted to determine the homozygosity or heterozygosity of the mutant ALS alleles when simple or double peaks were detected in the sequence chromatograms. In the dCAPS assay, a restriction enzyme

recognition site was created using PCR in a sequence where none exists. The technique allows nucleotide polymorphism detection on the basis of the gain (amplicon digested) or loss (amplicon undigested) of this site. The *A. palmeri* ALS sequence deposited in GenBank (accession number KT833339) was used to design dCAPS primers targeting codons Ala-122, Trp-574 and Ser-653 (Fig. 1) using dCAPS Finder 2.0 [33]. For primer selection, the rules previously described for dCAPS primer design by [34] were applied. Once the dCAPS primer was selected, the corresponding forward or reverse primer pair needed for the amplification of the desired region was designed using Primer 3 Plus software.

PCR was performed in a total volume of 50 μL containing 1 μg gDNA, 0.5 μM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl, 1.25 U Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 1x Taq buffer with KCl (Thermo Fisher Scientific, Waltham, MA, USA). The amplifications were performed as follows: 3 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60–74 °C, 1 min at 72 °C and a final step of 5 min at 72 °C. All primers and annealing temperatures are listed in Table 1. The reactions were performed on a Mastercycler Pro Thermocycler (Eppendorf, AG, Hamburg, Germany).

Subsequently, 5 μg of PCR product was digested with 1 μL of the corresponding FastDigest restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA) (Table 1) in a total volume of 30 μL according to the manufacturer's recommendations. Bands were analysed on a 3% agarose gel stained with SYBRSafe DNA gel stain (Thermo Fisher Scientific, Waltham, MA, USA) that ran for 1 h 15 min at 130 mA with 1× TAE buffer. The resulting electrophoresis gels were visualized under UV light in a GelDoc 2000 (Bio–Rad Laboratories Inc., Hercules, CA, USA). The digestion profile for each individual was compared with its respective, nondigested control profile, as well as the sensitive-control digestion profile.

2.3. In vitro ALS activity assays

ALS extraction and assays were performed according to [35], with modifications. Frozen leaf tissue was homogenized with a mortar and pestle in 10 volumes of grinding buffer containing potassium phosphate buffer (100 mM; pH 7.5), 5 mM sodium pyruvate, 5 mM MgCl, 5 mM EDTA and 10% glycerol (v/v). One hundred μM flavin adenine dinucleotide (FAD), 1 mM thiamine pyrophosphate (TPP) and 2% polyvinyl-polypyrrolidone were freshly added. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 20 min. The supernatant was fractionated by ammonium sulfate (25–50%) and allowed to stand with stirring for 30 min on ice. It was then centrifuged at 15,000*g* for 20 min, and the supernatant was discarded. The precipitate was resuspended in the same extraction buffer without sodium pyruvate and then desalted on a Sephadex G-25 column (PD 10 columns, GE Healthcare, Buckinghamshire, UK) equilibrated with the same buffer. The extract was used immediately for assays.

More specifically, ALS activity was measured by the estimation of the produced acetolactate after conversion by decarboxylation in the presence of acid to acetoin. Extract aliquots were added to the reaction mixture in a total volume of 0.3 mL, consisting of potassium phosphate buffer (50 mM; pH 7.0), 100 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM TPP and 10 μM FAD. Pyrithiobac concentrations used for enzyme activity testing were 0, 0.01, 0.1, 1, 10, 50 and 100 μM. Technical pyrithiobac was used (Dr. Ehrenstorfer LGC, Augsburg, Germany). The reaction mixture was incubated at 37 °C for 1 h. The reaction was stopped by adding 305 μL of H_2SO_4 (6 N), and the mixture was heated (60 °C for 15 min) to facilitate the decarboxylation of acetolactate to acetoin. Acetoin was detected as a colored complex (A525 nm) formed after the addition of 0.3 mL of creatine (6 g L^{-1}, freshly prepared in water) and 0.3 mL of α-naphthol (60 g L^{-1}, freshly prepared in 2.5 M of NaOH) and incubated (60 °C for 15 min). Background was determined using control wells in which the reaction was stopped before the incubation and subtracted. Appropriate checks for acetoin not derived from acetolactate were made by stopping the assay with 4 M sodium hydroxide. This was estimated in all assays and subtracted from the overall acetoin production to give actual ALS activities. Protein concentration was determined according to [36]. All spectrophotometric determinations were performed in a Synergy HT microplate reader (Biotek Instruments, Vermont, USA).

Three replicates per herbicide dose and individual were used. The activity values were expressed as the percentage of control values in which the reaction took place in the absence of herbicide. Enzyme

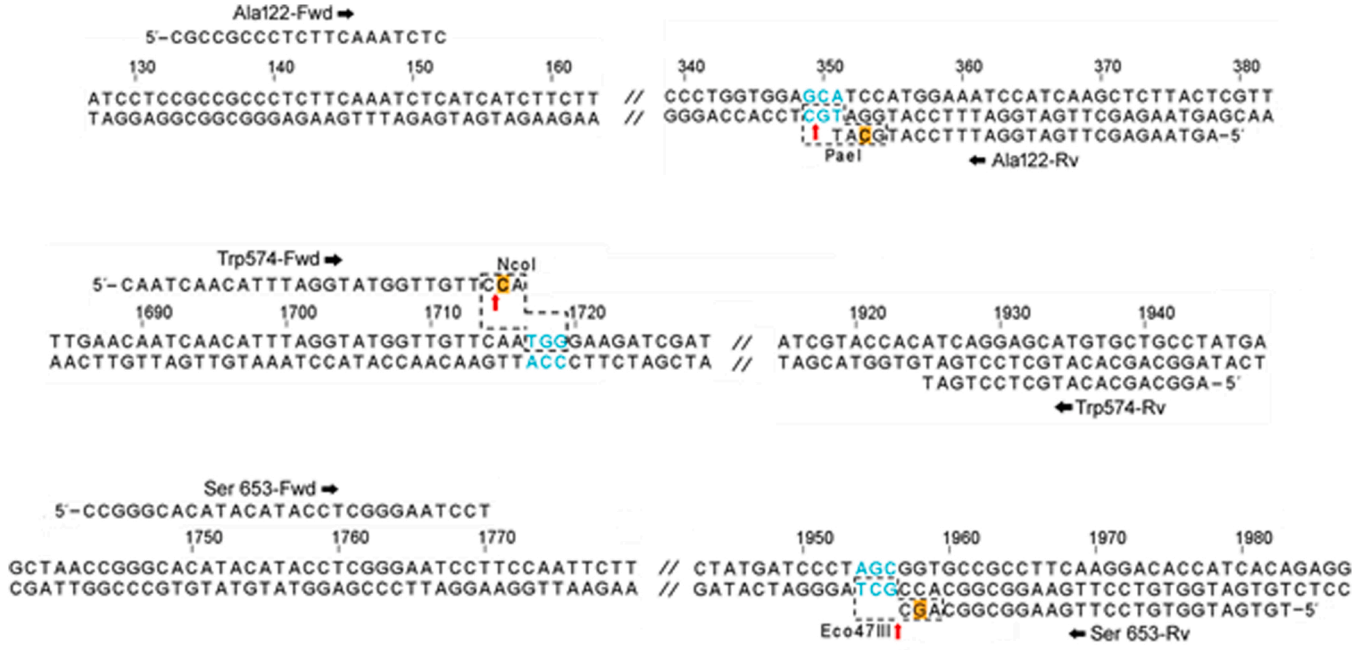

Fig. 1. Schematic representation of the dCAPS assays developed in this study. Nucleotide sequences and positions of forward and reverse primers are above the *Amaranthus palmeri* acetolactate synthase DNA plus strand and below the DNA minus strand, respectively. Targeted codons are indicated in blue letters. Mismatching nucleotides in dCAPS primers are highlighted in orange. Restriction enzyme recognition sites are boxed, and cutting site positions are indicated with a red arrow.

Table 1
Derived cleaved amplified polymorphic sequences (dCAPS) assay primers and restriction enzymes.

Target codons	Primer sequences		Annealing temperatures	Restriction enzymes	dCAPS patterns (fragment sizes)	
					wild-type	mutant
Ala122	Fwd 5′–3′	CGCCGCCCTCTTCAAATCTC	61 °C	PaeI	29 bp, 217 bp	246 bp
	Rv 5′–3′	AGTAAGAGCTTGATGGATTTCCATGCAT				
Trp574	Fwd 5′–3′	CAATCAACATTTAGGTATGGTTGTTCCA	60 °C	*Nco*I	26 bp, 230 bp	256 bp
	Rv 5′–3′	AGGCAGCACATGCTCCTGAT				
Ser653	Fwd 5′–3′	CCGGGCACATACATACCTCGGGAATCCT	74 °C	*Eco*47III	27 bp, 214 bp	241 bp
	Rv 5′–3′	TGTGATGGTGTCCTTGAAGGCGGCAGC				

inhibition was computed as I50, which represents the herbicide concentration required to reduce the enzyme activity by 50% compared to the untreated control. Resistance factors (Rf) of each individual MR plant were calculated using I50 concentrations of pyrithiobac in reference to the mean I50 of individuals of the S population (n = 6).

2.4. Relative gene expression of BCAA and AAA biosynthetic pathways

The relative transcript level was measured for nine genes of the AAA biosynthetic pathway and four genes of the BCAA biosynthetic pathway. RNA extraction and subsequent cDNA were performed as described previously [13]. qRT–PCR was conducted by using primers detailed before. The genes of D-arabinoheptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQSD), shikimate kinase (SK), EPSPS, chorismate synthase (CS), anthranilate synthase (AS) and two isoenzymes of chorismate mutase (CM) (plastidics 1–3 and cytosolic 2) were evaluated using the primers and annealing temperatures described previously [12]. Primers for the study of the relative expression of ALS, ketol-acid reductoisomerase (AHAIR), dihydroxyacid dehydratase (DHAD) and branched-chain amino acid transaminase (TA) were the same as indicated in [13]. Melting curve analysis was conducted to verify the amplification of single PCR products. β-tubulin was used as the normalization gene [12]. The relative transcript level was calculated using the $2^{-\Delta\Delta Ct}$ method [30]. Relative transcript abundance was normalized using the normalization gene β-tubulin and each population to its own control. Gene expression was monitored in at least four biological replicates.

2.5. EPSPS and DAHPS immunoblotting

Proteins were separated by 12.5% SDS–PAGE, and immunoblots were produced according to standard techniques. EPSPS and DAHPS immunoblotting was performed as described previously [12].

2.6. Shikimate content

Three leaf discs (4 mm diameter) were excised from the youngest leaf of each plant for shikimate content determination. Leaf discs were placed in 2 mL Eppendorf tubes and stored at − 80 °C until analysis. Shikimate was extracted as described previously, and the shikimate content was quantified spectrophotometrically [26].

2.7. Amino acid content

Amino acid content (AAA and BCAA) was determined in HCl extracts using capillary electrophoresis equipped with a laser-induced fluorescence detector, as previously described [37].

2.8. Carbohydrate content

The soluble carbohydrate (glucose, fructose, and sucrose) content was determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis [9]. Carbohydrate levels were analysed by ion chromatography as previously described [38].

2.9. Statistical analysis

For *ALS* and *EPSPS* gene copy number, each mean value was calculated using samples from different individual plants as replicates. The means were compared using a Student's or Welch *t*-test after checking homocedasticity by Levene's test and discussed in the text if significant.

For ALS activity and resistance factor comparison, one-way ANOVA was used to determine the significance of the differences using the type of mutation as a factor. The means were separated using Tukey's multiple comparison adjustment ($p < 0.05$).

For shikimate, carbohydrate and amino acid content and gene expression studies, each mean value was calculated using samples from different individual plants from the two performed experiments as replicates (minimum four replicates). The difference between untreated plants of each population was evaluated using a Student's or Welch t-test after checking homocedasticity by Levene's test and confirmed as significant when $p < 0.05$. Differences between treatments for each population were evaluated by one-way ANOVA (SPSS 27.0) with a multiple-comparison adjustment (Tukey or T3 Dunnett, depending on the Levene test of the homoscedasticity of variances) at $p < 0.05$. For each population, significant differences are highlighted in the figures by different letters.

3. Results and discussion

3.1. Visual symptoms and lethality

Glyphosate and pyrithiobac were applied alone or together at the recommended field dose for *A. palmeri* to the S and MR populations. The visual status of the plants was monitored for 20 days.

The visual comparison of both populations at the moment of harvest (3 days after herbicide application) is shown in Fig. 2. Almost no visual symptoms were detected in the MR population treated with glyphosate, while S plants were affected and showed growth arrest. Although growth arrest was elicited after pyrithiobac and the combined treatment in both populations, the effects were more evident in the S population.

The S population treated with glyphosate died in approximately 7–10 days, while plant death after pyrithiobac or the combined treatment was slower and took more than 10 days. Indeed, ALS inhibitors have been reported to need more time to cause plant death than glyphosate [39,40]. Although MR-treated plants were affected within the 20 days following the treatment, no treatment was lethal. MR plants treated with pyrithiobac or the combination of both herbicides were more affected than were plants treated with glyphosate alone.

3.2. Molecular and biochemical characterization of the resistance mechanisms

One of the increasingly widespread mechanisms of resistance to the herbicide glyphosate is *EPSPS* gene amplification. Gene amplification

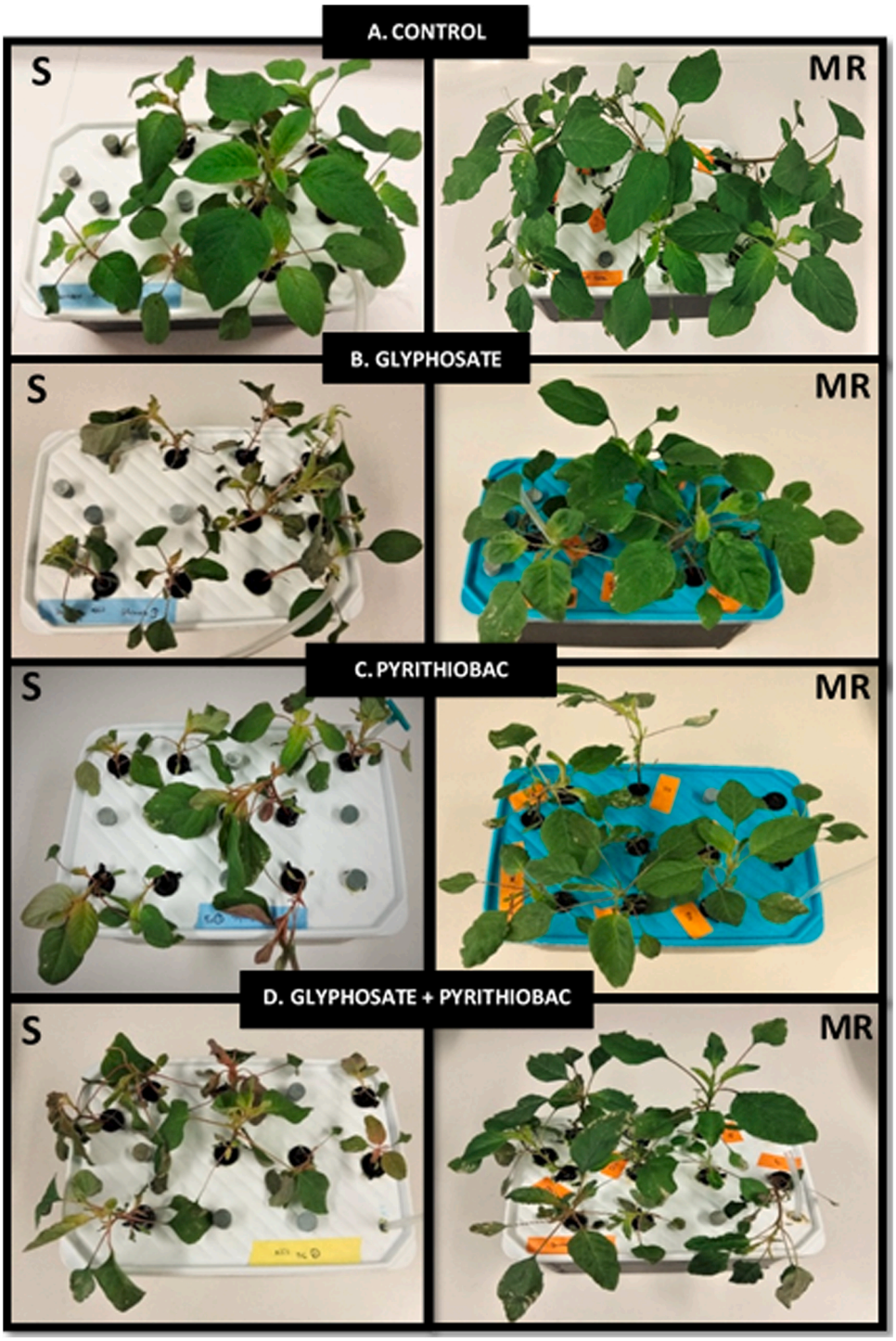

Fig. 2. Visual appearance of the *Amaranthus palmeri* plants in multiple resistant (MR) and sensitive (S) populations 3 days after the treatment. Plants were untreated (Control) (A) or treated with 0.84 kg ha^{-1}glyphosate (B), 89 g ha^{-1} pyrithiobac (C) or with glyphosate and pyrithiobac (D).

was first reported in an *A. palmeri* population from Georgia and has been reported in several grasses and dicotyledonous species [41]. In this study, to confirm this resistance mechanism, the *EPSPS* relative genomic copy number was calculated by quantitative real-time PCR using CPS as an internal standard (Fig. 3A). The genomes of the S plants exhibited a mean copy number of 0.80, while all MR-evaluated plants showed gene amplification. Relative copy numbers for MR plants varied from 30 to more than 90, with a mean of 58 relative copies (n = 71).

Shikimate content is a well-known marker, as it accumulates when the EPSPS enzyme is inhibited by glyphosate, and it can be used to distinguish resistant and sensitive plants [20]. Shikimate content was very low in the untreated and pyrithiobac-treated plants of both populations (Fig. 3C). Glyphosate treatment provoked substantial shikimate accumulation in the S population and lower but significant accumulation in the MR population. In both populations, the combined treatment provoked shikimate accumulation similar to that induced by herbicide treatment alone.

All S plants treated with glyphosate or the combined treatment accumulated shikimate, whereas MR plants did not accumulate or accumulated lower amounts of shikimate, indicating that EPSPS was still

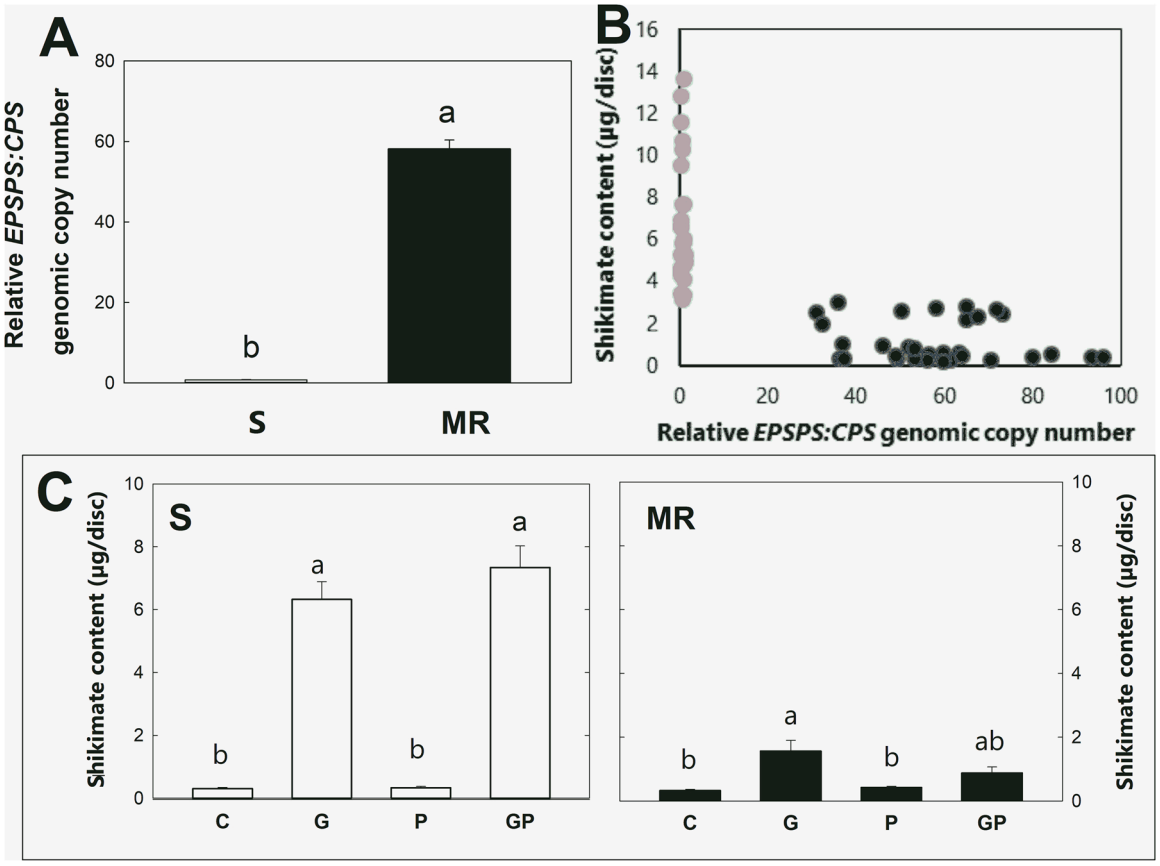

Fig. 3. (A) *Amaranthus palmeri* genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to carbamoylphosphate synthetase (CPS) in the multiple resistant (MR) and sensitive (S) populations (mean ± SE; n = 58 (S); n = 71 (MR)). (B) Correlation between genomic copy number of *EPSPS* and shikimate accumulation in individuals of the sensitive (grey circles, n = 26) and multiple resistant (black circles, n = 31) populations. Shikimate accumulation was measured after 3 days of treatment with glyphosate or glyphosate + pyrithiobac. (C) Shikimate content in sensitive (white bars, left; S) and multiple resistant (black bars, right; MR) populations. Plants were untreated (control, C) or sampled 3 days after treatment with glyphosate (G), pyrithiobac (P) or both herbicides (GP). (Mean ± SE; n = 11–27) Different letters indicate significant differences between treatments (ANOVA, HSD Tukey/T3 Dunnett test, p value < 0.05).

functioning (Fig. 3B). It was not possible to detect a direct correlation between the level of shikimate and the number of gene copies in the MR population, as independent of the gene copy number, some MR plants accumulated a modest amount of shikimate, and some showed shikimate levels only slightly above background levels.

ALS gene amplification, as a mechanism of resistance to ALS inhibitors, is rare in plant species. However, selection pressure with ALS inhibitors may isolate genotypes with increased *ALS* gene copies. This was not the case for the MR population we studied, as S and MR plants showed relative copy numbers of *ALS* to *CPS* of 1.52 ± 0.05 (n = 53) and 1.57 ± 0.03 (n = 71), respectively. In other studies [42,43], a slight increase in the *ALS* copy number was detected in resistant populations of *A. palmeri* and *Alopecurus aequalis*, but their main mechanism of resistance was attributed to ALS mutations, as has been detected in most resistant populations to ALS inhibitors [18,22].

To investigate whether this was the case for the MR population and whether mutations in the *ALS* gene contribute to pyrithiobac resistance, the *ALS* gene was amplified from individual plants, covering all eight codon positions that have been reported to be changed in mutated resistant *ALS* genes [15,24]. In the MR population, 69% of the plants showed single or double nucleotide polymorphisms, and the remaining plants (31%) did not.

In the mutated genomes, there were more individuals showing simple point mutations (40%) than double point mutations (29%) (Table 2).

Sequencing the *ALS* gene from both populations resulted in the identification of five mutations in the MR *ALS* gene sequence, resulting in a change from tryptophan to leucine at position 574 (W574L), serine to asparagine or threonine at position 653 (S653N/T), alanine to threonine at position 122 (A122T), alanine to aspartate at position 205 (A205D) and glycine to serine at position 654 (G654S) (Table 2).

Table 2
Types of mutations detected in the *ALS* sequences of individuals of the multiple resistant (MR) population. The amino acid changes are detailed. The frequency of each mutation type relative to the total mutated genotypes is included.

Mutation type	Mutation	% of total mutated individuals
Simple	W574L	19.22
Simple	S653N/T	13.10
Simple	A122T	7.86
Double	W574L + S653N	20.96
Double	W574L+ A122T	6.11
Double	W574L + A205D	0.87
Double	W574L + G654S	0.87

The mutations W574L and S653N/T were the most abundant substitutions in the plants, with only one simple point mutation, while the A122T mutation was not as common. All plants with a double mutation contained the W574L mutation, and most of them also showed the point mutation S653N. The double mutation at the level A205 or G654 was very rare and only detected in one individual each.

The W574L mutation has been described as the most common point mutation in *A. palmeri* resistant to ALS inhibitors [32,43,44]. It is known to confer broad resistance to imidazolinones, sulfonylureas and triazolopyrimidines [45–48] and specifically to pyrithiobac [44].

The S653N mutation is known to confer resistance mainly to

imidazolinones and not to sulfonylureas [49–51]. No studies about resistance to pyrimidinyl benzoates in weeds with simple mutations have been performed, but resistance to pyrithiobac was detected in an *A. palmeri* population from Arkansas carrying the double mutation W574L + S653N [43]. In this study, the substitution at the level of S653 was mainly with N, as has been previously detected in *A. palmeri* [32,43]; only in two cases did the S653T change occur.

The A122T mutation endows high resistance to imidazolinones but low resistance to sulfonylureas [50], and it was previously detected in *A. palmeri*, in Argentina [52] and Arkansas, where it was resistant to pyrithiobac [43]. The A205D and G654S mutations have not been reported previously in *A. palmeri*, although they have been found in other species [15,24]. The A205D mutation was found for the first time in 1996 in *Xanthium strumarium* L. [53], and more recently, it has been reported to confer broad resistance to ALS inhibitors, including pyrithiobac [54]. Each of these mutations was detected in only one plant carrying the W574L mutation, so the role of these mutations in the resistance trait is difficult to elucidate. The G654S mutation was only found in resistant *Setaria viridis* in 2008 [55].

ALS gene sequencing showed that resistant plants with resistance-endowing mutation(s) displayed double peaks or simple peaks in the sequencing chromatograms, indicating homozygosity and heterozygosity. To check the genotype, primers for dCAPS assays were designed after polymorphic nucleotide positions were identified. No assay was designed for codons 205 and 654 because of their low appearance. All dCAPS assays were set up using most of the MR plants and some S plants. Digested and undigested amplicons were subjected to electrophoresis side-by-side, and gels are shown in Suppl. Fig. S2.

The plants were classified as heterozygous resistant (RS) or homozygous resistant (RR) for each position and are indicated in the genotype column of Table 3. All MRs were heterozygous for W574L, while in another multiple resistant population of Argentina, both homozygous and heterozygous individuals were described [46]. All individuals were heterozygous for A122T. Interestingly, for position S653, heterozygosity was only detected if the individual carried the double mutation with W574L. The MR plants with the simple mutation S653N were all homozygous, as has been reported recently in other *A. palmeri* populations [32]. In general, there are a great variety of amino acid changes that can confer resistance, and the outcrossing nature of *A. palmeri* can contribute to the high number of polymorphisms observed [52].

After sequencing and genotyping the MR plants, leaves were used to evaluate the *in vitro* ALS sensitivity to pyrithiobac to correlate each mutation type with the functionality of the enzyme. The *A. palmeri* MR plants with only one point mutation presented similar ALS basal activity profiles to the S plants (Fig. 4A), while plants with the double mutation W574L + S653N showed higher ALS activity. Additionally, in resistant *Lolium rigidum* plants carrying the W574L mutation, higher extractable basal ALS activity was shown [56], although in this MR population, the increase was not detected when the W574L mutation appeared alone.

The herbicide sensitivity of the ALS extracted from MR and S plants was determined using the herbicide pyrithiobac. As shown for representative individuals in Fig. 4C, wild-type ALS isolated from plants of the S population was, as expected, strongly inhibited by pyrithiobac, with I_{50} values averaged at 0.038 μM (n = 6). However, ALS extracted from MR plants for the various resistance mutations was clearly resistant, with I_{50} values greater than those of the S population.

The Rf of each MR plant was calculated (I_{50}/mean S I_{50}) (Table 3). Although the Rf of each genotype was variable and there were not significant differences, there was still a difference in range among them. The mean Rf of MR plants with the simple mutations A122T or S653N/T was lower than the Rf of the plants carrying the mutation W574L (Fig. 4B), both alone or with S653N. Previous studies have characterized W574L as the mutation conferring the highest resistance to the sulfometuron (sulfonylurea) trait in *Lolium rigidum* [56] and broad spectrum resistance to ALS-inhibiting herbicides in *Poa annua* [57]. The presence of the S653N mutation as a double point mutation did not significantly affect the Rf of the MR plants. Moreover, the Rf of the homozygous plants with the S653N mutation was the lowest, so it can be proposed that the point mutation S653N confers a low resistance to pyrimidinyl benzoates.

Table 3
Relationship between specific mutations, genotype and Rf (resistance factor) of each individual plant of the multiple resistant (MR) population (identified with a number in the first column). After dCAPS genotyping, plants were classified as homozygous mutant (RR) or heterozygous mutant (RS). Resistance factors (Rfs) were calculated using the concentrations of inhibitors required to reduce ALS activity by 50% (I_{50}) divided by the mean I_{50} of the sensitive population (0.038 μM, n = 6). Individuals with the same mutation(s) were arranged by increasing Rf.

Individual plant number	Mutation type	Mutation (s)	Genotype (dCAPS assay)	Rf
85	SIMPLE	W574L	RS	2.2
72		W574L	RS	28.4
87		W574L	RS	40.4
66		W574L	RS	61.7
107		W574L	RS	144.3
44		W574L	RS	363.5
25		W574L	RS	521.8
28		W574L	RS	573.7
61		W574L	RS	591.2
89	SIMPLE	S653N	RR	3.23
88		S653N	RR	3.41
1		S653N/T	RR	5.2
45		S653N	-	7.87
101		S653N	RR	7.87
51		S653N	RR	8.29
86		S653N	RR	11.33
6		S653N/T	RR	11.6
73		S653N	RR	36.84
77		S653N	RR	195.67
27	SIMPLE	A122T	RS	4.98
80		A122T	RS	9.44
76		A122T	RS	250.91
29	DOUBLE	W574L+S653N	RS+RS	97.31
19		W574L+S653N	RS+RS	109.40
18		W574L+S653N	RS+RS	119.19
56		W574L+S653N	-	380.75
46		W574L+S653N	-	426.20
60		W574L+S653N	RS+RS	476.30
63	DOUBLE	W574L+A122T	RS+RS	> 500
112	DOUBLE	W574L+A205D	RS +-	> 500

After confirming the TSR mechanisms in the MR population from Arizona, the primary metabolism was analysed at three points: AAA and BCAA pathways, AAA and BCAA, and total amino acid and carbohydrate content. Leaf samples were taken after three days. This time point was chosen to evaluate the physiological and biochemical plant responses induced by the herbicides but not directly resulting from cell death. Physiological analysis was performed in confirmed MR plants, which were previously checked to possess point mutations at the *ALS* gene and *EPSPS* gene amplification. The physiological comparison was performed at two levels. First, to detect additional changes in the MR plants, primary metabolism between untreated plants of both populations was compared. Second, the physiological effects induced by both herbicides (alone or combined) on both populations were compared.

3.3. Expression of the targeted pathways and primary metabolism in multiple resistant plants

To study the impact of the multiple resistance mechanism on the regulation of the AAA biosynthetic pathway (Suppl. Fig. S1), transcript levels of nine enzymes were analysed by qRT–PCR. The *EPSPS* transcript level in the MR population was 130-fold the level in the S population (Table 4). Similar increases have been reported in other *A. palmeri* populations with EPSPS gene amplification [20,58]. *DAHPS*, *DQSD*, *CM1–3* and *CM2* transcription levels were 2–3 times higher in the MR

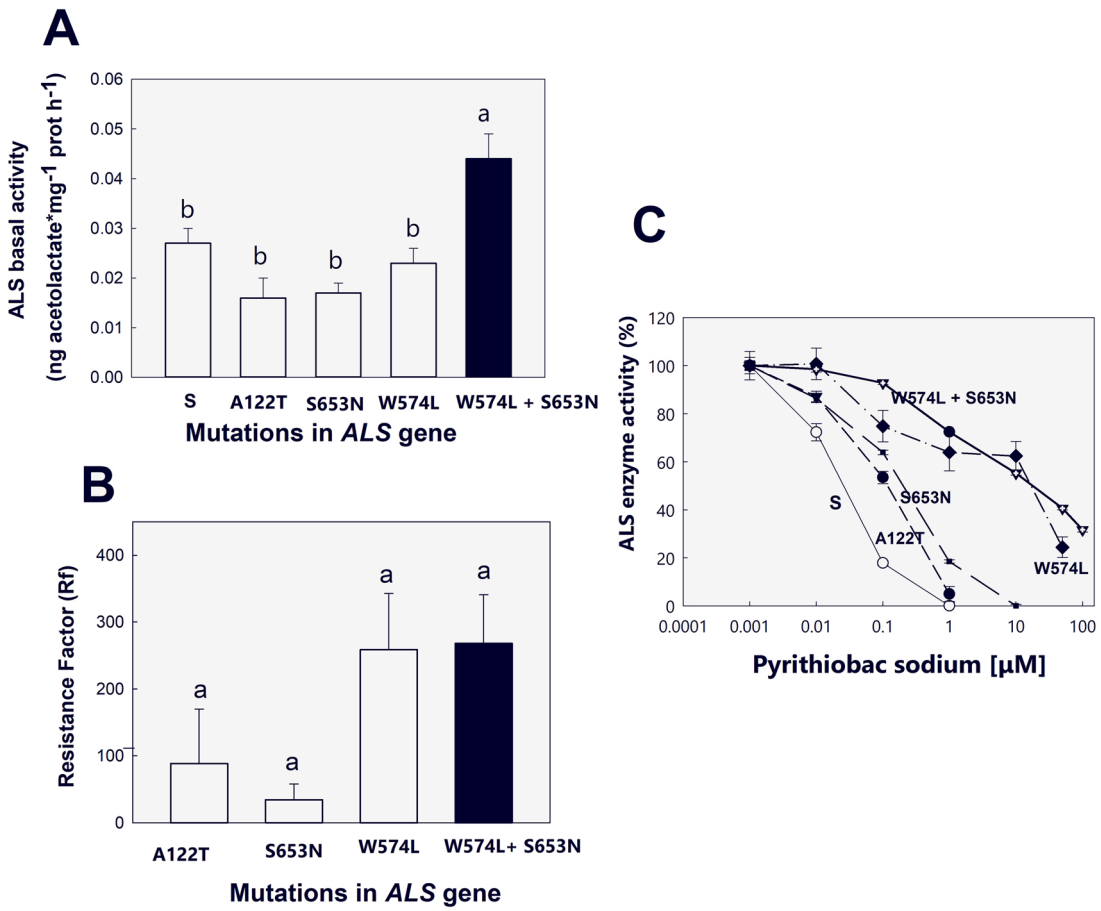

Fig. 4. (A) Basal ALS activity in the leaf extracts of untreated plants of the sensitive population (S) or multiple resistant (MR) population with simple (white bars) or double (black bars) mutations. (Mean ± SE; n = 6 (S); n = 3–9 (MR)). Different letters indicate significant differences between genotypes (p value <0.05. Tukey). (B) Resistance factors (Rfs) of MR plants with simple (white bars) or double (black bars) mutations. (Mean ± SE; n = 3–9). Rf was calculated by dividing the pyrithiobac concentration required to reduce ALS activity by 50% (I_{50}) by the mean I_{50} of the sensitive population (0.038 µM. n = 6). (C) *In vitro* dose–response curves of leaf extracts in the presence of pyrithiobac. Each line shows a representative individual extract of the sensitive population (S) or multiple resistant population with simple or double mutations.

Table 4
Ratio of MR to S relative transcript abundance measured with qRT–PCR (n = 2–11). Asterisks indicate significant differences between populations (p value < 0.05, Student's/Welch's *t*-test).

DAHPS	DHQS	DQSD	SK	EPSPS	CS	CM2	CM1–3	AS
3.31* ± 0.31	0.86 ± 0.13	2.35* ± 0.17	0.59* ± 0.08	130.27*± 14.95	1.09 ± 0.14	2.06* ± 0.14	2.93* ± 0.37	1.18 ± 0.19

population. This general increase in the expression level of the biosynthetic pathway was not detected in another population that was only glyphosate-resistant [13], suggesting a particular physiological trait of the MR populations. *SK* expression was the only transcript level lower in the MR population than in the S population.

The expression pattern of the genes involved in the BCAA pathway (Suppl. Fig. S1) was also studied in untreated plants of both populations (Table 5). The results indicate that the MR population showed a very remarkable increase in the transcript levels of two of the four enzymes of the biosynthetic pathway: *AHAIR* and *TA* (Table 5). In a previous work, a glyphosate-resistant population of *A. palmeri* due to *EPSPS* overexpression did not show any change in BCAA expression [13], indicating that the expression changes detected in the MR population are related to ALS gene mutations and not to *EPSPS* amplification.

Table 5
Ratio of MR to S relative transcript abundance measured with qRT–PCR (n = 3–9). Asterisks indicate significant differences between populations (p value < 0.05, Student's/Welch's *t*-test).

ALS	AHAIR	DHAD	TA
1.01 ± 0.09	10.69* ± 0.66	1.11 ± 0.15	6.58* ± 0.72

Basal levels of carbohydrates and amino acids were compared between both populations to detect additional physiological traits in the MR population. Both populations showed similar total amino acid, AAA, TSS and starch contents in nontreated plants (Table 6).

BCAA contents between control plants of both populations were compared, and resistant plants showed a higher BCAA content (Table 6). In the same way, previous works have described the accumulation of BCAAs in resistant populations of *Lactuca serriola* and *Solanum ptychanthum* or transgenic recombinant rice lines with mutated *ALS* genes [59–61]. Indeed, it has been proposed that mutations at position 574 do not drastically affect ALS functionality but alter ALS sensitivity to BCAA feedback inhibition, resulting in the accumulation of these amino acids

Table 6
Amino acid and carbohydrate contents in the S and MR populations (n = 4–12). Asterisks indicate significant differences between populations (p value < 0.05, Student's /Welch's t-test).

Population	Total free amino acid (μmol g-1 FW)	AAA (nmolg-1 FW)	BCAA (nmol/g FW)	Total soluble sugars (mg g-1 FW)	Starch (mg glucoseg-1 DW)
S	17.61 ± 0.97	113.72 ± 17.77	128.59 ± 16.86	1.02 ± 0.25	31.47 ± 14.5
MR	19.04 ± 0.43	168.66 ± 23.11	249.78 ± 22.03*	0.65 ± 0.09	20.09 ± 3.11

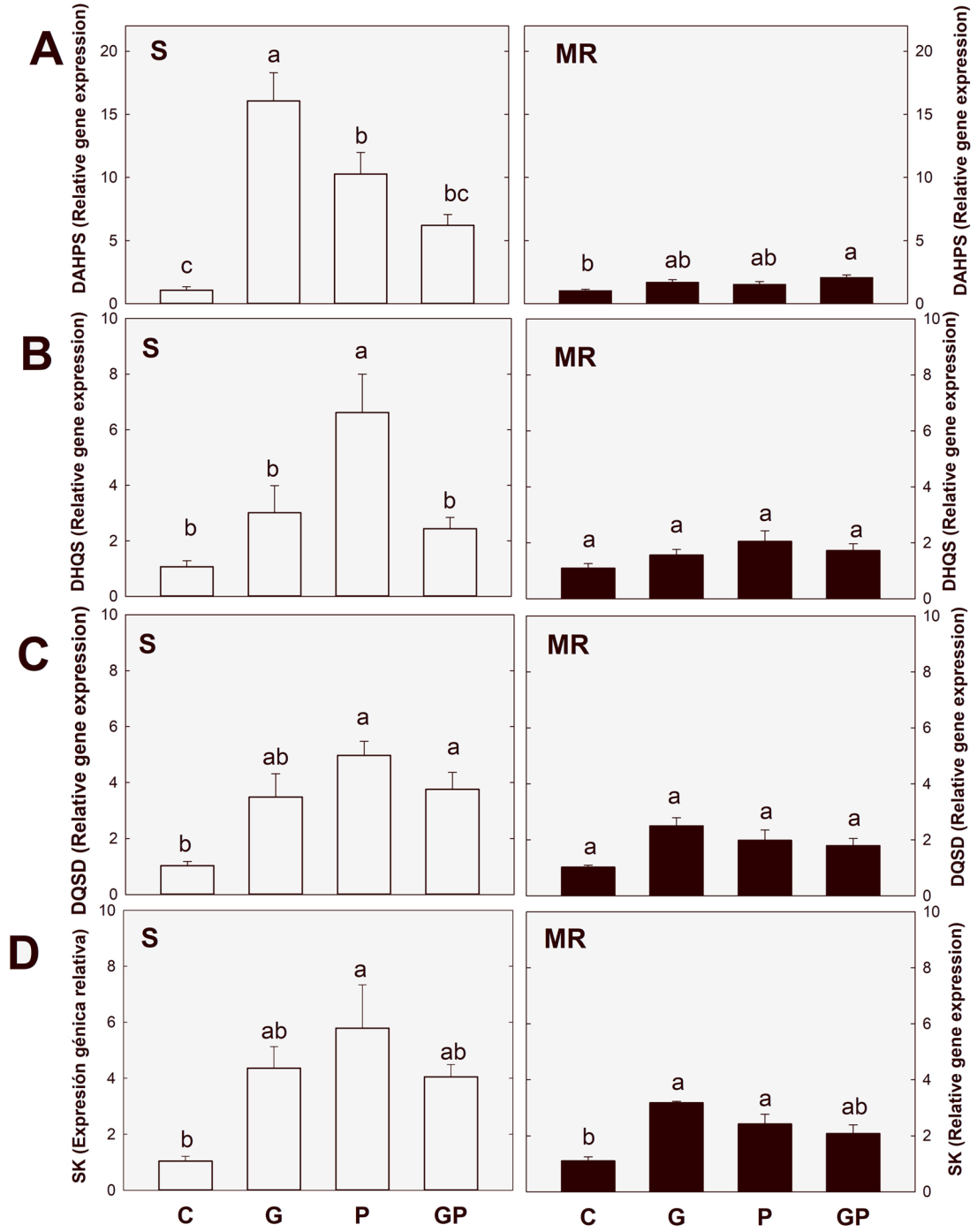

Fig. 5. Transcript abundance of genes in the aromatic amino acid (AAA) biosynthetic pathway in sensitive (white bars, left, S) and multiple resistant (black bars, right, MR) populations. Plants were untreated (control, C) or sampled 3 days after treatment with glyphosate (G), pyrithiobac (P) or both herbicides (GP). Relative transcript abundance was normalized using the normalization gene β tubulin and each population to its own control. (A) 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), (B) dehydroquinate synthase (DHQS), (C) 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQSD) and (D) shikimate kinase (SK). (Mean ± SE; n = 2–11). In each graph, the different letters indicate statistically significant differences between treatments in each population (ANOVA, HSD Tukey/Dunnett's T3, p value < 0.05).

[56]. In this sense, the point mutations detected in the *ALS* genes of the MR population would provoke changes in the feedback sensitivity inducing BCAA accumulation and specific enhancement of half of the genes of the BCAA pathway.

3.4. Effect of glyphosate and/or pyrithiobac treatments on targeted pathways and primary metabolism in multiple resistant and sensitive populations

The effect of glyphosate and/or pyrithiobac in the expression of AAA and BCAA biosynthetic pathways, AAA and BCAA, total amino acid and carbohydrate content was determined in the same replicates.

The expression pattern of the nine genes of the shikimate pathway genes is shown in Fig. 5 (upstream EPSPS) and 6 (EPSPS and after). Graphs show the relative expression of each gene to the untreated group of each population. In the S population, the glyphosate treatment applied alone caused a general increase in the expression of the genes of the AAA pathway, although the increase was only significant in *DAHPS*, *EPSPS* and *CS* (Figs. 5 and 6). Previous studies reported that glyphosate treatment provoked increased transcript abundance in the AAA pathway in glyphosate-sensitive and glyphosate-resistant plants [13]. In contrast, gene expression of the AAA pathway in the MR population in this study was not affected by glyphosate, with the exception of the residual increase in *SK* (Figs. 5 and 6).

While pyrithiobac alone did not affect the majority of transcript abundance of the MR plants, with the exception of *SK* and *AS*, in the S population, it provoked a general accumulation of the transcripts corresponding to genes of the AAA pathway, with the exception of *CM2* (the cytosolic isoform of CM) and *CM1–3*. This general increase was not detected after other ALS-inhibiting herbicides [62], which suggests that it can be considered a physiological effect of pyrithiobac not directly related to target site inhibition and that it was abolished in the MR population.

The increased expression detected after pyrithiobac in the S population was attenuated in the GP mixture, and the induced increase was only significant for *DQSD* and both isoforms of *CM* and not for the other genes (Figs. 5 and 6). In contrast, a glyphosate-resistant population treated with a mixture of glyphosate and another ALS inhibitor (an imidazolinone) showed an enhancement of the transcriptional pattern after the mixture compared to when treated with glyphosate alone [62]. *DAHPS* expression was the only one that increased after the combined treatment in the MR population.

The protein contents of two important enzymes of the AAA pathway (DAHPS and EPSPS) were measured (Suppl. Fig. S3) after herbicide treatments. The only significant change in the protein content was detected in EPSPS in the MR population, where pyrithiobac treatment provoked a significant increase (Suppl. Fig. S3) after herbicide treatments. The only significant change in the protein content was detected. The increased EPSPS content in the MR population after pyrithiobac indicated posttranscriptional regulation, as the EPSPS expression level was not affected (Fig. 6A). Both populations showed no effect in response to glyphosate alone or the mixture, contrary to previous studies performed with a glyphosate-resistant population due to *EPSPS* gene amplification [26,62].

The expression pattern of the genes involved in the BCAA pathway was also studied in plants treated with glyphosate and/or pyrithiobac (Fig. 7). When glyphosate was applied alone, there was only a significant change in the expression of *TA* transcript levels in the S population (Fig. 7D). No changes in BCAA gene expression were detected after the inhibition of one of the enzymes (ALS) of the BCAA pathway with pyrithiobac in the S population, as previously described [62]. In the MR population, the expression of the BCAA pathway was not affected by glyphosate or pyrithiobac when applied alone (with the exception of TA after pyrithiobac treatment). Interestingly, the herbicide mixtures induced significant effects on BCAA pathway expression: in both populations, *DHAD* and *TA* transcript levels increased (Fig. 7C and D). However, *AHAIR* expression decreased in the MR population after mixing (Fig. 7B). The enzyme TA catalyses the final step in the biosynthesis of BCAAs. However, TA activity is involved not only in biosynthesis but also in the degradation of these three amino acids [63], so these contrasting pathways must be carefully balanced to maintain the homeostasis of the pool of this important group of amino acids [64]. The involvement of TAs in the plant stress response has been reported [65,66]. Increased levels of BCAAs under drought conditions initiate a defense mechanism that stimulates the catabolic activity of TA [67]. In this sense, it can be proposed that the higher BCAA availability detected in MR plants (Table 6) would be related to an increased catabolism mediated by TA.

The effect of treatments on the most significant parameters of the free amino acid profile was evaluated (Fig. 8): AAA and BCAA (products of the targeted pathways) and free amino acid content. Since both herbicides induced an increase in the content of free amino acids and, as cross regulation in the amino acid metabolic pathways might exist [68,69], the use of the herbicides on an MR plant could lead to a different response than the responses to individual herbicides.

The total free amino acid content was determined as a physiological marker of the effect of the herbicides. In the S population, all treatments induced a significant increase in total free amino acid content with respect to the control (Fig. 8A), as has been reported before with glyphosate and other ALS inhibitors [26,37,62,70,71]. Due to the resistance to glyphosate and pyrithiobac, in the MR population, only the combined treatment induced free amino acid accumulation.

AAA accumulation was detected in the S population treated with glyphosate alone (Fig. 8B),and it has been reported as a physiological marker of damage [13,26,37,72], evidencing a higher level of damage to the S population due to its higher sensitivity to the herbicide. The increased expression of AAA pathway genes does not appear to be the cause of AAA levels because, while AAA accumulated more with glyphosate (Fig. 8A), the expression was more affected after pyrithiobac alone (Figs. 5 and 6). The higher AAA content detected in treated plants is related to an increase in protein turnover [73,74], which is supported by the higher free amino acid pool (Fig. 8). Further research is needed to understand the signal(s) that upregulate the AAA pathway following glyphosate or pyrithiobac treatment.

In the MR population only the combined treatment of glyphosate and pyrithiobac provoked AAA accumulation (Fig. 8B). Our study shows different patterns in the regulation of the AAA pathway between S and MR plants. Only the S population, not the MR population, showed a general and important increase in AAA pathway transcript levels after pyrithiobac. In addition, in the MR population, only the EPSPS content increased after pyrithiobac and AAA, and the total free amino acid content significantly accumulated after the herbicide mixture. These results indicate that the EPSPS and ALS modifications in the MR population involve changes in the physiological response to glyphosate or ALS inhibitors.

In the S population, there was a significant increase in BCAA content after glyphosate compared to the control (Fig. 8C), as has been reported before [37,62]. This effect was not observed in the MR population (Fig. 8C), evidencing a lower level of damage due to its lower sensitivity to the herbicide. The BCAA content was not affected by pyrithiobac or the mixture in any of the populations.

Carbohydrate accumulation is induced by the application of glyphosate and ALS inhibitors [9,10,72] and thus can be used as a physiological marker of herbicide toxicity. Total soluble sugars (TSS, the sum of glucose, fructose and sucrose) and starch contents were measured in the leaves of both populations (Fig. 9). A general increase in TSS and starch content was observed in the S population after the three treatments (Fig. 9A and B). The most remarkable increase was detected after treatment with pyrithiobac. Previous studies have reported carbohydrate accumulation with glyphosate or other ALS inhibitors, such as imazamox [26,62].

TSS did not accumulate in MR plants after glyphosate, in

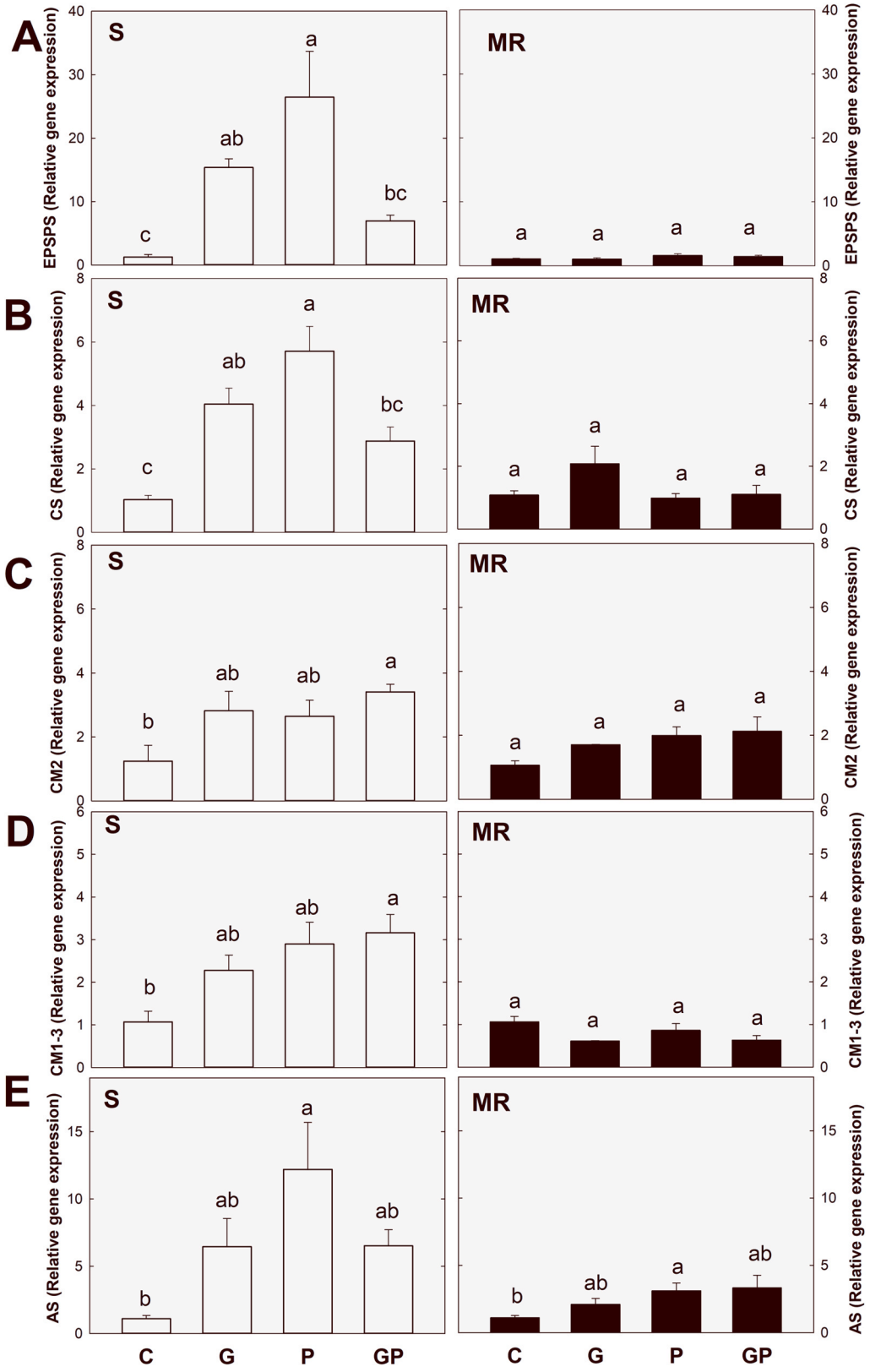

Fig. 6. Transcript abundance of genes in the aromatic amino acid biosynthetic pathway in sensitive (white bars, left, S) and multiple resistant (black bars, right, MR) populations. Plants were untreated (control, C) or sampled 3 days after treatment with glyphosate (G), pyrithiobac (P) or both herbicides (GP). Relative transcript abundance was normalized using the normalization gene β-tubulin and each population to its own control. (A) 5-Enolpyruvylshikimate 3-phosphate synthase (EPSPS), (B) chorismate synthase (CS), (C) chorismate mutase isoform 2 (CM2), (D) chorismate mutase isoforms 2 and 3 (CM 1–3) and (E) anthranilate synthase (AS). (Mean ± SE; n = 2–11). In each graph, the different letters indicate statistically significant differences between treatments in each population (ANOVA, HSD Tukey/ Dunnett's T3, p value < 0.05).

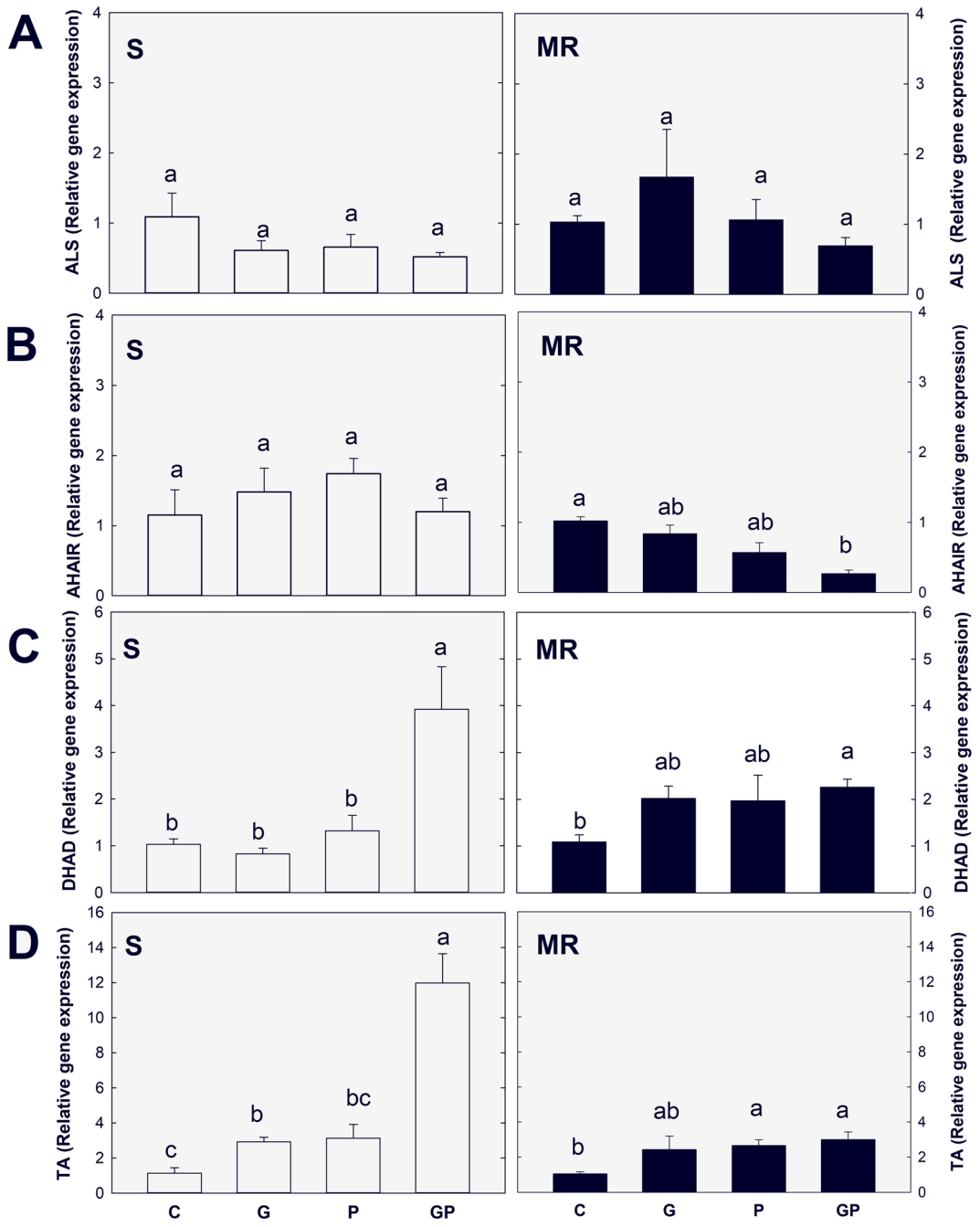

Fig. 7. Transcript abundance of genes in the branched-chain amino acid biosynthetic pathway in sensitive (white bars, left, S) and multiple resistant (black bars, right, MR) populations. Plants were untreated (control, C) or sampled 3 days after treatment with glyphosate (G), pyrithiobac (P) or both herbicides (GP). Relative transcript abundance was normalized using the normalization gene β tubulin and each population to its own control. Acetolactate synthase (ALS) (A), ketol-acid reductoisomerase (AHAIR) (B), dihydroxyacid dehydratase (DHAD) (C) and branched-chain amino acid transaminase (TA) (D). (Mean ± SE; n = 2–11). In each graph, the different letters indicate statistically significant differences between treatments in each population (ANOVA, HSD Tukey/T3 Dunnett, p value < 0.05).

concordance with the expected resistance to the herbicide, and, as has been reported in other populations, it was only glyphosate-resistant [26, 62]. In the same way, while MR plants accumulated TSS after pyrithiobac, the level of accumulation was much lower than the accumulation detected in S plants (Fig. 9A). Both herbicides applied alone induced starch accumulation in S plants (Fig. 9B), and the mixture of both herbicides attenuated starch accumulation. Interestingly, starch accumulation was the only physiological effect that was not attenuated or abolished in the MR population in comparison with the S population. Similar accumulation of TSS and starch was detected in sensitive and glyphosate-resistant populations, indicating that carbohydrate content was not affected by the resistance trait [26]. Future research will be necessary to unravel the specific role of the contrary patterns between starch and TSS in the carbon metabolism of the MR population.

In general, the changes detected in the primary metabolism in response to herbicide mixtures were similar or less than the changes detected after individual treatments. In the S population, the increased expression of the AAA biosynthetic pathway and the AAA and BCAA and carbohydrate accumulations induced by glyphosate or pyrithiobac alone were attenuated after the mixture (Figs. 5, 6 and 9). In the MR population, the increased EPSPS enzyme and TSS contents induced by pyrithiobac were attenuated after the mixture (Suppl. Fig. 3 and Fig. 9A). In

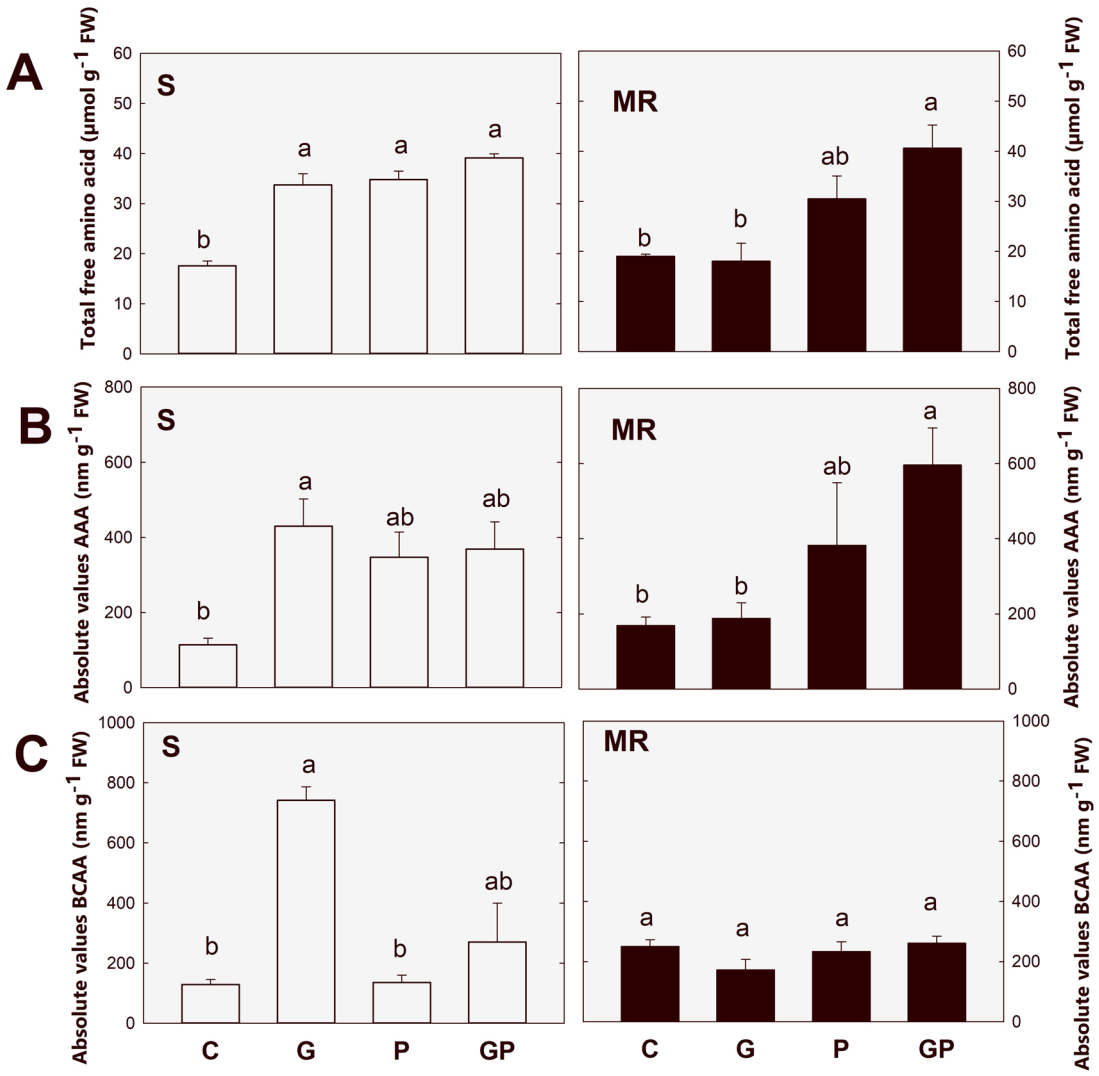

Fig. 8. Total free amino acid (A), aromatic amino acid (AAA) (B), and branched-chain amino acid (BCAA) (C) contents in sensitive (white bars, left, S) and multiple resistant (black bars, right, MR) populations. Plants were untreated (Control, C) or sampled 3 days after treatment with glyphosate (G), pyrithiobac (P) or both herbicides (GP). (Mean ± SE; n = 5–13). In each graph, different letters indicate significant differences between treatments (ANOVA, HSD Tukey/T3 Dunnett, p value < 0.05).

addition, lethality was not exacerbated by the combination of both herbicides.

In this study, physiological parameters were used to evaluate the interaction of the herbicide mixture. As in both populations, the mixture effects were mostly less than the sum of the individual effects, evidencing some kind of antagonistic effect. This interaction could have practical implications in the widespread use of a mixture of glyphosate and ALS inhibitors [62]. When both herbicides are applied together, the recommended rates have to be fully applied as toxic effects are not additive. As mixtures inhibit two different target sites, they are usually used to control resistant populations to one type of herbicides. Nevertheless, care should be taken when using mixture treatments, as they will increase the risk of selecting multiple resistant populations to both types of herbicides.

4. Conclusions

The mechanism of resistance detected in the MR population used in this study was TSR. All plants of the MR population used in this study were glyphosate resistant due to *EPSPS* gene amplification. While not all individuals were resistant to pyrithiobac, *ALS* point mutations at five positions were the TSR mechanism found in the resistant individuals. W574L was the most common mutation and conferred the highest resistance level to pyrithiobac.

MR plants showed a different basal primary metabolism and a different physiological response to the herbicides than sensitive plants. Untreated plants of the MR population showed higher levels of AAA and BCAA expression and BCAA content, which implies that additional effects of *EPSPS* gene amplification and ALS mutation were apparent. With the exception of starch accumulation, all physiological changes detected in the S population after both herbicides were abolished or attenuated in MR-treated plants, in concordance with the expected lower sensitivity to the treatments.

Contributions

MR and AZ designed the experiments. MGM, MVE and MB-A performed the experiments. MB-A analysed the data. AZ oversaw the project and wrote the paper with input from MR, MGM and MBA. MR supervised the project and funding acquisition. All authors have read and agreed to the published version of the manuscript.

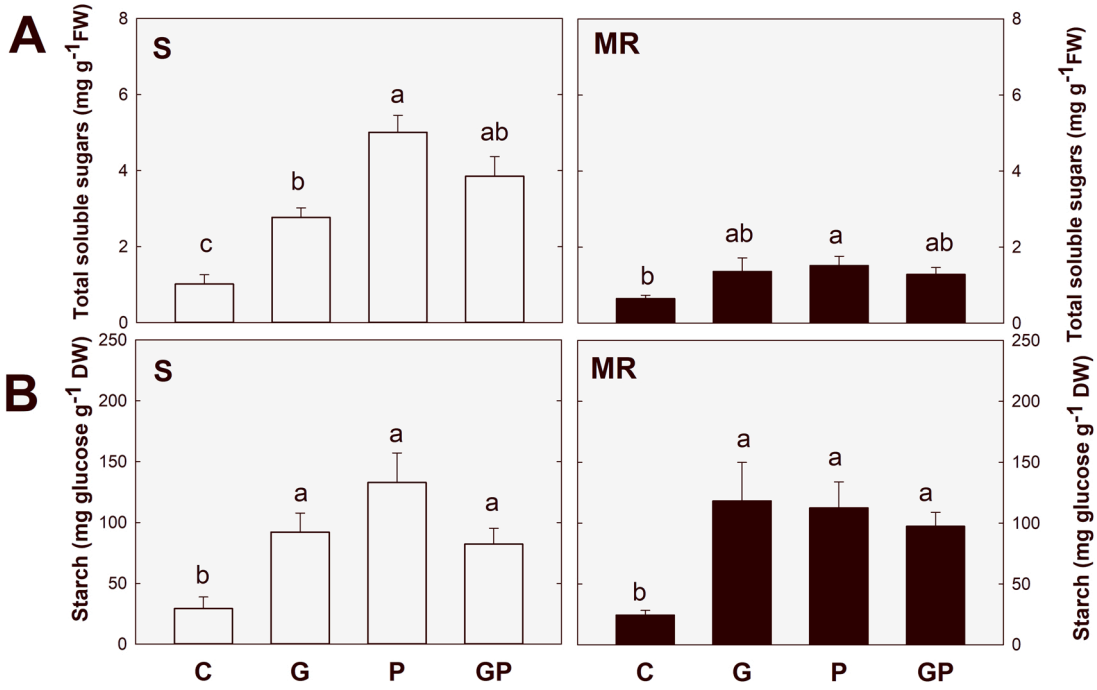

Fig. 9. Total soluble sugar (A) and starch (B) contents in sensitive (white bars, left, S) and multiple resistant (black bars, right, MR) populations. Plants were untreated (control, C) or sampled 3 days after treatment with glyphosate (G), pyrithiobac (P) or both herbicides (GP). (Mean ± SE; n = 5–13). In each graph, different letters indicate significant differences between treatments (ANOVA, HSD Tukey/T3 Dunnett, p value < 0.05).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111212.

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