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ORIGINAL RESEARCH

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Cysteine proteases are activated in sensitive *Amaranthus palmeri* populations upon treatment with herbicides inhibiting amino acid biosynthesis

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Abstract

The herbicides glyphosate and pyrithiobac inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the aromatic amino acid biosynthetic pathway and acetolactate synthase (ALS) in the branched-chain amino acid biosynthetic pathway, respectively. Here we characterise the protease activity profiles of a sensitive (S), a glyphosate-resistant (GR) and a multiple-resistant (MR) population of *Amaranthus palmeri* in response to glyphosate and pyrithiobac. Amino acid accumulation and cysteine protease activities were induced with both herbicides in the S population and with pyrithiobac in the GR population, suggesting that the increase in cysteine proteases is responsible for the increased degradation of the available proteins and the observed increase in free amino acids. Herbicides did not induce any changes in the proteolytic activities in the populations with target-site resistance, indicating that this effect was only induced in sensitive plants.

1 | INTRODUCTION

Although new methods are being developed and implemented in integrated agriculture, the use of herbicides continues to be the most extensively used method for weed control (Westwood et al., 2018). Among all the herbicides, amino acid biosynthesis inhibitors are currently outstandingly important. The aromatic amino acid (AAA) biosynthesis pathway transforms the inputs of carbon into the amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) through the shikimate pathway (Tzin & Galili, 2010). 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) is a key enzyme in this pathway and is the target of the herbicide glyphosate (Steinrücken & Amrhein, 1980). Glyphosate is a once-in-a-century herbicide and is currently the most widely used herbicide in the world (Duke et al., 2018). The branched-chain amino acid biosynthesis pathway leads to the formation of valine (Val), leucine (Leu) and isoleucine (Ile). Acetolactate synthase (ALS, EC 2.2.1.6; also termed acetohydroxyacid synthase) plays a key role in the pathway, and it has been used as a target for herbicides, known as ALS-inhibitors (Tan et al., 2006). Several chemical families have ALS as their site of action, and their commercialisation was a milestone in weed control history (Wittenbach & Abell, 1999).

The metabolic roadblock caused by EPSPS or ALS inhibition carries physiological consequences that eventually lead to plant death (Gomes et al., 2014). Although the two types of herbicides target different enzymes, common physiological consequences have been detected, suggesting that these herbicides kill plants by similar mechanisms. Growth is arrested, and carbon metabolism is impaired following the application of both types of herbicides (Orcaray et al., 2012; Zabalza et al., 2004).

Additional specific common biochemical effects of ALS and EPSPS inhibitors are an increase in the free amino acid pool and a decrease in the soluble protein content (Fernández-Escalada et al., 2016; Orcaray et al., 2010). Free amino acid pool accumulation is due to an increased protein turnover as a result of increased degradation and reduced synthesis, which means that protein synthesis occurs by using the amino acids scavenged from protein degradation and not generated from newly incorporated nitrogen (Maroli et al., 2016; Rhodes et al., 1987; Zabalza et al., 2006). Several proteolytic activities are involved in protein degradation upon ALS or EPSPS inhibition (Zulet et al., 2013).

In all living cells, the breakdown of functional proteins and the recycling of non-functional, misfolded or obsolete polypeptides to amino acids are fundamental regulatory physiological and developmental processes involving proteolytic activities, which also participate in signalling pathways, mediating diverse biological functions (Fanourakis et al., 2020; Tornkvist et al., 2019; van der Hoorn, 2008). Proteases include diverse classes (e.g., cysteine-, serine-proteases) based on the nature of the active sites that catalyse the hydrolysis of peptide bonds (van der Hoorn, 2008). Cysteine proteases have physiological roles in germination, development, senescence, immunity and stress responses (Sueldo & van der Hoorn, 2017; van der Hoorn, 2008). Among them, papain-like cysteine proteases (PLCPs) are one of the most abundant groups of cysteine proteases and are normally secreted into the vacuole, vesicles or apoplast (Liu et al., 2018; Sueldo & van der Hoorn, 2017). Vacuolar processing enzymes (VPEs), also known as asparaginyl endopeptidases or legumains, are cysteine proteases found in the vacuole of plants, animals and protozoans (Sueldo & van der Hoorn, 2017; Yamada et al., 2020). Serine hydrolases (SHs) are a large, diverse class of enzymes that carry an active site serine residue and are involved in immune responses, stomatal density regulation, detoxification and secondary metabolism (Kaschani et al., 2012).

Acetolactate synthase inhibitors are 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 (Zhou et al., 2007). Glyphosate is a wide-spectrum, nonselective post-emergence herbicide widely used in non-agricultural areas, but biotechnology and glyphosate-tolerant crops (maise, soybean, cotton and canola) have exacerbated its use (Nandula, 2010). However, the repeated use of glyphosate and ALS inhibitors has selected corresponding resistances in weed populations. One of the most problematic weed species resistant to both types of herbicides is *Amaranthus palmeri*, which is one of the most competitive troublesome and economically damaging weeds due to its high genetic diversity, seed production and vigour (Ward et al., 2013). The most common resistance mechanism in *A. palmeri* populations resistant to glyphosate is target-site amplification of the *EPSPS* gene (Chandi et al., 2012; Gaines et al., 2010). When this gene is overexpressed, the EPSPS enzyme accumulates so much that the recommended field dose of glyphosate is not sufficient to kill the plant. By contrast, the most commonly reported mechanisms conferring resistance to ALS inhibitors are point mutations in the *ALS* gene in weed species, including *A. palmeri* (Küpper et al., 2017; Nakka et al., 2017; Yu & Powles, 2014). The 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 (Heap, 2023; Nandula et al., 2012).

The resistance mechanism of the resistant populations is usually well characterised, but the possible physiological implications and the physiological effects triggered by the exposure to glyphosate or an ALS-inhibiting herbicide in these resistant weeds are not known. The availability of a glyphosate-resistant population overexpressing *EPSPS* (Fernández-Escalada et al., 2016) and of another population with multiple resistance to glyphosate and ALS inhibitors (due to *EPSPS* gene amplification and *ALS* point mutations) (Barco-Antoñanzas et al., 2022), offers the opportunity to study the physiological responses of target-site resistant biotypes to glyphosate and ALS inhibitors, and specifically the role of proteolytic activities in the mode of action and the resistance to these herbicides.

Protease activity profiling (also called activity-based protein profiling [ABPP] of proteases) is an easy and powerful method that can be used to monitor activity levels in a broad range of species (Morimoto & van der Hoorn, 2016), although it has not been used until now in *A. palmeri*. In this versatile method, specific probes react with the active site of specific proteases in an activity-dependent manner. The irreversible and covalent bond formed allows the subsequent separation on protein gels and detection by fluorescence scanning.

In this study, we investigated protease activities in sensitive and pyrithiobac and or glyphosate-resistant *A. palmeri* plants treated with glyphosate and/or pyrithiobac (ALS inhibitor). In addition, amino acid accumulation was determined, and the activities of several classes of proteases were monitored with ABPP.

2 | MATERIALS AND METHODS

2.1 | Plant material and treatment application

The seeds of the *A. palmeri* biotypes were kindly provided by Dr Gaines (Colorado State University, Fort Collins, CO, USA). The seeds of sensitive (S) standard and glyphosate-resistant (GR) *A. palmeri* biotypes were originally collected from North Carolina (USA), and the seeds of the multiple resistant (MR) population to glyphosate and pyrithiobac were originally collected from Arizona (USA). Seeds were stored at 4°C until use. The resistance mechanisms of both populations were target-site. The mechanism of the GR population was *EPSPS* gene amplification, as GR individuals had a mean 47.5-fold increase in the number of copies of the *EPSPS* gene (Chandi et al., 2012; Fernández-Escalada et al., 2016). Multiple resistant plants were glyphosate-resistant due to *EPSPS* gene amplification (48-fold

increase), while resistance to ALS inhibitors was related to point mutations at five positions in the *ALS* gene sequence (Barco-Antoñanzas et al., 2022). No other phenotypic differences have been detected between populations (Barco-Antoñanzas et al., 2022; Fernández-Escalada et al., 2016, 2017, 2019). Resistance of the GR population to glyphosate and the MR population to glyphosate and pyrithiobac was previously shown by visual symptoms and lethality (Barco-Antoñanzas et al., 2022).

Plants were cultivated and grown hydroponically, according to Fernández-Escalada et al. (2016). Briefly, seeds were surface-sterilised before germination (Labhilili et al., 1995). For germination, the seeds were incubated for 4 days at 4°C in the dark and a 60 h light/dark cycle of 16/8 h at 30/18°C. After germination, plants were transferred to aerated 2.7 L hydroponic tanks in a phytotron. The Hoagland nutrient solution with 15 mM KNO_3 (Hoagland & Arnon, 1950) was used, and growth conditions were set according to procedures described earlier (Fernández-Escalada et al., 2016). The plants remained at the vegetative phenological stage throughout the experiment.

All treatments were applied to three-week-old plants after selecting individuals of similar size and vigour after reaching the growth stage defined as BBCH 14.35. Treatments were performed as described before (Barco-Antoñanzas et al., 2022). Plants of all populations were divided into four groups: control (C), glyphosate (G), pyrithiobac (P) or both herbicides together (G + P). Glyphosate (commercial formula, FORTIN Green, Key, Lleida, Spain) was applied at the recommended field rate (0.84 kg ha^{-1}) (Culpepper et al., 2006). The ALS inhibitor pyrithiobac, which belongs to the pyrimidinyl-benzoate chemical family (analytical grade, Dr Ehrenstorfer LGC, Augsburg, Germany), was also applied at recommended field rate (89 g ha^{-1}) (Wise et al., 2009) with 5.4% sodium lauryl sulphate adjuvant (commercial formula Biopower 27.65% [p/v]) (Bayer Crop Science, Madrid, Spain) (Barco-Antoñanzas et al., 2022). Plants of the combined treatment were sprayed first with glyphosate and then with pyrithiobac. Control plants were only sprayed with adjuvant. The leaves were sampled 3 days after herbicide treatment, frozen in liquid N_2, and stored at −80°C. The samples were powdered using a Retsch mixer mill (MM200, Retsch, Haan, Germany), and the amount of tissue required for each analytical determination was separated.

The experiment was repeated twice. Samples were taken from both experiments and individual plants were considered biological replicates.

2.2 | Soluble protein and amino acid content determination

Samples (0.05 g) were homogenised in a 0.15 mL extraction buffer comprising 100 mM phosphate-buffer (pH 6.5), and total soluble protein was measured in the extracts (Bradford, 1976). A Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) was used for absorbance measurements.

Amino acid content was determined in HCl extracts using capillary electrophoresis equipped with a laser-induced fluorescence detector, as previously described (Orcaray et al., 2010).

2.3 | Labelling, ABPP and detection

Labelling was performed on aliquots (0.1 g) of ground *A. palmeri* leaves. Extraction was performed in 50 mM MES buffer 1-hydrate (pH 5); 5 mM DTT (6 mL g^{-1} FW). Samples were centrifuged twice (5 min; 12,000 *g*), and supernatants were collected. Protein concentration in the supernatants was quantified using the RC/DC Protein Assay (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Labelling was done by incubating the proteic sample (1.5 mg mL^{-1}) with a specific probe diluted in 50 mM MES buffer 1-hydrate (pH 5); 5 mM DTT at a concentration of 1.5 mg mL^{-1}.

For ABPP of the VPEs, labelling was performed by incubating the protein with the probe JOPD1 at 0.25 μM (Lu et al., 2015) for 3 hours. The labelling of PLCPs was done with E64 conjugated with a fluorophore (E64-Cy5), incubated at 2.5 μM for 3 h. E64-Cy5 (TK09) was generated by coupling Bodipy 630/650 alkyne (Lumiprobe) to N3Le (Toronto Research Chemicals, A522545) via copper-catalysed click chemistry.

To confirm the identities of the bands detected after co-labelling with JOPD1 or E64-Cy5, a competitive assay was performed. In this assay, a representative mixture of all the control and treated samples were pre-incubated with the inhibitors: E64 (PLCP inhibitor) or YVAD-cmk (VPEs inhibitor). Competition of protease labelling was performed by pre-incubating the extracts for 30 min at room temperature before the labelling. Inhibitors E64 and YVAD-cmk were used at a concentration of 200 μM and 100 μM, respectively. The different size of the bands allowed their discrimination in the image after scanning.

For ABPP of the SHs, FP-TAMRA labelling was performed by incubating the protein and the probe at 0.5 μM for 90 min. FP probes are extremely powerful probes that easily label >50 different SHs in a single proteome (Morimoto & van der Hoorn, 2016) by reacting with the serine active site nucleophile.

All labelling reactions were performed at room temperature in the dark. Equal volumes of DMSO were added to the no-probe controls. The incubation reactions were stopped by adding 200 μL acetone and incubated for 30 min at 4°C. After protein precipitation, acetone was removed, and samples were dried at room temperature for 15 min. Fifty microlitres of 1× SDS electrophoresis gel loading buffer (280 mM SDS, 400 mM Tris, 40% glycerol, 3 mM β-mercaptoethanol, 0.6 mM bromophenol blue, pH 6.8) was added. Proteins were separated on 12% SDS polyacrylamide gels. Gels were washed three times for 15 min with distilled water and then scanned on an Amersham™ Typhoon™ Biomolecular Imager (GE Healthcare, Munich, Germany) scanner. The laser excitation wavelength and filters used were: JOPD1: 532 nm laser and Cy3 570BP20 filter; E64-Cy5: 635 nm laser and Cy5 670BP30 filter and FP-TAMRA: 532 nm laser and Cy3 570BP20 filter. Emission was measured using a TAMRA filter (580 nm). For the band quantification, the Image J software was used.

2.4 | Statistical analysis

For all measured parameters, Student's *t*-test was used to compare pairs of untreated plants of GR or MR populations and S population, and it was confirmed as significant when $p < 0.05$. Significant differences are highlighted in the graphs with *.

In each population, differences between treatments 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 $p < 0.05$ using the treatment as the factor. Significant differences are highlighted in the graphs with different letters for each population. All statistical analyses were performed using Excel or SPSS 27 software.

3 | RESULTS

3.1 | Soluble protein and free amino acid profile

The soluble protein (Figure 1) and free amino acid (Figure 2) contents were evaluated. The plants of the MR population showed a lower basal soluble protein content than plants of the S population.

Figure 2 shows the profile of free amino acid content. Proteinogenic amino acids can be classified from the intermediates they are derived from. The carbon skeletons used by plants for amino acid biosynthesis are derived from only a handful of intermediates of glycolysis, photosynthetic carbon reduction, the oxidative pentose phosphate pathway and the citric acid cycle: 3-phosphoglycerate, pyruvate, phosphoenolpyruvate, α-ketoglutarate and oxaloacetate.

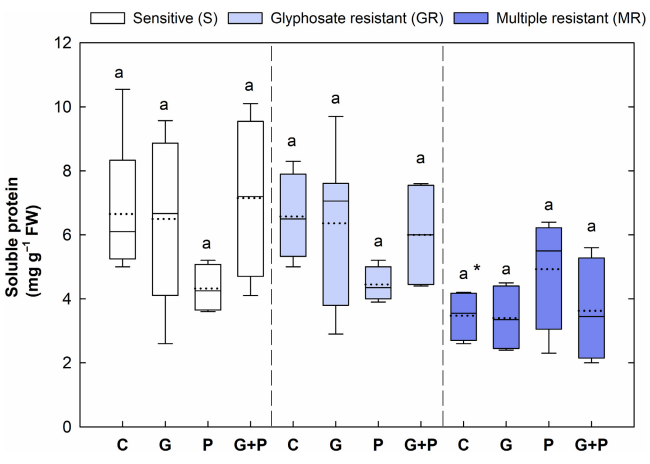

FIGURE 1 Soluble protein content in leaves of three populations of *Amaranthus palmeri*, sensitive (S, white boxes), glyphosate-resistant (GR, light blue boxes) and multiple-resistant (MR, dark blue boxes), 3 days after herbicide treatments: glyphosate (0.84 kg ha^{-1}), pyrithiobac (89 g m.a ha^{-1}), the mixture of both herbicides (GP) and plants without treatment (C). The symbol * indicates significant differences between the control of this population and S population. In each population the different letters indicate statistically significant differences between treatments (ANOVA, HSD Tukey/Dunnett's T3, *p* value <0.05).

The mean contents of each amino acid in the leaves and the significant differences among them are included in Table S1.

Serine and glycine (amino acids derived from 3-phosphoglycerate) were accumulated in all populations only after pyrithiobac and the mixture of glyphosate and pyrithiobac. Amino acids derived from pyruvate were the lowest affected by the treatments, and accumulation was only detected after glyphosate in the S population. The accumulation of amino acids derived from oxaloacetate was evident only in treated S plants. Amino acids derived from phosphoenolpyruvate and α-ketoglutarate were mostly accumulated after the three treatments in the S population, after pyrithiobac and the mixture in the GR population, and only after the mixture in the MR population. Although the differences were not very evident, it can be proposed that amino acids with a carbon skeleton of oxaloacetate were the groups most accumulated in sensitive treated plants.

3.2 | VPEs are activated upon treatment

To establish activity profiling of VPEs in *A. palmeri*, a leaf extract was labelled from a mix of all three populations, separated on protein gels and scanned for fluorescence. Three fluorescent signals between 35 and 40 kDa were detected, which were absent in the no-probe control (Figure 3A). These signals are consistent with active VPE isoforms detected in *Arabidopsis* (Lu et al., 2015; Misas-Villamil et al., 2013). To confirm these signals were from VPEs, we pre-incubated the extracts with the tetrapeptide chloromethyl inhibitor YVAD-cmk, which inhibits VPEs (Lu et al., 2015; Misas-Villamil et al., 2013), and with E-64, which inhibits PLCPs, but not VPEs (Lu et al., 2015). JOPD1 labelling was blocked by YVAD-cmk but not by E-64 (Figure 3A), confirming that these signals were derived from VPEs.

VPE activities in leaves of *A. palmeri* treated with herbicides were monitored by labelling extracts of sensitive and resistant *A. palmeri*. Interestingly, the top VPE signal increased about 4-fold in intensity in all five sensitive plants treated with herbicides. Because individual plants were not genetically identical, this experiment was performed on five individuals from each *A. palmeri* population, showing similar results (Figure S1) and one representative gel is shown in Figure 3B. Quantification of fluorescence intensities of the top VPE bands over these individual plants demonstrated that the increased VPE activity detected in the first band was statistically significant compared to control in all treated sensitive plants (S after all treatments and GR after P and GP) (Figure 3C). Collectively, these data demonstrate that active VPEs are significantly activated in sensitive plants treated with herbicides.

3.3 | PLCPs induction suggests a general activation of cysteine proteases in treated sensitive plants

After the use of the fluorogenic probe E64-Cy5 to label PLCP activity, two signals between 25 and 35 kDa were detected. Preincubation of the extracts with the inhibitors YVAD-cmk (VPE inhibitor) or E-64 (PLCP inhibitor) was performed to confirm the signals

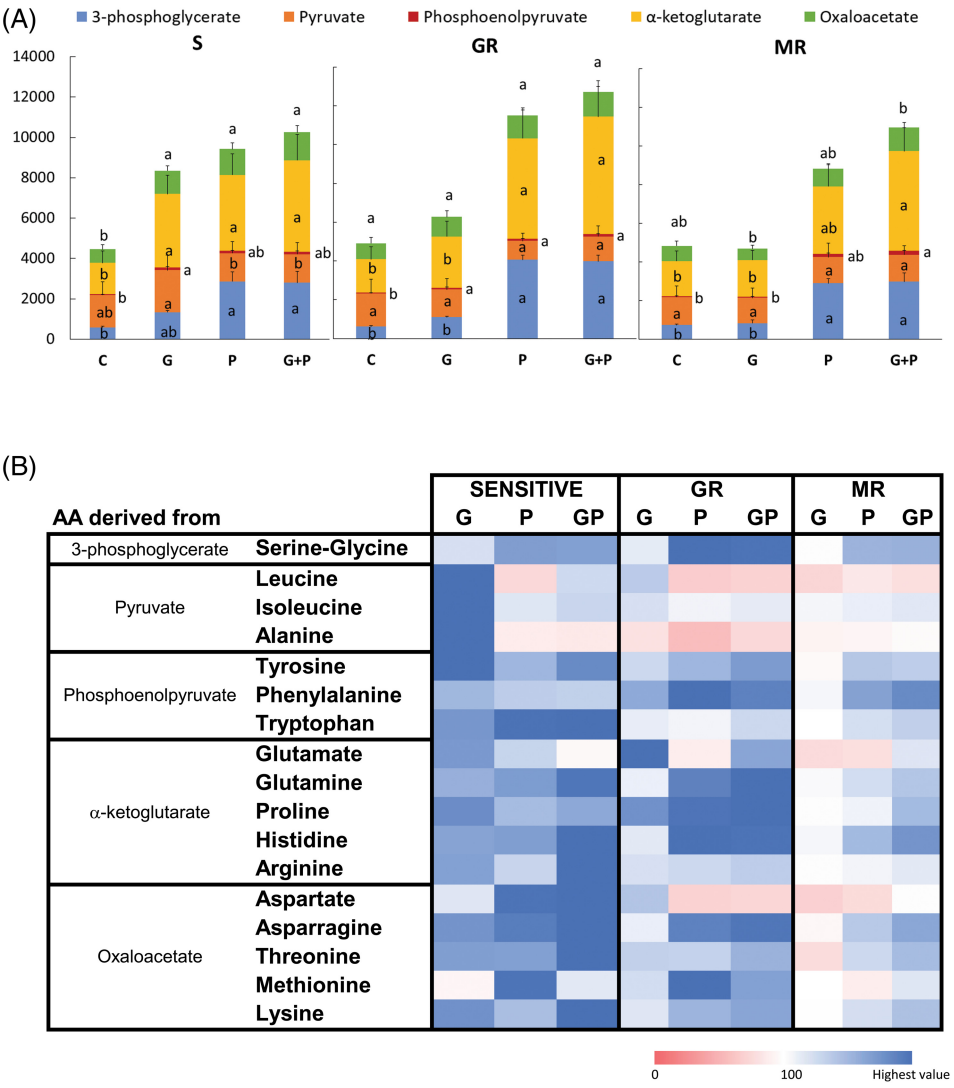

FIGURE 2 Free amino acid pattern in leaves of three populations of *Amaranthus palmeri*, S (Sensitive), GR (Glyphosate-resistant) and MR (Multiple-resistant), 3 days after herbicide treatments: glyphosate (0.84 kg ha^{-1}), pyrithiobac (89 g m.a ha^{-1}), the mixture of both herbicides (GP) and plants without treatment (C). (A) Amino acids are classified by the intermediates they are derived from. In each population for each amino acid type, the different letters indicate statistically significant differences between treatments (ANOVA, HSD Tukey/Dunnett's T3, p value <0.05). (B) Heatmap of each relative amino acid content (% of the mean to the mean of control plants of the respective population).

as PLCPs. The inhibitor E-64 blocked E64-Cy5 labelling while YVAD-cmk did not (Figure 4A), confirming that these signals were derived from PLCPs.

The activity of PLCPs was evaluated to determine whether their activities were affected by herbicide treatment in *A. palmeri* plants. As in VPEs, because plants were not genetically identical, this experiment was performed on five individuals from each *A. palmeri* population, showing similar results (Figure S2), and Figure 4B shows a representative gel. Quantification of fluorescence intensities of the first band demonstrated that the pattern detected in the PLCPs response was similar to VPEs. Only in the S population was PLCPs activity induced upon G, P and GP herbicide treatment, but the signal was only significant with the mixture (Figure 4C). In GR, a significant increase with P or GP was observed. On the contrary, the proteolytic pattern of PLCPs in the MR population was not modified upon herbicide treatment. Thus, both VPEs and PLCPs are activated upon herbicide treatment in sensitive plants.

3.4 | Serine hydrolases

The role of SHs was evaluated by ABPP to determine whether this activity was also affected by herbicide treatment. No consistent pattern was observed between replicates (Figure S3), and a representative gel is shown in Figure 5. In each set of individuals from the three populations (S, GR and MR), the responses were very different in each treatment. A first group of signals was observed between 35 and 40 kDa, and a second group of signals were detected at 25 kDa (Figure 5). These patterns detected in the fluorescent protein gel were very different. In some cases, one band was detected, and in others, up to 4 bands, so it was not possible to establish a clear pattern of these proteases to the different herbicide treatments in the three populations. To detect a pattern of activation or inactivation on the whole enzymatic activity the total intensity of the bands in each was quantified (Figure S4) and no clear effect was detected. These different profiles are probably caused by genetic heterogeneity of the used populations.

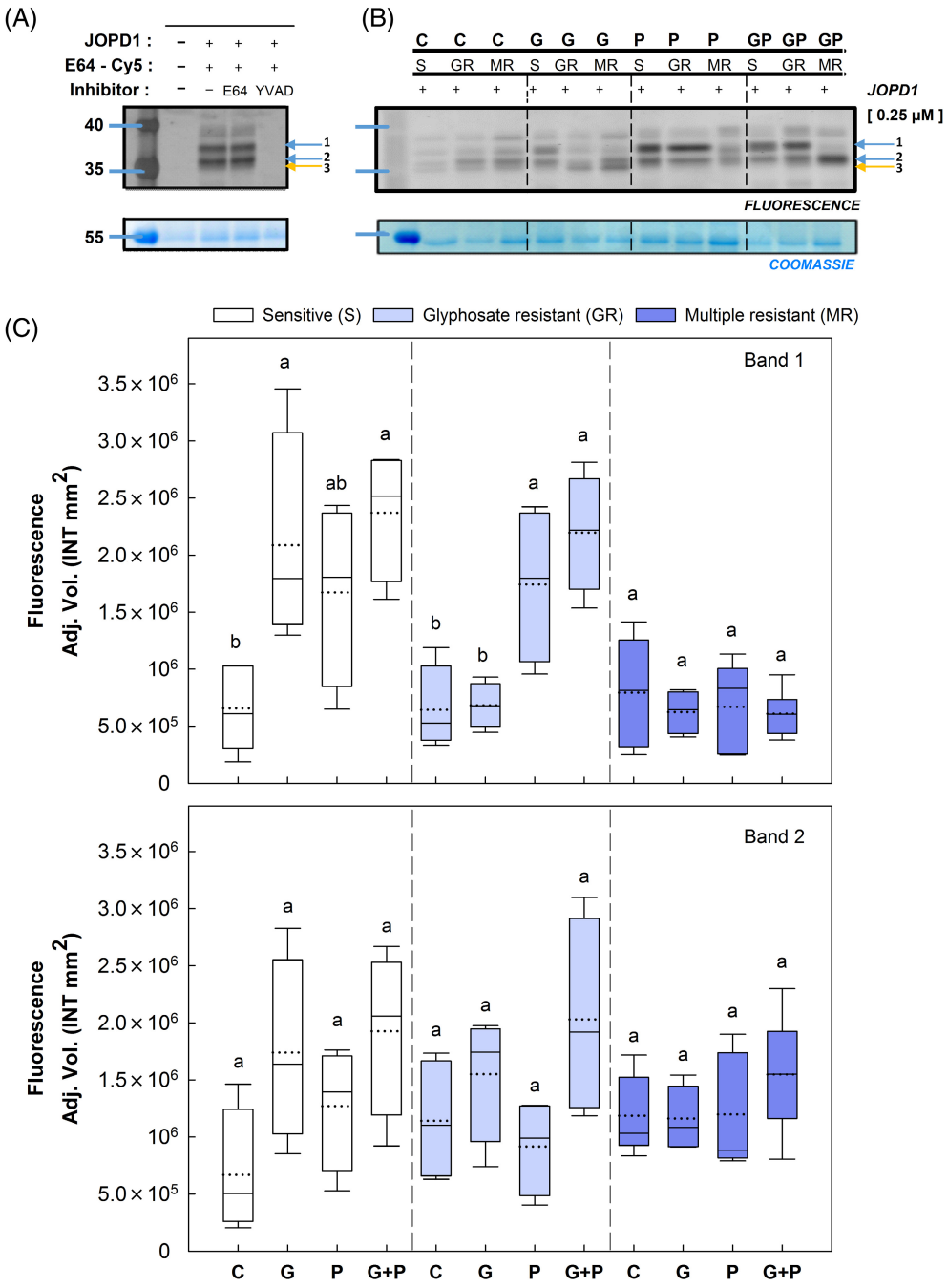

FIGURE 3 Vacuolar processing enzymes (VPEs) are activated upon herbicide treatment in sensitive plants. Leaves of three populations of *Amaranthus palmeri*, sensitive (S, white boxes), glyphosate-resistant (GR, light blue boxes) and multiple resistant (MR, dark blue boxes), 3 days after herbicide treatments; glyphosate (G, 0.84 kg ha^{-1}), pyrithiobac (P, 89 g m.a ha^{-1}), the mixture of both herbicides (GP) and plants without treatment (C). (A) Competitive assay with leaf extracts pre-incubated with E64 and YVAD-cmk inhibitors labelling with the probe JOPD1. A representative mix gel (made up of control and treated individuals) is shown. Arrows indicate VPEs. In the coomassie-stained protein gel, the band corresponding to the rubisco (55 kDa) is shown as the loading control band, showing the total amounts of input proteins. (B) Labelling of control and treated plants. A representative gel is shown. (C) The signals corresponding to the first and second band (from the top) were quantified using a densitometer and the relative values are shown in the box-plots ($n = 5$). In each population the different letters indicate statistically significant differences between treatments (ANOVA, HSD Tukey/Dunnett's T3, p value <0.05).

4 | DISCUSSION

4.1 | VPEs and PLCPs induction in the mode of action of the herbicides

Even though research on plant proteases is relevant for characterising resistance and physiological mechanisms, few studies have analysed the role of proteases in protein degradation in response to herbicide treatment, even less in resistant populations. So, the present study has evaluated the effects of glyphosate, pyrithiobac and their mixture on protease activity of *A. palmeri* sensitive and resistant populations to glyphosate or to glyphosate and pyrithiobac.

The two upper bands of VPEs and the upper band of PLCPs of the S population showed a greater signal after individual herbicide treatments and mixture GP, indicating that the induction of the VPEs and PLCPs is a common response to both types of herbicides, as has been proposed for carbon and nitrogen metabolism for these two types of amino acid biosynthesis inhibitors targeting different enzymes (Orcaray et al., 2010, 2012; Zabalza et al., 2011; Zulet et al., 2015). This result confirms previous studies with pea roots, which showed a common proteolytic profile upon glyphosate or imazamox (another ALS inhibitor) treatment (Zulet et al., 2013). However, contrary to our results, proteolytic activities of VPEs in Zulet et al. (2013) decreased after herbicide treatment instead of displaying an increase.

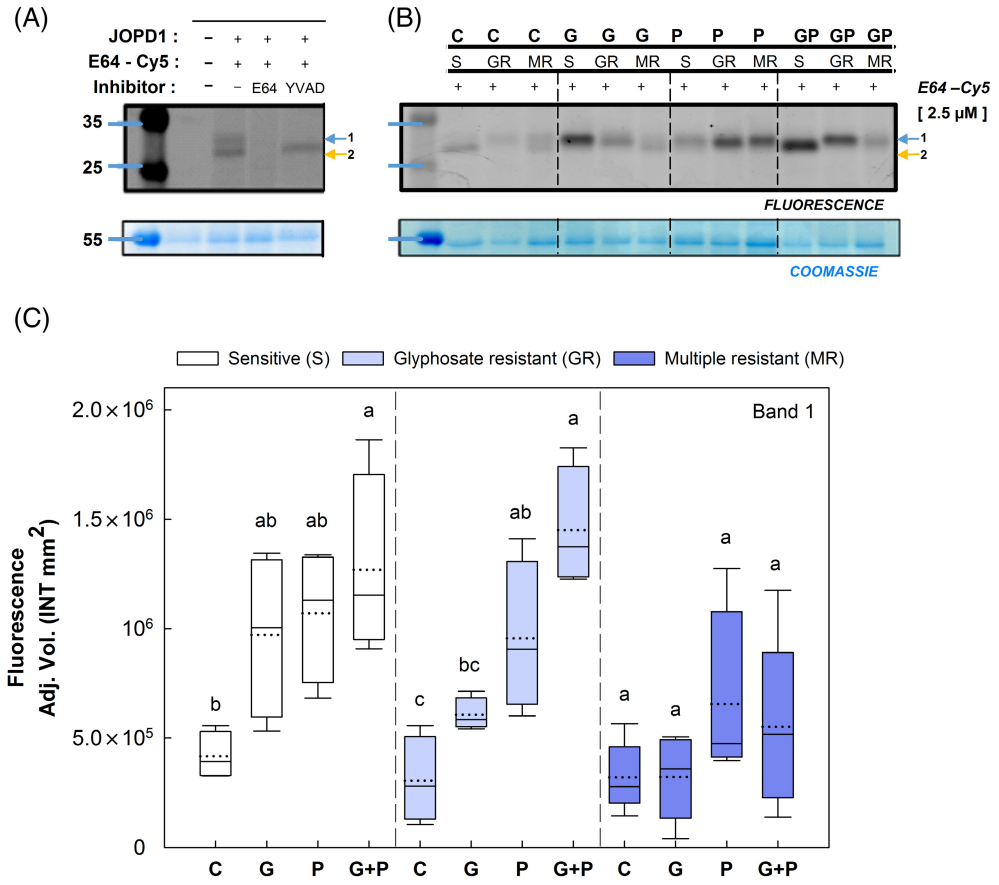

FIGURE 4 Identification and labelling of Papain-like cysteine proteases (PLCPs) in leaves of three populations of *Amaranthus palmeri*, sensitive (S, white boxes), glyphosate-resistant (GR, light blue boxes) and multiple resistant (MR, dark blue boxes), 3 days after herbicide treatments; glyphosate (G, 0.84 kg ha^{-1}), pyrithiobac (P, 89 g m.a ha^{-1}), the mixture of both herbicides (GP) and plants without treatment (C). (A) Competitive assay with leaf extracts pre-incubated with E64 and YVAD-cmk inhibitors labelling with the probe E64-Cy5. A representative mix gel (made up of control and treated individuals) is shown. Arrows indicate PLCPs. In the coomassie-stained protein gel, the band corresponding to the rubisco (55 kDa) is shown as the loading control band, showing the total amounts of input proteins. (B) Labelling of control and treated plants. A representative gel is shown. The coomassie-stained gel is identical to the one of Figure 3 as it is the same gel but labelled with another probe with signal in other size. (C) The signals corresponding to the first band (from the top) were quantified using a densitometer and the relative values are shown in the box-plots ($n = 5$). In each population the different letters indicate statistically significant differences between treatments (ANOVA, HSD Tukey/Dunnett's T3, p value <0.05).

Serine hydrolases are the largest class of plant proteases (Schaller et al., 2018), and they are involved in many biological processes, including metabolism and detoxification processes (Kaschani et al., 2012). We did not find any consistent pattern in the proteolytic pattern of SHs (Figure 5). On the contrary, a consistent pattern of three signals was found in pea roots treated with glyphosate or imazamox and labelled with FP-based probes, although the response was not equal for the three bands detected (Zulet et al., 2013). The heterogeneity detected in this study could be related to the high genetic variation of the dioecious weed *A. palmeri*.

The decrease in the soluble protein and the increase in the amino acid contents are previously known effects of herbicides inhibiting amino acid biosynthesis (Anderson & Hibberd, 1985; Gaston et al., 2002; Orcaray et al., 2010; Shaner & Reider, 1986; Zabalza et al., 2006, 2011, 2013). An increase in the protein turnover rate after herbicide treatment has been proposed to explain the increase in the free amino acid pool and the decrease in total soluble proteins (Rhodes et al., 1987). Indeed, in *A. palmeri*, isotopic studies revealed that both de novo synthesis of amino acids and protein turnover contribute to amino acid accumulation in response to glyphosate (Maroli et al., 2016). Although there was a general increase of individual amino acids in treated sensitive plants, amino acids derived from oxaloacetate were the group most accumulated. Such specific accumulation has been reported after heat shock (Xu & Fu, 2022) or high temperatures (Yamakawa & Hakata, 2010). The concomitant increase of amino acid content and VPEs and PLCPs after herbicide treatments detected in this study suggest that cysteine proteases might be involved in protein degradation to provide plants with amino acids that cannot otherwise be synthesised due to herbicide inhibition.

The activity or expression of cysteine protease genes can be induced under abiotic stresses (Cilliers et al., 2018; Khanna-Chopra et al., 1999) and is related to the degradation of damaged or

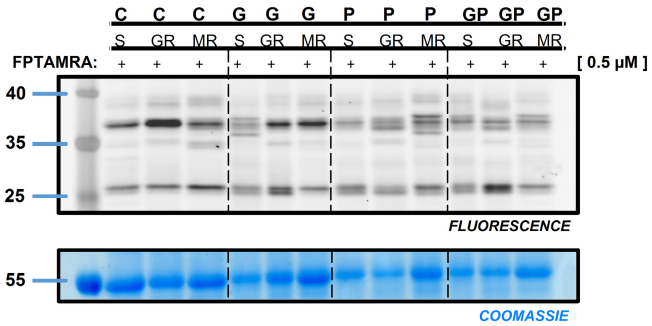

FIGURE 5 Representative example of the labelling profile of serine hydrolases (SHs) in leaves of three populations of *Amaranthus palmeri*, sensitive (S), glyphosate-resistant (GR) and multiple resistant (MR), 3 days after herbicide treatments; glyphosate (G, 0.84 kg ha^{-1}), pyrithiobac (P, 89 g m.a ha^{-1}), the mixture of both herbicides (GP) and plants without treatment (C). The incubation was performed with 0.5 µM FP-TAMRA probe for 90 min at RT in the dark. Fluorescently labelled proteins were detected in protein gels via fluorescence scanning. In the coomassie-stained protein gel, the band corresponding to the rubisco (55 kDa) is shown as the loading control band, showing the total amounts of input proteins.

unnecessary proteins (Martínez et al., 2012). Although the specific knowledge about VPEs role in weeds is still rather limited, it is widely accepted that VPEs are enzymes that play an important role in environmental (biotic and abiotic) stress responses (Christoff & Margis, 2014; Vorster et al., 2019). PLCPs are involved in a variety of proteolytic physiological functions such as plant defence, senescence and response to biotic stresses (Martínez et al., 2012; McLellan et al., 2009; van der Hoorn, 2008). The induction of cysteine proteases detected in this study can be considered as the effect of abiotic stresses, such as herbicides, although the role of the physiological response of cysteine proteases is not yet completely elucidated.

In this study, amino acid content and proteolytic activities were used to evaluate the interaction of the herbicide mixture. The changes detected in amino acid content, VPEs and PLCPs in response to herbicide mixtures were similar to those detected after individual treatments and less than the sum of the individual effects. This evidenced that this physiological perturbation was not exacerbated if both herbicides were applied together, an interaction that could have practical implications in the widespread use of a mixture of glyphosate and ALS inhibitors (Barco-Antoñanzas et al., 2022; Fernández-Escalada et al., 2019). When both herbicides were applied together, the recommended rates had to be fully applied, as toxic effects were not synergic.

4.2 | Proteolysis was not induced in the resistant populations

In order to detect additional physiological traits in the resistant populations, the comparison of basal levels of amino acid content among populations was compared. In the absence of herbicide treatments, basal levels between the S and the GR population were similar, as previously reported (Fernández-Escalada et al., 2016, 2017). Only the basal content of branched-chain amino acids was higher in MR than in the S population, probably due to the altered ALS sensitivity to branched-chain amino acid feedback inhibition, as was reported before (Barco-Antoñanzas et al., 2022; Yu et al., 2010).

No different pattern or signal intensity in VPEs (Figure 3) and PLCPs (Figure 4) was observed among the untreated individuals of the three populations, suggesting that the target-site resistance mechanisms of the resistant populations (*EPSPS* gene amplification and/or *ALS* sequence mutation) have no major additional effect on the proteolytic profile of the untreated resistant plants.

The common effects of both herbicides on VPEs and PLCPs were not observed in the resistant populations treated with the herbicide they are resistant to (Figures 3 and 4). In the GR and MR populations, the cysteine proteases induction was not detected after glyphosate treatment and after all treatments, respectively. So, it seems that changes in the proteolytic profile are not contributing to the resistance physiology of GR or MR populations.

A clear induction of cysteine proteases activity was detected when EPSPS or ALS were inhibited, suggesting that the increase in proteolytic activities is related to the toxicity of both herbicides as it is not detected in resistant plants with resistance mechanisms related to the enzyme. However, the exact mechanisms linking proteolysis induction and EPSPS/ALS inhibition remain unclear and may involve some other physiological aspects of the mode of action of the herbicides.

5 | CONCLUSIONS

A common induction after pyrithiobac and glyphosate has been detected in the VPEs and PLCPs proteolytic activities in the sensitive population. The increase in these cysteine protease activities could be responsible for a higher degradation of the available proteins and, consequently, to the observed increase in free amino acids. No consistent pattern was observed in the profile of SHs.

In the resistant populations, changes in the proteolytic profiles are not relevant as a resistance mechanism, since the control samples of the three populations were similar. Herbicides did not change the proteolytic activities in the resistant populations with target-site resistance mechanisms to glyphosate (in the GR population) or to glyphosate and ALS inhibitors (in the MR population), indicating that the proteolytic response is elicited after EPSPS and/or ALS inhibition.

AUTHOR CONTRIBUTIONS

Mercedes Royuela, Ana Zabalza and Renier A. L. van der Hoorn designed the experiments. Maria Barco-Antoñanzas and Maria Font-Farre performed the experiments. Maria Barco-Antoñanzas analysed the data. Ana Zabalza oversaw the project and wrote the paper with input from Mercedes Royuela, Miriam Gil-Monreal, Mikel V. Eceiza and Renier A. L. van der Hoorn. Mercedes Royuela

supervised the project and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article and supplementary material.

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