Research Article

Received: 5 September 2017 Revised: 16 October 2017 Accepted article published: 26 October 2017 Published online in Wiley Online Library: 8 December 2017

(wileyonlinelibrary.com) DOI 10.1002/ps.4774

Multiple resistance to glyphosate, paraquat and ACCase-inhibiting herbicides in Italian ryegrass populations from California: confirmation and mechanisms of resistance

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Abstract

BACKGROUND: Glyphosate, paraquat and acetyl CoA carboxylase (ACCase)-inhibiting herbicides are widely used in California annual and perennial cropping systems. Recently, glyphosate, paraquat, and ACCase- and acetolactate synthase (ALS)-inhibitor resistance was confirmed in several Italian ryegrass populations from the Central Valley of California. This research characterized the possible mechanisms of resistance.

RESULTS: Multiple-resistant populations (*MR1*, *MR2*) are resistant to several herbicides from at least three modes of action. Dose–response experiments revealed that the *MR1* population was 45.9-, 122.7- and 20.5-fold, and the *MR2* population was 24.8-, 93.9- and 4.0-fold less susceptible to glyphosate, sethoxydim and paraquat, respectively, than the susceptible (*Sus*) population. Accumulation of shikimate in *Sus* plants was significantly greater than in *MR* plants 32 h after light pretreatments. Glyphosate resistance in *MR* plants was at least partially due to Pro106-to-Ala and Pro106-to-Thr substitutions at site 106 of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS gene copy number and expression level were similar in plants from the *Sus* and *MR* populations. An Ile1781-to-Leu substitution in ACCase gene of *MR* plants conferred a high level of resistance to sethoxydim and cross-resistance to other ACCase-inhibitors. Radiolabeled herbicide studies and phosphorimaging indicated that *MR* plants had restricted translocation of ^{14}C-paraquat to untreated leaves compared to *Sus* plants.

CONCLUSION: This study shows that multiple herbicide resistance in Italian ryegrass populations in California, USA, is due to both target-site and non-target-site resistance mechanisms.

Keywords: herbicide resistance; resistance mechanism; glyphosate; shikimate accumulation; EPSPS copy number; ACCase; cross-resistance; paraquat; translocation; *Lolium perenne* ssp. *multiflorum*; *Lolium multiflorum*

1 INTRODUCTION

Italian ryegrass (*Lolium perenne* spp. *multiflorum*) is a major annual grass weed in several cropping systems worldwide.[1] It thrives under mild environmental regimes and is highly competitive in several annual and perennial crops.[2–4] To date, Italian ryegrass populations have evolved resistance to eight herbicide mechanisms of action worldwide.[5] Within the USA, resistance to acetyl CoA carboxylase (ACCase), acetolactate synthase (ALS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), glutamine synthetase (GS), microtubule assembly and very long-chain fatty acid (VLCFA) inhibitors has been confirmed in several populations from 14 states. In 1998, multiple resistance to ACCase and ALS herbicides was identified in several wheat fields in Arkansas[6] and was the first confirmed case of Italian ryegrass populations with resistance to two modes of action.[5] However, evolved multiple resistance to dissimilar herbicide groups in Italian ryegrass is now reported in at least eight states.

In California, glyphosate has been the primary herbicide used for weed control in orchards, vineyards, field edges and ditches for decades.[7] Glyphosate is a non-selective herbicide used to control a broad spectrum of annual and perennial weeds by inhibiting the plastidic enzyme EPSPS (EC 2.5.1.19). This inhibition causes shikimate accumulation in glyphosate-sensitive plants, blocking the aromatic amino acid biosynthesis pathway.[8–10] Glyphosate resistance was first reported in California in 1998 when glyphosate failed to control a rigid ryegrass (*Lolium rigidum* Gaud.) population in an almond orchard.[11] In 2005, glyphosate-resistant plants were identified in approximately half of the 118 Italian ryegrass

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populations sampled from orchards, vineyards, roadsides and crop fields across the Central Valley.[12] In 2015, > 75% of the populations contained glyphosate-resistant plants (unpublished data). The increased incidence of glyphosate-resistant Italian ryegrass in California is likely in response to the high grower use of glyphosate over other herbicide mechanisms of action.

In addition to being retested for glyphosate resistance, Italian ryegrass populations resampled in 2015 were tested for resistance to glufosinate, paraquat and sethoxydim at one and two times the recommended field rate (unpublished data). These herbicides are commonly used to control California ryegrass suspected of being resistant to glyphosate (Brad Hanson, University of California Cooperative Extension Specialist, pers. commun.). Multiple resistance to glyphosate, sethoxydim and/or paraquat (in at least two or three combinations) was confirmed in several populations.[5,13] Until this study, multiple resistance to glyphosate, paraquat and ACCase-inhibiting herbicides in *Lolium* species was only reported in Australia.[14]

For several years, ACCase inhibitors have provided excellent control of ryegrass species in both winter cereals and broadleaf crops worldwide.[15] However, less than a decade after the introduction of these herbicides (1980s), diclofop-methyl-resistant Italian ryegrass was identified in the USA.[16] To date, resistance to aryloxyphenoxypropionate (APP) herbicides has been reported in several Italian ryegrass biotypes in cropping regions across the USA.[5] Based on chemical structure, APP, cyclohexanedione (CHD) and phenylpyrazolin (PPZ) herbicides specifically target the carboxyl transferase domain of the homomeric plastidic isoform of the ACCase enzyme (E.C. 6.4.1.2) in almost all grass weeds.[17] Several molecular and biochemical studies on ACCase inhibitor-resistant ryegrass populations from different geographical regions of the world revealed both enhanced metabolism and target site alterations as the mechanisms of resistance.[17]

Paraquat is a fast-acting contact herbicide commonly used to control grass and broadleaf weeds.[14,18] It is an alternative to glyphosate and one of the most widely used bipyridylium (1,1′-dimethyl-4,4′-bipyridynium dichloride) herbicides with rapid activity in the light and no activity in soil.[19,20] To date, resistance to paraquat has been reported in 33 weed species worldwide, including rigid ryegrass.[5] Recently, paraquat at the labeled field rate also failed to control an Italian ryegrass population from a prune orchard in California.[21]

In this study, we selected two multiple-resistant populations with negligible/zero glyphosate, sethoxydim and paraquat injury to elucidate the underlying resistance mechanisms. The objectives of this study using the two multiple-resistant populations and one susceptible population were to: (1) assess the level of resistance to glyphosate, sethoxydim and paraquat; (2) determine the cross-resistance patterns to ACCase-inhibiting herbicides across two different chemical families; and (3) characterize the mechanisms of resistance to each herbicide.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Seeds of 83 Italian ryegrass populations were collected in 2015 as part of a survey evaluating the distribution of glyphosate resistance in Italian ryegrass from a diversity of crops in northern California (unpublished). Seeds were bulked from 30–50 mature individuals in each sampling location. Based on preliminary herbicide-screening experiments (data not shown), several Italian ryegrass populations exhibited multiple resistance to glyphosate, sethoxydim and paraquat. The multiple herbicide-resistant populations from an almond orchard (*MR1*) and an alfalfa field (*MR2*) in Glenn County used in this study were resampled for seed in 2016 and retested for multiple resistance. A known glyphosate-susceptible population from a vineyard in Sonoma County was used as the susceptible population (*Sus*). The *Sus* population was confirmed to be susceptible to glufosinate, paraquat, and ACCase at the respective recommended rates in a greenhouse study.

Seeds of the three populations (*Sus*, *MR1* and *MR2*) were placed on moist filter paper in Petri dishes (150 × 15 mm) and kept at 4 °C for 24 h to maximize germination. Subsequently, seeds were inoculated with 1% v/v Captan 80 WDG (Agri Star, Ankeny, IA, USA) and incubated at ambient temperature under a 12: 12 h photoperiod provided by fluorescent lights (160 μmol m^{-2} s^{-1}). Seedlings were transplanted at the one- to two-leaf stage into plastic pots (50 mm height × 4.5 mm diameter) filled with UCD soil mix (1: 1: 1: 3 sand/compost/peat/dolomite) and maintained in a greenhouse set at 25/18 °C day/night temperature and 14: 10 h photoperiod provided by high pressure sodium lights (400 μmol m^{-2} s^{-1}). Seedlings were watered daily and fertilized once a week using a water-soluble commercial fertilizer (Miracle-Gro Products, Marysville, OH, USA). Plants from each population were treated independently with different herbicides (Table 1) at the three- to four-leaf stage (88.5–110.5 mm height) using an automated spray chamber equipped with a flat-fan 8001E nozzle (TeeJet Technologies, Springfield, IL, USA). The sprayer was calibrated to deliver 187 L ha^{-1} of herbicide solution at a pressure of 296 kPa.

2.2 Whole-plant bioassays

2.2.1 *Dose–response experiments*

Experiments were arranged in a randomized complete block factorial design (RCBD) with three populations and 25 replications of individual seedlings for each herbicide dose. Each experiment was repeated twice. *MR* plants were treated at 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 times the labeled field rate of 867 g ae ha^{-1} for glyphosate and 515 g ai ha^{-1} for sethoxydim; *Sus* plants were treated with 0, 0.03, 0.06, 0.12, 0.25, 0.5, 1 and 2 times the labeled field rates of these herbicides. All sethoxydim treatments contained a Pro Crop Oil (Integrated Agribusiness Professionals, Fresno, CA, USA) at 1% v/v. Both *MR* and *Sus* plants were treated with paraquat at 0, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 times the labeled field rate (560.4 g ai ha^{-1}) containing 1% v/v crop oil concentrate. Treated plants were maintained in the greenhouse under the same conditions as described above and plant mortality was recorded 21 days after treatment (DAT). All data were subjected to analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (v. 9.4, SAS Institute Inc., Cary, NC, USA). No significant treatment by experiment interactions were observed; therefore, the data obtained from the repeated experiments for each herbicide were pooled. Mortality data were subjected to probit analysis using PROC PROBIT in SAS to evaluate the amount of each herbicide required to kill 50% (LD_{50}) of the resistant and susceptible Italian ryegrass populations. Fold resistance ratios (R/S) were calculated by dividing the LD_{50} values of the *MR* populations by the LD_{50} values of the *Sus* population.

2.2.2 *Cross-resistance to ACCase-inhibiting herbicides*

The experiment was conducted in a RCBD factorial with three Italian ryegrass populations by five herbicide treatments to evaluate the efficacy of APP and CHD herbicides commonly used in California cropping systems. The experiment was repeated once. Plants

Table 1. List of herbicides and labeled field rates used in dose–response and cross-resistance studies

Common name	Trade name	Herbicide family	Field rate (g ai/ae ha^{-1})	Adjuvant	Manufacturer
Glyphosate	RoundupPowerMax	Glycines	867*	–	Monsanto
Paraquat	Gramoxone SL 2.0	Bipyridyliums	560	0.25% NIS	Syngenta Crop Protection
ACCase inhibitors					
Sethoxydim	Poast	Cyclohexanediones	515	1% COC	BASF Corporation
Clethodim	SelectMax	Cyclohexanediones	146	1% COC	Valent
Fluazifop-P-ethyl	Fusilade DX	Aryloxyphenoxypropionates	448	1% COC	Syngenta Crop Protection
Fenoxaprop-P-ethyl	Acclaim Extra	Aryloxyphenoxypropionates	128	–	Bayer CropScience
Cyhalofop-butyl	Clincher SF	Aryloxyphenoxypropionates	313	1% COC	Dow AgroScience

ACCase, acetyl CoA carboxylase; COC, crop oil concentrate; NIS, nonionic surfactant.
*Acid equivalent.

were treated individually with each herbicide solution at the recommended rate as shown in Table 1. Above-ground biomass of all Italian ryegrass individual plants was harvested 21 DAT, oven dried at 60 °C for 48 h and weighed. Dry weight reduction percentage was calculated based on the mean of the non-treated control. A normality test was conducted using PROC UNIVARIATE and then data were subjected to PROC MIXED in SAS. No significant differences were observed between the two runs; therefore, pooled data were analyzed and means separated using Fisher's LSD at $\alpha = 0.05$.

2.3 Shikimate accumulation assays

Leaf disc assays[22,23] were conducted to measure the accumulation of shikimate in young leaves of plants from the *Sus*, *MR1* and *MR2* populations. The experiment was conducted in a split-split-plot in a RCBD with six light pretreatments (0, 2, 4, 8, 16 and 32 h) as the main plots, three Italian ryegrass populations (*Sus*, *MR1*, *MR2*) as subplots and 12 glyphosate doses as sub-subplots with eight replications. The experiment was conducted twice. *Sus* and *MR* plants were grown until the three-tiller stage in the greenhouse under the growing conditions described earlier. Each of the three tillers of each individual were separated and transplanted into new pots, and allowed to regrow to the three- to four-leaf stage. Individuals were cloned to obtain enough young leaf tissue for each individual of the same genotype. Leaf discs (4 mm in diameter) were excised from the third fully expanded leaf of each clone using a single-hole paper puncher and placed in each well of a 96-well microplate (Thermo Scientific, Pittsburgh, PA, USA) containing solutions with glyphosate doses ranging from 0 to 1250 μM. Solutions also contained 10 mM ammonium phosphate monobasic and 0.1% v/v Tween 80 surfactant. Plates were covered with 120 × 90 mm Parafilm®, topped with a lid, and sealed with a strip of Parafilm prior to the light pretreatments provided by fluorescent lights (160 μmol m^{-1} s^{-1}) at 25 °C. After incubation, plates were transferred to a – 20 °C freezer and kept frozen until further analysis. Subsequent biochemical experiments were performed based on procedures described by Shaner *et al.*[22] The herbicide concentrations used in this assay were 0, 5, 10, 25, 50, 100, 200, 400, 500, 700, 1000 and 1250 μM of glyphosate. Known amounts of shikimic acid (i.e. 0, 1, 2.5, 5, 10, 25, 50, 75, 100, 125, 150 and 175 μM) were added to each plate containing non-treated leaf discs to develop a standard curve. Shikimate level was spectrophotometrically (380 nm) determined as micrograms per milliliter HCl for each individual using a DYNEX MRX II microplate reader (Magellan Biosciences, Chantilly, VA, USA). Background absorption (optical density) measured in the control wells was subtracted from the absorbances measured for the glyphosate treated wells. All data were subjected to ANOVA using PROC MIXED in SAS. Means were separated for each treatment using Fisher's LSD at $\alpha = 0.05$. No significant differences were observed between runs. Therefore, to determine the glyphosate dose (μM) resulting in 50% shikimate accumulation (IC_{50}), we fit the following three-parameter sigmoidal model to pooled data using SigmaPlot (v. 12.5, Systat Software Inc., San Jose, CA, USA):

$$y = \frac{a}{1 + e^{-\left(\frac{X/IC50}{b}\right)}} \quad (1)$$

where y is shikimate level, a is maximum shikimate accumulated, and b is the slope of the curve around IC_{50}.

2.4 *EPSPS* gene sequencing

Young leaf tissue was harvested from non-treated *Sus* individuals and from *MR1* and *MR2* individuals that survived the 4× rate (3468 g ae h^{-1}) of glyphosate 21 DAT. RNA was extracted from six plants of each population using RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). RNA quantity was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and cDNA constructed using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, Petaluma, CA, USA). To amplify the region covering the Pro_{106} codon of the EPSPS gene, a primer pair (forward: 5′-AACGGTCGTGGATAACCTGT-3′ and reverse: 5′-CCAGCCAAGAAATAGCTCGC-3′) was designed based on EPSPS mRNA sequence of *L. multiflorum* (GeneBank accession number DQ153168.2) using NCBI/Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Primers were synthesized by Integrated DNA Technologies (Redwood City, CA, USA).

Polymerase chain reaction (PCR) was performed in 25 μL reaction mixture containing ~ 16 ng cDNA, 12.5 μL GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.5 μL of each 10 μM primer and Nuclease-Free Water (Promega). A Bio-Rad T100 Thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) was programmed as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C (denaturation) for 30 s, 57 °C (annealing) for 45 s, 72 °C (elongation) for 60 s, and a final elongation at 72 °C for 5 min. The PCR amplicons were fractioned on 1.5% agarose gel pre-stained by a fluorescent nucleic acid dye (GelRed™, Biotium, Fermont, CA, USA) and visualized under UV light. PCR

products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol and used in BigDye (Terminator v. 3.1 Cycle Sequencing Kit, Thermo Fisher Scientific, Waltham, MA, USA) PCR amplification reactions. Subsequently, products were precipitated using Applied Biosystems ethanol and sodium acetate precipitation protocol and sequenced with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were edited and trimmed in BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA) and aligned along with *L. multiflorum* sequence (GeneBank accession number DQ153168.2) using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo). Sequences were submitted to NCBI. Individuals with single peaks at each base position were considered homozygous for that base while individuals with multiple overlapping peaks at a base position were considered heterozygous for the two bases.

2.5 *EPSPS* genomic copy number and expression

Quantitative PCR (StepOnePlus™ real-time detection system, Thermo Fisher Scientific) was performed to determine EPSPS genomic copy number and expression in the glyphosate-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass. A glyphosate-resistant Italian ryegrass (*LOLR*) known to have increased EPSPS copies and gene expression, based on earlier studies,[24,25] was included as a positive control. For EPSPS copy number analysis, genomic DNA was extracted using Qiagen DNEasy Plant Mini Kit following the manufacturer's provided protocol. DNA was quantified using a NanoDrop 1000 spectrophotometer. Genomic DNA was normalized to 20 ng μL^{-1} for direct use in quantitative PCR (qPCR) reactions. For EPSPS gene expression, we used the cDNA samples that were synthesized for the EPSPS gene sequencing. The cDNAs were diluted 1: 5 v/v for direct use in the qPCR reactions. The qPCR reaction mix for determination of both EPSPS copy number and expression consisted of 8 μL of Power SYBR green (Life Technologies, Grand Island, NY, USA), 2 μL each of forward and reverse primers (5 μmol), and 2 μL of the diluted genomic DNA or cDNA to make a final reaction volume of 14 μL. A StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) was programmed for an initial denaturation at 94 °C for 10 min and 30 cycles of 15 s denaturation at 94 °C and 30 s annealing at 60 °C. A melt curve profile was included at the end of the cycle to determine the specificity of the reaction. The EPSPS primer pair (EPSPS *F2*: 5′-CTGATGGCTGCTCCTTTAGCTC-3′ and EPSPS *R2*: 5′-CCCAGCTATCAGAATGCTCTGC-3′) designed by Salas et al.[24,25] were used for the *MR1*, *MR2*, and *Sus* EPSPS gene amplifications. *β-tubulin* primers (forward: 5′ ATGTGGG ATGCCAAGAACATGATGTG-3′ and reverse 5′ TCCACTCCACAAAGTA GGAAGAGTTCT-3′)[26] were used to normalize EPSPS copy number, whereas *actin* primers (forward: 5′-CTGACTGAGGCACCCCTGAA-3′ and reverse: 5′-GCTGACACCATCACCAGAATCCAAC-3′) were used to normalize EPSPS gene expression. The *actin* primers were designed against the *Populus euphratica* (accession number LOC105117324), *Sesamum indicum* (accession number LOC105170653) and *Tarenaya hassleriana* (accession number LOC104823416) sequences obtained from NCBI GenBank. The relative fold increase in EPSPS copy number and gene expression of *MR1* and *MR2* individuals was determined by the comparative C_t method[27] known as $2^{\Delta Ct}$ [$\Delta Ct = C_t$ β-tubulin or *actin* $- C_t$ EPSPS)] relative to the *Sus*. Results for three replicates (individuals) from each population were used to calculate the mean and standard error for each reaction.

2.6 ACCase gene sequencing

RNA was extracted from fresh shoots of six confirmed sethoxydim-resistant (*MR1*, *MR2*) and -susceptible (*Sus*) individuals and cDNA synthesized, as described above for EPSPS gene sequencing. Two sets of primers were designed based on the full chloroplastic ACCase sequence of blackgrass (accession number AJ310767) to amplify the ACCase gene encompassing all seven known mutation sites.[17] The first primer pair (ACCF1: ATGGTAGCCTGGATCTTGGACA and ACCR1: AACTACTGTCTTCGCCCATCC) amplified ~ 730 bp spanning the Ile1781 codon. The second pair (ACCF2: ATCCTCGTGCAGCCATAAGTG and ACCR2: TGCATTCTTGGAGTTCCTCTG) amplified ~ 510 bp spanning the Trp1999, Trp2027, Ile2041, Asp2078, Cys2088 and Gly2096 codons. Primers were designed using NCBI/Primer-Blast program and synthesized by IDT. PCR conditions and sequencing procedures were the same as described above for EPSPS gene sequencing. All sequence chromatograms were checked for potential sequencing errors and trimmed using the BioEdit software. The *MR1*, *MR2* and *Sus* sequences were aligned against blackgrass mRNA for ACCase and sequences of the sethoxydim-resistant plants submitted to NCBI.

2.7 ^{14}C-paraquat absorption, translocation and phosphorimaging

Two- to three-leaf stage plants of the three populations (*Sus*, *MR1*, *MR2*) were grown in the greenhouse, as described above, and transferred to a growth chamber for acclimatization 7 days prior to application of ^{14}C-paraquat. The growth chamber was maintained at 25/20 ± 3 °C with a 14: 10 h light/dark photoperiod (300 μmol m^{-2} s^{-1}) provided by fluorescent and incandescent bulbs.

A solution containing paraquat at a final concentration of 0.84 kg ai ha^{-1} in 190 L ha^{-1} was made by using ^{14}C-paraquat (paraquat-methyl-[^{14}C] dichloride hydrate, 11.84 MBq $mmol^{-1}$ specific activity; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA), a commercial formulation of paraquat (Gramoxone SL 2.0, Syngenta Crop Protection, LLC, Greensboro, NC, USA), and 1% v/v crop oil concentrate. A 1 μL volume of the solution (~ 2.1 kBq of ^{14}C-paraquat) was applied to the middle 3 cm adaxial surface of the second fully expanded leaf of each plant with a micro-applicator in the form of one or two droplets. Plants were harvested 5, 24 and 48 hours after treatment (HAT). At each harvest, the treated leaf (TL) was removed and only the middle 3 cm portion that received ^{14}C-paraquat solution was excised and rinsed in 10 mL of 0.1% v/v Triton X-100 (Sigma-Aldrich) for 20 s to remove the unabsorbed ^{14}C-paraquat from the leaf surface. A 1 mL aliquot of each leaf wash was mixed with 10 mL scintillation cocktail (Ecolume; ICN, Costa Mesa, CA, USA) to measure the unabsorbed ^{14}C-paraquat. After removing the treated leaf, each plant was divided into the shoot above the treated leaf (SATL), the shoot below the treated leaf (SBTL), and the roots for measuring translocation. Each of the four plant parts (treated leaf, SATL, SBTL, roots) were wrapped individually in a single layer of tissue paper (Kimwipes; Kimberly-Clark Corp., Roswell, GA, USA), placed in a glass vial and oven-dried at 60 °C for 48 h. The oven-dried plant samples were combusted in a biological oxidizer (Packard Instruments Co., Downers Grove, IL, USA) and the evolved $^{14}CO_2$ was trapped in 10 mL of Carbosorb E (Packard BioScience Co., Meridian, CT, USA) and 10 mL of Permaflour E+ (Packard BioScience). Levels of radioactivity from the leaf washes and oxidations were quantified by using liquid scintillation spectrometry (Packard Tri-Carb 2100TR; Packard Instruments). The average recovery of

the applied ^{14}C-paraquat was 96% (absorption, expressed as a percentage of the applied ^{14}C), based on the sum of the radioactivity that was measured in all the plant parts and leaf washes. The total level of radioactivity recovered from all plant parts, except the treated leaf, was designated as the translocated ^{14}C and expressed as a percentage of the absorbed ^{14}C-paraquat. The experiment was conducted twice with four replicates per harvest time per population for each experiment.

For each experiment, population and harvest time, the fourth replicate was used for phosphorimaging. As described above, the treated leaf from each of the fourth replicate plants was removed, the middle 3 cm portion that received ^{14}C-paraquat solution was excised and rinsed in 10 mL of 0.1% v/v Triton X-100 (Sigma-Aldrich) for 20 s to remove any unabsorbed radioactivity, and set aside. The remaining above-ground part of each fourth replicate plant was excised from the roots and mounted on a 27 × 21.25 cm piece of plain white paper. Shoot parts were spread out evenly and kept in place with thin strips of clear office tape. The washed treated leaf was also mounted. Care was taken to avoid contact of the washed treated leaf with the other parts of the plant. Prior to mounting with the shoot, roots were gently rinsed with water to remove the soil and blotted dry with paper towels. The mounted plant parts were pressed between one or more layers of newspaper, bound with two hard cardboard sections, held together with large binder clips and stored at −20 °C until drying. Plant samples were dried in a gravity convection oven at 60 °C for 1 h. After cooling the dried sample to room temperature, the plant was placed in a 20 × 40 cm exposure cassette (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and brought into contact with a storage phosphor screen (BAS IPSR 2025 E; GE Healthcare Bio-Sciences) under diffused lighting. The apparatus was placed in a dark cabinet for 24 h. A phosphorimager (Typhoon FLA 7000; GE Healthcare) was used to detect the distribution of ^{14}C-paraquat and to develop an image. After image development, plant parts were carefully dismounted and dried in an oven in preparation for oxidation using the methods described above.

All experiments were conducted using a completely randomized design (CRD). Data were analyzed by ANOVA using PROC GLM in SAS. No significant differences ($P < 0.05$) were detected between experiments therefore data from the two experiments were pooled. Differences among populations were tested using Fisher's Protected LSD test at $P = 0.05$.

3 RESULTS

3.1 Resistance levels

Glyphosate controlled 100% of the *Sus* individuals at the labeled field rate. In contrast, both MR populations exhibited a high level of resistance to glyphosate. Glyphosate LD_{50} values were 93.21, 4280 and 2318 g ae ha^{-1} for the *Sus*, *MR1* and *MR2* populations, respectively (Table 2). In contrast to *MR1* individuals, all *Sus* and *MR2* individuals were killed at the highest rate of glyphosate (27744 g ae ha^{-1}) tested. The resistance index (R/S) calculated from LD_{50} values revealed that the *MR1* and *MR2* populations were 45.9- and 24.8-fold, respectively, less susceptible to glyphosate than the *Sus* population.

Sethoxydim, at the labeled field rate, controlled 100% of the *Sus* individuals whereas both *MR* populations exhibited high levels of resistance to this herbicide (Table 2). LD_{50} values were 93.74, 11 503 and 8803 g ai ha^{-1}, for the *Sus*, *MR1* and *MR2* populations, respectively. Even the highest rate of sethoxydim (16 800 g ai ha^{-1}) used in the dose–response experiment did not kill 90% of *MR1* and *MR2* individuals 21 DAT. Based on the R/S calculated from the LD_{50} values, *MR1* and *MR2* populations were 122.7- and 93.9-fold, respectively, less susceptible to sethoxydim than the *Sus* population.

Table 2. Dose of glyphosate, sethoxydim and paraquat required for 50% (LD_{50}) mortality of the multiple herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass populations

Herbicide	Population	LD_{50}	LD_{50} R/S†
		g ai/ae ha^{-1}	
Glyphosate*	*Sus*	93.21 (71.67–119)	
	MR1	4280 (3421–5370)	45.9
	MR2	2318 (1938–2777)	24.8
Sethoxydim	*Sus*	93.74 (80.94–111)	
	MR1	11 503 (8694–16 918)	122.7
	MR2	8803 (7069–11 269)	93.9
Paraquat	*Sus*	25.04 (20.29–30.07)	
	MR1	514.12 (437.05–589.41)	20.5
	MR2	103.47 (79.78–133.10)	4

Values in parentheses are 95% fiducial limits.
†Resistant/susceptible ratio.
*Acid equivalent.

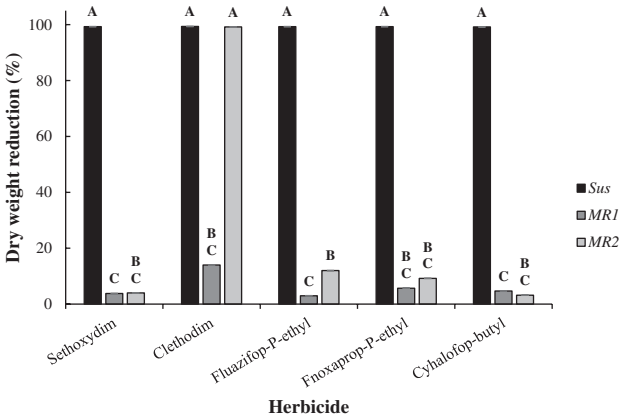

Figure 1. Percentage of above-ground biomass dry weight reduction of plants from herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass populations in response to ACCase-inhibiting herbicides at 21 days after treatment. Mean values for each herbicide followed by different letters indicate significant differences ($\alpha = 0.05$).

Paraquat controlled 100% of the *Sus* individuals at 0.125× the labeled field rate. *Sus* plants showed rapid desiccation 6 h after treatment and were killed within 24 h. All *MR* plants were controlled at the highest paraquat rate (2240 g ai ha^{-1}) tested. Only a few *MR1* individuals survived the 2× rate of paraquat, whereas all *MR2* plants died. The paraquat rates estimated to achieve 50% of the *Sus*, *MR1* and *MR2* were 25, 514 and 103 g ai ha^{-1} (Table 2). Based on the LD_{50} values, the *MR1* and *MR2* populations were 20.5- and 4.1-fold less responsive to paraquat compared to *Sus*. These results suggest that >50% of the *MR1* population would escape the labeled field rate of the herbicide.

3.2 Cross-resistance to ACCase-inhibiting herbicides

Sus individuals were effectively controlled with essentially 100% reduction in above-ground biomass (Fig. 1) at the labeled field rates of all ACCase-inhibiting herbicides used in this study

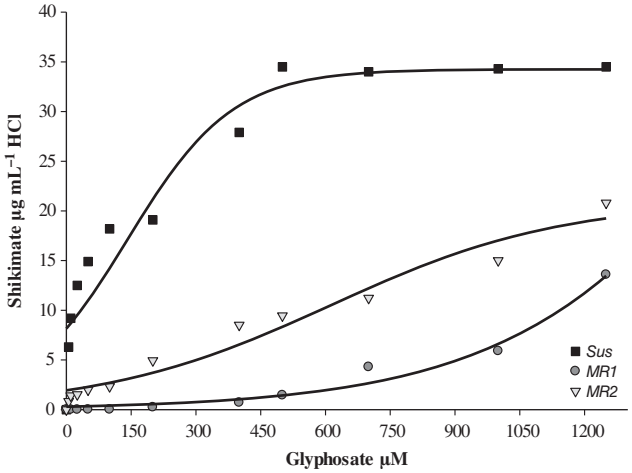

Figure 2. Shikimate accumulation of leaf discs from herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass populations over a range of glyphosate doses. Each data point indicates the averaged amount of shikimate accumulation from two runs pooled over seven leaf discs. Plotted lines are predicted from equation 1 as follows: *Sus*: $y = 34.2/(1 + \exp(-(x/141.5)/121.6)$, $R^2 = 0.92$; *MR1*: $y = 92/(1 + \exp(-(x/1804.2)/314)$, $R^2 = 0.97$; *MR2*: $y = 21/(1 + \exp(-(x/613.2)/267.8)$, $R^2 = 0.95$.

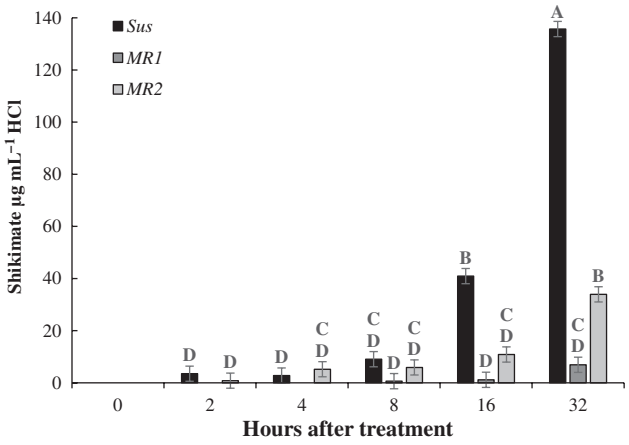

Figure 3. Accumulation of shikimate in herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) leaf discs inoculated in 500 μM glyphosate. The amount of shikimate was averaged over seven replicates. Means followed by the same letters are not significantly different ($\alpha = 0.05$).

(Table 1). In contrast, the above-ground biomass reduction of *MR1* and *MR2* plants was considerably lower ranging from 3% to 14% and 3.2% to 12%, respectively, in response to treatment with sethoxydim, fluazifor-*P*-ethyl, fenoxaprop-*P*-ethyl and cyhalofop-butyl. Interestingly, the response of *MR1* and *MR2* plants to clethodim was significantly different ($\alpha = 0.05$). At the labeled field rate, clethodim resulted in only a 14% biomass reduction in *MR1* individuals whereas *MR2* individuals were effectively controlled with a biomass reduction of < 99% (Fig. 1).

3.3 Resistance mechanisms

3.3.1 Glyphosate

3.3.1.1 Shikimate accumulation. Shikimate accumulation was observed in leaf discs of both *Sus* and *MR* plants over a range of glyphosate doses although leaf discs from *MR* population plants revealed significantly lower shikimate accumulations at all doses than *Sus* plants. Shikimate accumulation in *Sus* leaf discs reached a maximum at 500 μM glyphosate (Fig. 2). IC_{50} values calculated at this dose were 142 μM for *Sus* leaf discs, 1010 μM for *MR1* leaf discs, and 570 μM for *MR2* leaf discs.

Shikimate levels detected in untreated leaf discs from the three populations (*Sus*, *MR1* and *MR2*) were similar over all six light pretreatments (0, 2, 4, 8, 16 and 32 h). However, treated leaf discs differed in shikimate accumulation among populations with *MR1* leaf discs exhibiting shikimate levels significantly lower at all light pretreatments than *MR2* and *Sus* leaf discs (Fig. 3). At 16 HAT, *Sus* leaf discs accumulated 35.2- and 3.7-fold more shikimate than *MR1* and *MR2* leaf discs, respectively. At 32 HAT, *Sus* leaf discs accumulated 19.6 and 3.9 times more shikimate than the *MR1* and *MR2*, respectively. This difference in shikimate accumulation was consistent with the results of the glyphosate dose–response experiments where *MR1 and MR2* plants were 20.5- and 4-fold respectively less responsive to glyphosate at the labeled rate than *Sus* plants.

3.3.1.2 Partial *EPSPS* gene sequencing. Partial EPSPS sequences of 540–590 bp were obtained for six individuals from the *MR1* (accession number MF163392), *MR2* (accession number MF163393), and *Sus* populations via Sanger sequencing. Sequences were trimmed and 506 bp of each sequence aligned against the available EPSPS sequence of Italian ryegrass (accession number DQ153168.2) in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Missense mutations at the Pro106 codon were detected in all *MR* glyphosate-resistant individuals, which were heterozygous at the first base of the codon in contrast to *Sus* individuals, which were homozygous. Based on our results, glyphosate resistance in both *MR* Italian ryegrass populations is due, or at least partially due, to target site mutations corresponding to Pro106-to-Thr (*MR1*) and Pro106-to-Ala (*MR2*) amino acid substitutions (Fig. 4).

3.3.1.3 EPSPS copy number and expression. Genomic EPSPS copy number in all *Sus* plants ranged from 0.95 to 1.2, based on the relative EPSPS: *β-tubulin* genomic copy number. Similarly, all *MR* plants also had a single EPSPS copy (Table 3) in contrast to the *LOLR* plant (positive control) that was shown to have increased EPSPS copies (~ 65 copies) in previous studies.[24,25] Consistent with the *EPSPS* gene copy number results, there was no difference in relative EPSPS: actin gene expression between *Sus* and *MR* plants, whereas the *LOLR* positive control with increased EPSPS copies showed increased expression of the EPSPS gene (Table 3). These results suggest that EPSPS gene amplification does not contribute to glyphosate resistance in the *MR* populations.

3.3.2 Sethoxydim

3.3.2.1 Partial ACCase gene sequencing. Two fragments of the plastidic ACCase gene spanning seven codons where point mutations are known to confer resistance to ACCase-inhibiting herbicides[17] were amplified and sequenced in six plants from each of the three populations. Sequences were trimmed to 660 bp (including Ile1781codon) and 490 bp (including Trp1999, Trp2027, Ile2041, Asp2078, Cys2088, Gly2096). Sequence alignment revealed no insertions or deletions in either fragment. However, sequences did reveal non-synonymous single nucleotide polymorphisms (SNPs) among *Sus*, *MR1* (accession number MF163394) and *MR2* (accession number MF163395) individuals in the 660 bp fragment. Nucleotide polymorphisms at Ile1781 (ATA to TTA) were found in all *MR1* and *MR2* resistant individuals (Fig. 5).

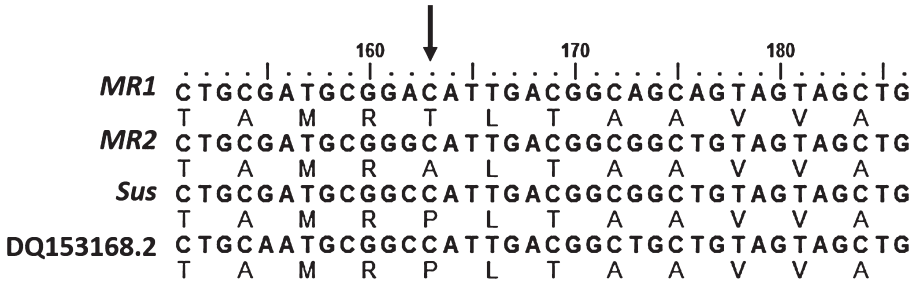

Figure 4. Sequence comparison of plants from herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass populations in California with *Lolium multiflorum* (GeneBank accession number DQ153168.2) around EPSPS codon Pro106. The black arrow shows the amino acid substitutions at Pro106.

Table 3. Relative EPSPS genomic copy number and expression compared with *β-tubulin* and *actin* genes, respectively, for the herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass populations, and the positive control (*LOLR*). Values are averages of three replicates.*

Population	Mean		Min	Max	SE
	EPSPS: *β-tubulin* genomic copy no.				
Sus 1	1.03	b	0.84	1.88	0.32
MR1.1	0.58	bc	0.49	1.44	0.29
MR 2.1	0.49	c	0.48	1.72	0.41
LOLR	75.41	a	58.94	64.06	4.86
	EPSPS: *actin* gene expression				
Sus 1	2.10	b	0.10	1.92	2.3
MR1.3	1.43	b	0.15	1.17	1.73
MR 2.2	2.24	b	0.18	1.94	2.5
LOLR	104.42	a	12.75	84.25	100.96

*Means for each population followed by the same letter are not significantly different ($\alpha = 0.05$).

Chromatograms revealed an A/T overlapping double peak at the first base of the Ile1781 codon in some sequences of resistant individuals, indicating heterozygosity at this codon. Sequences of the 490 bp fragment revealed some, but not all, *MR2* individuals with non-synonymous SNPs at Cys1709, Trp 1712, Ile1715 and His1741, and one *MR1* individual with a SNP at Leu1742 resulting in a substitution of Leu1742-to-Pro (data not shown). The SNPs identified in the 490 bp have not been previously shown to be associated with resistance to ACCase-inhibiting herbicides.

3.3.3 *Paraquat*

3.3.3.1 Paraquat uptake and translocation. Paraquat absorption ranged from 98% to 99% of applied radioactivity across *Sus*, *MR1* and *MR2* populations and harvest times. Translocation of ^{14}C-paraquat was similar in all three populations at 5 HAT (Fig. 6). Thereafter, *Sus* plants transported more paraquat out of the treated leaves at both 24 and 48 HAT (8.84% and 8.45% of absorbed, respectively) than plants from the *MR1* and *MR2* populations (3.69% and 0.9% of absorbed at 24 HAT, and 1.48% and 3.44% of absorbed at 48 HAT, respectively). Similar results were reported by Yu *et al.*,[28] where a resistant rigid ryegrass (*Lolium rigidum* Gaud.) population had less ^{14}C-paraquat movement out of the treated leaf compared to a susceptible population at 24 and 48 HAT.

The distribution of absorbed ^{14}C-paraquat in *Sus*, *MR1* and *MR2* plants is summarized in Table 4. At 5 HAT, there were no differences in ^{14}C-paraquat accumulation in all tissues among the three populations. Treated leaves of *MR1* and *MR2* plants retained more ^{14}C-paraquat than *Sus* plants at both 24 and 48 HAT. Consequently, ^{14}C-paraquat levels were higher in the SATL and SBTL tissues of *Sus* plants than *MR1* and *MR2* plants at 24 and 48 HAT with the following exceptions. Paraquat levels were similar in SATL of *Sus* and *MR1* plants at 24 HAT, and in SBTL of *Sus* and *MR2* plants at 48 HAT. Paraquat distribution in the roots at 24 HAT was greatest in *Sus* followed by *MR1* and *MR2* but there were no differences in the root distribution levels of paraquat between the three populations at 48 HAT.

The phosphorimaging results (Fig. 7) were consistent with the translocation pattern for the most part. At 5 HAT, most of the absorbed ^{14}C-paraquat remained in the treated leaf of plants from all three populations. At 24 HAT, paraquat movement was evident in the youngest leaves as well as in the roots of the *Sus* plants, but not in the youngest leaves or roots of the resistant (*MR1* and *MR2*) plants. However, by 48 HAT, paraquat translocated to youngest leaves and growing points of plants from all three populations but the roots of the *Sus* and *MR2* plants only.

4 DISCUSSION

Resistance in the two *MR* Italian ryegrass populations examined in this study from the Central Valley of California exists across multiple herbicide modes of action (MOAs), including glyphosate, ACCase inhibiting herbicides and paraquat. Both the *MR1* and *MR2* populations exhibited a high level of resistance (R/S > 45 and >24, respectively) to glyphosate and (R/S > 122 and >93, respectively) to sethoxydim (Table 2). *MR1* also exhibited a high level of resistance (R/S > 20) to paraquat, whereas *MR2* exhibited a low (R/S > 4) resistance level. Results further indicated cross-resistance of both *MR* populations to APP and CHD ACCase-inhibitors but a slightly different cross-resistance pattern in the two populations (Fig. 1). This is the first report of ACCase-inhibiting herbicide-resistant Italian ryegrass populations in California with cross-resistance to APP and CHD herbicides.

We did not find evidence for a single, common mechanism of multiple resistance in Italian ryegrass. Rather, results indicated similar mechanisms of resistance to each herbicide MOA in both *MR* populations, albeit with internal variation that points to independent evolutionary origins of resistance in each population. Based on the results of EPSPS gene sequencing, glyphosate resistance in *MR1* and *MR2* populations is due, at least partially, to target site mutations leading to amino acid substitutions at codon site 106 of EPSPS. Mutations resulting in Pro106-to-Thr substitutions in EPSPS were detected in *MR1* individuals, whereas mutations causing Pro106-to-Ala substitutions were detected in *MR2* individuals (Fig. 4). The differences between populations point to independent evolutionary origins of target-site glyphosate resistance

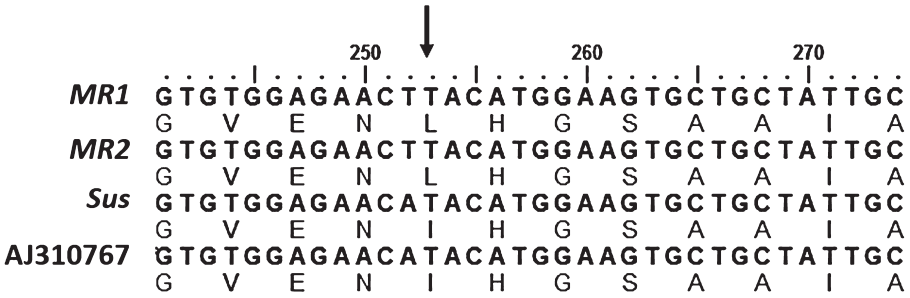

Figure 5. Sequence comparison of plants from herbicide-susceptible (*Sus*) and -resistant (*MR1*, *MR2*) Italian ryegrass populations in California with *Alopecurus myosuroides* (GeneBank accession number AJ310767) around ACCase codon Ile1781. The black arrow shows the amino acid substitutions at Ile1781.

Table 4. Distribution of ^{14}C-paraquat in plants from resistant and susceptible Italian ryegrass populations*

	% absorbed											
	TL			SATL			SBTL			Root		
Population	5 h	24 h	48 h	5 h	24 h	48 h	5 h	24 h	48 h	5 h	24 h	48 h
Sus	99.61 a	91.55 a	91.16 a	0.00 a	1.81 b	2.50 b	0.00 a	4.65 b	3.66 b	0.39 a	1.99 b	2.67 a
MR1	99.54 a	96.31 b	98.52 b	0.05 a	1.32 b	0.16 a	0.04 a	0.85 a	0.54 a	0.37 a	1.52 ab	0.77 a
MR2	99.29 a	99.10 b	96.56 b	0.02 a	0.02 a	0.49 a	0.11 a	0.48 a	1.2a b	0.58 a	0.40 a	1.74 a

SATL, shoot above treated leaf; SBTL, shoot below treated leaf; TL, treated leaf.
*Similar letters indicate no difference between means within the same column ($\alpha = 0.05$).

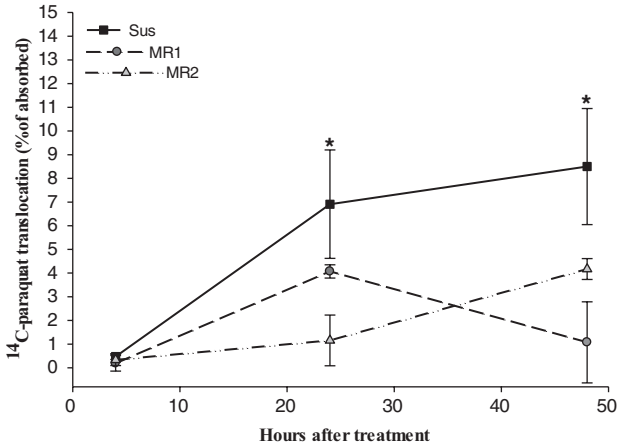

Figure 6. Translocation of ^{14}C-paraquat in plants from resistant and susceptible Italian ryegrass populations. An asterisk at a data point indicates significant difference between the susceptible population and the resistant populations (*MR1*, *MR2*) within the harvest time according to Fisher's LSD at $\alpha = 0.05$.

in each population. The specific gene mutations and associated substitutions were previously detected in EPSPS sequences of California Italian ryegrass.[12,29] Non-synonymous EPSPS gene mutations were also identified in glyphosate-resistant Italian and rigid ryegrass in areas outside California.[30–32] It has been suggested that EPSPS point mutations conferring resistance to glyphosate provide only a moderate level of resistance, whereas EPSPS gene amplification and altered glyphosate translocation confer higher levels of glyphosate resistance in weeds.[33,34] However, despite the high levels of glyphosate resistance detected (Table 2), we found no evidence of increased EPSPS gene copy numbers or expression in *MR1* and *MR2* plants compared with *Sus* plants (Table 3), which indicates that EPSPS gene amplification is not conferring resistance to glyphosate in the populations examined in this study. Altered glyphosate translocation was not found to be a mechanism of glyphosate resistance in early studies of California rigid ryegrass, but remains to be investigated in the *MR* Italian ryegrass populations.[11,35]

Sequencing of two chloroplastidic *ACCase* gene regions revealed nucleotide polymorphisms at Ile1781 (ATA to TTA) in individuals resistant to ACCase-inhibiting herbicides from both *MR1* and *MR2* populations (Fig. 5). The target site mutation and resulting amino acid substitution have previously been shown to be directly correlated with a high level of resistance to APP and CHD herbicides in several weed species, including Italian ryegrass.[17,36,37] Based on the cross-resistance results obtained in our study, however, the Ile1781-to-Leu substitution did not cause clethodim resistance in the *MR2* population. This result is similar to earlier findings on the genetic basis of ACCase inhibiting herbicide resistance in Amazon sprangletop biotypes[38] where an Asp2078-to-Gly substitution did not result in resistance to either sethoxydim or clethodim. However, according to Yu *et al.*,[39] a mutant Asp2078-to-Gly allele endows cross-resistance to APP and CHD herbicides in ryegrass populations in Australia.

Varying levels of cross-resistance to sethoxydim and clethodim have been reported in ryegrass species worldwide,[14,40–42] but the underlying genetic basis of the phenotypic variation in resistance to the two herbicides, when it occurs, is not known. Considering previous studies on several ACCase inhibitor-resistant grass weeds in different cropping systems, cross-resistance to APP and CHD herbicides is generally due to point mutations at the CT domain of the plastidic ACCase gene, resulting in altered amino acid residues on the binding site of the enzyme.[17] Nevertheless, metabolism-based cross-resistance to low-dose herbicides with the same site of action in grass weeds, such as rigid ryegrass and wild oat, have been documented.[42–44]

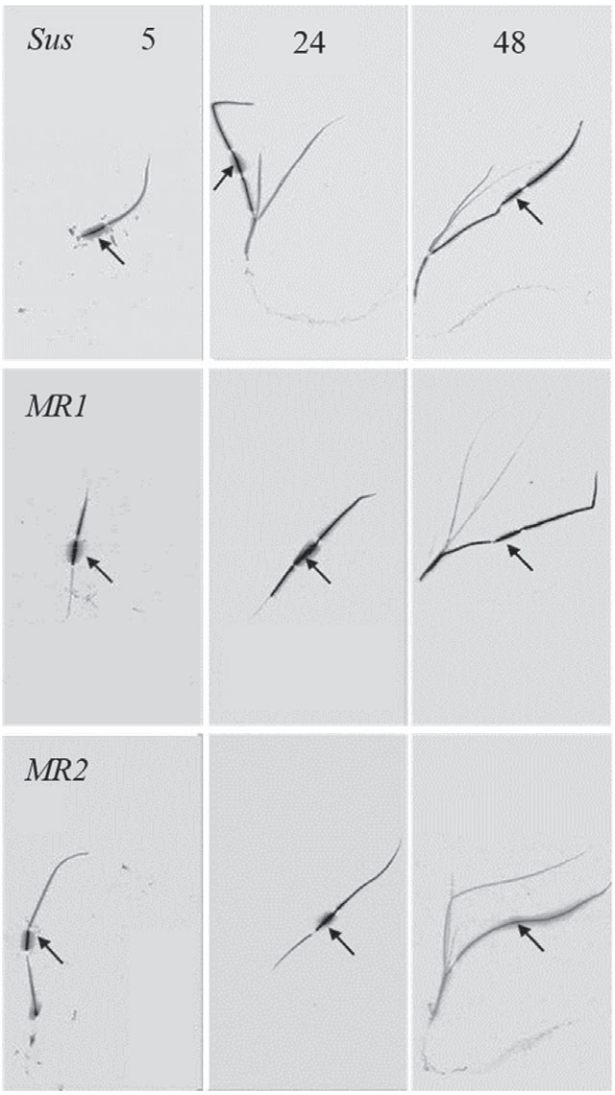

Figure 7. Phosphorimages of Italian ryegrass plants from the herbicide-susceptible (*Sus*: upper) and resistant (*MR1*: middle; *MR2*: lower) populations treated with ^{14}C-paraquat at 5, 24 and 48 HAT (left to right). Arrows indicate the treated area of the second fully expanded leaf.

Substantially more paraquat was retained in, and substantially less paraquat was translocated out of treated leaves of *MR1* and *MR2* plants than *Sus* plants (Table 4 and Fig. 6) pointing to reduced paraquat translocation as a mechanism of resistance. Similar results were reported by Yu *et al.*,[14,28] where a resistant rigid ryegrass population had less ^{14}C-paraquat movement out of the treated leaf compared with a susceptible population at 24 and 48 HAT. These researchers[28] did not observe wilting and/or desiccation of either treated or untreated leaves in the resistant population, whereas we did observe such symptoms in all three populations, in particular, the *Sus* population (data not shown). The level of paraquat resistance differed between the *MR2* (> 20-fold) and *MR1* (> 4-fold) populations, but we could not determine the underlying reason for the difference. More recently, paraquat resistance was confirmed in a multiple herbicide-resistant Italian ryegrass population from a prune orchard in California with vacuolar sequestration of paraquat as the probable mechanism of resistance.[21] The level of paraquat resistance (RI = 30-fold) in this population was higher than that of the *MR* populations in the current study. Various levels of field-evolved paraquat resistance have been reported for several weed species.[45]

In summary, we have shown that multiple resistance to glyphosate, ACCase-inhibiting herbicides and paraquat is present in some Italian ryegrass populations of California, and that a single, common mechanism does not underlie resistance to the multiple herbicide MOAs. Rather, resistance to each herbicide group appears to have evolved independently, based on the mechanisms identified, and the differences in resistance levels and variation observed in each mechanism (e.g. different specific mutations) between the two multiple-resistant (*MR1* and *MR2*) populations. Given the independent mechanisms of resistance to glyphosate, ACCase inhibitors, and paraquat identified in *MR* populations and the temporal pattern of usage of these herbicides in California orchards and vineyards (e.g. paraquat was used to control ryegrass after it became difficult to control with glyphosate), it is likely that resistance to each herbicide evolved sequentially within each population to produce the multiple resistant populations we have today.

ACKNOWLEDGEMENTS

This research was funded by USDA-NIFA-AFRI Award No. 2015-67013-22949. The authors would also like to thank Chad Fautt and Lena El-Mahmoud for greenhouse and laboratory assistance, and Ian Baker for greenhouse maintenance.

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