Molecular basis of resistance to clethodim in Australian ryegrass (*Lolium rigidum*) populations

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Summary Annual ryegrass (*Lolium rigidum* Gaudin) is one of the most troublesome herbicide resistant weeds in Australia. It is an obligate cross-pollinated species, which has so far evolved resistance to herbicides with nine different modes of action. Clethodim, an inhibitor of acetyl-coenzyme A carboxylase (ACCase), is a selective post-emergence herbicide used to control annual and perennial grasses in a wide variety of broadleaf crops. Clethodim has the ability to control ryegrass populations with resistance to other ACCase-inhibiting herbicides and has been used by many farmers to manage annual ryegrass in continuous cropping rotations. However, repeated use of this herbicide during the last two decades has resulted in the appearance of populations that are highly resistant to clethodim, which is causing serious concern in the grains industry. In this study, we have identified clethodim resistance in annual ryegrass collected from different locations in Australia and investigated the mechanism of clethodim resistance. Pot trials were conducted on four populations of annual ryegrass collected from Western Australia and Victoria. Clethodim provided inadequate control of these populations in the field and resistance was suspected. All four populations were confirmed to be resistant to clethodim (3 to 33-fold) and butroxydim (2 to 3-fold). Sequencing of the target-site ACCase gene identified several known ACCase mutations (aspartate-2078-glycine, isoleucine-1781-leucine, and isoleucine-2041-asparagine) in these populations. The Asp-2078-Gly mutation was detected in three populations, Ile-1781-Leu and Ile-2041-Asn mutations were detected in one population each.

Keywords *Lolium rigidum*, herbicide resistance, clethodim, butroxydim, resistance mechanism, ACCase.

INTRODUCTION

Annual ryegrass is the most important and widely distributed herbicide-resistant grass weed in Australian cropping systems (Neve and Powles 2005). This species has evolved resistance to at least nine different modes of action of herbicides (Heap 2012). The most important classes of herbicides used for the control of annual ryegrass are the aryloxyphenoxypropionates (APP/fops) and cyclohexanediones (CHD/dims), phenylpyraxoline (PPZ/dens), which act specifically on grass weeds by inhibiting the Acetyl-coenzyme A carboxylase (ACCase; EC 6.4.1.2) enzyme (Devine 1997).

ACCase catalyses the first committed step in de novo fatty acid biosynthesis in plants, which are essential components in cell membranes and secondary plant metabolites (Gronwald *et al.* 1992). In grasses, ACCase-inhibiting herbicides inhibit the homomeric plastidic ACCase; however, in most dicotyledonous species, their heteromeric chloroplastic ACCase is insensitive to ACCase-inhibiting herbicides. This allows these herbicides to be used for grass control in dicotyledonous crops (Devine 1997, Powles and Yu 2010). ACCase-inhibiting herbicides have been used extensively worldwide to control grass weed species (Devine and Shimabukuro 1994) and this frequent and widespread use has resulted in the evolution of resistance in many grass weed species including blackgrass (*Alopecurus myosuroides* Huds.), annual ryegrass and wild oat (*Avena fatua* L.) (Heap 2014).

Clethodim, a CHD herbicide, is a selective post-emergent herbicide typically used to control annual and perennial grasses infesting dicot crops (Burke *et al.* 2004). Clethodim has the ability to control annual ryegrass populations with resistance to other ACCase-inhibiting herbicides and has been used extensively for the control of annual ryegrass. This has resulted in the evolution of clethodim resistance in annual ryegrass populations in Australia (Yu *et al.* 2007), with as many as 60% of fields across south eastern Australia having some level of clethodim resistance (Boutsalis *et al.* 2012). In an effort to achieve acceptable control of such populations, farmers are using increasing rates of clethodim because many of the populations have only low-level resistance to clethodim. The objective of this study was to quantify the level of clethodim resistance in four annual ryegrass populations and to elucidate whether resistance in these annual ryegrass populations was associated with an alteration in the ACCase enzyme.

MATERIALS AND METHODS

The annual ryegrass populations used in this study (61-12, 52-12, 58-12 and 91-12) were collected from

different sites in Western Australia and Victoria where clethodim had provided inadequate weed control in the field and resistance was suspected. A known herbicide susceptible (S) ryegrass populations SLR4 and known resistant (R) population L739 were used as standards to compare with the populations being investigated.

Seeds of resistant and susceptible populations were germinated on 0.6% (w/v) agar in an environmentally controlled cabinet at 12 h light and 12 h dark periods at 20°C/15°C with 30 µmol m^{-2} s^{-1} light intensity during the light period (Lorraine-Colwill *et al.* 2001). After seven days, seedlings at the one leaf stage were transferred to 9.5 cm × 8.5 cm × 9.5 cm punnet pots (Masrac Plastics, South Australia, Australia) containing standard potting mix with a density of nine seedlings per pot and there were three replicates for each herbicide dose. The plants were maintained outdoors during May 2012 and watered and fertilised as required.

At the two to three leaf stage these seedlings were sprayed with different rates of clethodim (0, 7.5, 15, 30, 60, 120 g a.i. ha^{-1} for S plants, 0, 30, 60, 120, 240, 480 g a.i. ha^{-1} for R plants) and butroxydim (0, 5.6, 11.3, 22.5, 45, 90 g a.i. ha^{-1}). The recommended dose of clethodim for annual ryegrass control is 60 g a.i. ha^{-1} and butroxydim 45 g a.i. ha^{-1}. Herbicides were applied as commercial formulations plus 0.2% v/v Hasten spray adjuvant. The herbicides were applied by using a laboratory moving boom sprayer equipped with twin nozzles (Tee-jet 1100 flat fan, Spraying Systems, Wheaton, IL) with an output volume of 103 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1}. Control plants were not treated with herbicide. Plants were maintained outdoors after treatment and assessed for survival 21 days after treatment. Plants were recorded as alive if they had tillered since herbicide application and plants showing chlorosis, stunting and mortality were considered as susceptible (Powles *et al.* 1998). Treatments were arranged in a randomised complete block design with three replications per dose. All the dose response data were analysed by using log logistic equation (Graphpad Prism v.6.0; GraphPad Software, San Diego, California) to compare the differences among herbicide treatments and the dose of herbicide required to kill 50% of the plants (LD_{50}) was calculated.

Sequencing of ACCase gene Fresh leaf material (~1 cm^2) was harvested from young leaves of a single resistant plant of each resistant population, snap frozen in liquid nitrogen and stored at −20°C until its use. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. The concentration of nucleic acids was determined spectrophotometrically on a NanoDrop ND-1000 (Thermo Scientific, USA) at 260 nm. Primers were designed to amplify regions in the carboxyl tranferase (CT) domain known to be involved in sensitivity to ACCase herbicides. Two sets of primers covering all seven known mutation sites (1781, 1999, 2027, 2041, 2078, 2088 and 2096) were designed against the *A. myosuroides* (accession number AJ310767) ACCase gene sequence (Table 1) were used to amplify a 1.5 kb fragment covering nearly the entire CT domain without any intron. The range of amino acids covered by the fragment was equivalent to codons 1658–2157 in *A. myosuroides*. Twelve individual plants from each population were genotyped.

A nested polymerase chain reaction (PCR) approach was employed with oligo set Acclr9 and Acclr6 (Zhang and Powles 2006) followed by oligo set AccCT 2F and AccCT 2R. PCR reactions of 25 µL contained 20 ng DNA, 1 × High Fidelity buffer [60 mM Tris-SO_4 pH 8.9, 18 mM $(NH_4)_2SO_4$], 2 mM $MgSO_4$, 0.2 mM each dNTP, 0.2 µM of each specific primer and 1 unit Platinum Taq High Fidelity DNA Polymerase enzyme mix (Invitrogen, Australia). Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) with PCR conditions as follows: 3 min denaturing at 94°C; 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 56°C and 2 min elongation at 68°C, and a final extension for 7 min at 68°C.

The PCR products were examined on 1.5% agarose gels stained with 1 × SYBR® Safe DNA gel stain. Samples were electrophoresed in 1 × TAE Buffer (40 mM Trizma base, 1 mM Na_2EDTA, pH to 8 with glacial acetic acid) at 90 volts and photographed under UV light (λ302 nm). DNA fragment sizes were estimated by comparing their mobility to bands of known sizes in a low mass molecular weight marker (Invitrogen, Australia). PCR products were sequenced by Australian Genome Research Facility (AGRF Ltd., Australia) using primers CT Mid F and CT Mid R

Table 1. Primers sequences used for amplification and sequencing of the CT domain of the ACCase gene in *Lolium rigidum* from genomic DNA.

Primer	Sequence 5' 3'
Acclr9	ATGGTAGCCTGGATCTTGGACATG
Acclr6	GGAAGTGTCATGCAATTCAGCAA
AccCT 2F	CCACTCCTGAATTTCCCAGTGG
AccCT 2R	CGCGATTTGAGTGTACAAAGGCTG
AccCT MidF	CCTGAGAATACATGTGATCCTCGTG
AccCT MidR	CCATTTCCCTTGGCTGTCATCAATGCC

(Table 1) to obtain sequence data covering the full CT domain fragment. DNA sequence data were assembled compared and analysed using ContiExpress from the Vector-NTi Advance 11.5 programs (Invitrogen).

RESULTS AND DISCUSSION

Dose response experiment The dose response experiment confirmed that all the four annual ryegrass populations were resistant to both clethodim and butroxydim (Table 2). Of the four populations, three populations showed high-level resistance to clethodim and one population was less resistant to clethodim. The plants of the susceptible population SLR4 were completely killed by clethodim at the recommended rate of 60 g ha^{-1} and even at lower rates, while the known resistant population L739 had little or no mortality at this rate. All the putative resistant populations were also markedly less affected by the recommended field rate. The clethodim rate causing 50% mortality (LD_{50}) for the susceptible population was calculated as 2.7 g ha^{-1} (Table 2). The LD_{50} for the known resistant population L739 was 29.1 g ha^{-1}, giving resistance level (R/S) 10.9-fold higher than the susceptible population. The LD_{50} values for the resistant populations ranged from 10.2 to 89.3 g ha^{-1}. Therefore, the resistant populations were 3.8 to 33.6 fold more resistant to clethodim than the susceptible population. Similarly, varying level of clethodim resistance has been observed in populations of *L. rigidum* (Yu *et al.* 2007) and *Avena* spp. from the Western Australian grain belt (Ahmad-Hamdani *et al.* 2012).

All four populations were also resistant to butroxydim. Butroxydim at the normal use rate of 45 g ha^{-1} totally controlled the susceptible population (Table 2). In contrast, this rate provided less control of resistant populations. The LD_{50} for the susceptible population was 2.3 g ha^{-1}, whereas the LD_{50} values for the resistant population varies from 3.1 to 6.3 g ha^{-1}. Therefore, the resistant populations were 1.7 to 2.7-fold resistant to butroxydim. Previous research has shown that some clethodim resistant populations of annual ryegrass with different ACCase target site mutations have cross resistance to the CHD herbicide butroxydim (Yu *et al.* 2007). The resistance level in butroxydim is not high and these populations can still be controlled by using higher rates of butroxydim.

Table 2. The dose of clethodim and butroxydim required for 50% mortality (LD_{50}) of resistant and susceptible *Lolium rigidum* populations. R/S is the ratio of LD_{50} of resistant and susceptible populations.

Population	LD_{50} (g a.i. ha^{-1})	R/S
Clethodim dose response		
91-12	89.3	33.6
58-12	33.5	12.6
61-12	36.0	13.5
52-12	10.2	3.8
L739	29.1	10.9
SLR4	2.7	–
Butroxydim dose response		
91-12	6.3	2.7
58-12	6.3	2.7
61-12	3.1	1.3
52-12	3.9	1.7
L739	5.2	2.2
SLR4	2.3	–

Sequencing of ACCase gene DNA fragments of 1600 bp of the CT domain of the plastidic ACCase gene from ten resistant plants of each population were amplified by PCR and then sequenced from both ends. The sequences were then aligned to each other and to the chloroplastic ACCase gene of *A. myosuroides.* Nucleotide sequences from all the populations were compared to each other, as well as to the susceptible population, SLR4. The presence of nucleotide substitutions at seven previously characterised positions in the CT domain (1781, 1999, 2027, 2041, 2078, 2088 and 2096), according to the sequence of *A. myosuroides* ACCase (Délye 2005) known to cause resistance was analysed. The sequencing showed a target site mutation within ACCase was present in all the resistant populations. Sequencing results revealed three known ACCase mutations (aspartate-2078-glycine, isoleucine-1781-leucine, and isoleucine-2041-asparagine) in these populations. Four populations had the Asp-2078-Gly mutation and population 56-12, which is less resistant to clethodim, had the Ile-1781-Leu mutation. In population 91-12, two mutations: Ile-2041-Asn and Asp-2078-Gly were present. Annual ryegrass is a widespread obligate outcrossing species (Preston *et al.* 1999) and so multiple mutations in a single resistant population can be expected (Malone *et al.* 2014). All these three substitutions endowing clethodim resistance have been previously reported in *L. rigidum* (Yu *et al.* 2007), *A. myosuroides* (Délye 2005) and *A. fatua* (Cruz-Hipolito *et al.* 2011). Amino acid modification at position 2078 is known to provide strong resistance to clethodim and all other substitutions provide weak or no resistance to this herbicide (Délye *et al.* 2008). Malone *et al*. 2014 observed that there was an increase in the frequency of

individuals carrying multiple amino acid substitution in resistant individuals of annual ryegrass, which may be related to the increased frequency of clethodim resistance. The continuous use of clethodim over an extended period on annual ryegrass populations that already had resistance to other ACCase-inhibiting herbicides might be selecting for the accumulation of amino acid modifications within ACCase that contribute to clethodim resistance.

The high levels of clethodim resistance identified in some of the annual ryegrass populations will make management of this weed considerably more difficult in cropping systems of Australia. Growers will need to employ alternative management strategies for the control of this weed in future.

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