

Spread of herbicide resistance alleles in *Lolium rigidum* Gaud. (annual ryegrass)

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Summary Herbicide resistant weeds are a major problem in grain cropping areas of southern Australia. Resistance to the acetyl-coenzyme A carboxylase inhibiting herbicides is widespread in this area. In order to better understand how resistance to ACCase-inhibiting herbicides in populations of *Lolium rigidum* in South Australia has changed with time, the carboxyl-transferase (CT) domain of the ACCase gene from resistant *L. rigidum* populations collected in random surveys over 10 years was sequenced and mutations that cause resistance characterised. Mutations were identified in more than 80% of resistant individuals, indicating target site mutation is the most common mechanism of resistance in *L. rigidum* to this herbicide mode of action. Mutations at seven previously characterised positions in the ACCase gene were identified, and individuals containing multiple mutations were also found. Mutations at position 2041 occurred at the highest frequency in all years. However, other mutations and individuals carrying two mutations have increased in frequency. This study has shown that target site resistance to ACCase-inhibiting herbicides in *L. rigidum* is widespread across South Australia and has evolved numerous times.

Keywords *Lolium rigidum*, ACCase, herbicide resistance.

INTRODUCTION

Lolium rigidum Gaud. (annual ryegrass) is the most significant weed of southern Australian cropping systems, making it the most targeted weed species for control in grain production in this area. However, the intensive use of herbicides has resulted in the evolution of widespread herbicide resistance, with *L. rigidum* populations in Australia having evolved resistance to at least nine dissimilar herbicide chemistries (Preston *et al.* 1996).

Acetyl-CoA carboxylase (ACCase) is a key enzyme in fatty acid biosynthesis, catalysing the carboxylation of acetyl-CoA to produce malonyl-CoA. ACCase-inhibiting herbicides selectively target grasses by binding to the carboxyl-transferase (CT) domain of the ACCase enzyme, inhibiting fatty acid biosynthesis and ultimately causing plant death (Secor *et al.* 1989, Gronwald 1991).

There are three different herbicide chemistries that inhibit ACCase: aryloxyphenoxypropionates, cyclohexanediones (Devine 1997) and phenylpyrimidines (Muehlebach *et al.* 2009). Since their introduction in the late 1970s, ACCase-inhibiting herbicides have been used worldwide to selectively control grass weeds (Délye 2005) and were quickly adopted as the main herbicide to target *L. rigidum* in winter cereal crops in Australia (Heap and Knight 1990). However, as a consequence of the extensive use of herbicides from this mode of action, resistance to these herbicides has rapidly evolved in many grass weeds, with the first case of resistance to an ACCase inhibitor in *L. rigidum* in Australia reported in 1982 (Heap and Knight 1982). Previous studies have shown the major cause of ACCase-based resistance to be mutations in the CT domain of the ACCase gene leading to single amino acid substitutions in the plastidic ACCase (Holtum *et al.* 1991, Devine 1997, Zhang and Powles 2006).

Information from farmer complaints and resistance testing services has indicated that the number of ACCase-herbicide resistance cases in *L. rigidum* has been increasing across southern Australia (Boutsalis and Broster 2006). In addition, the spectrum of resistance appears to be changing with time. Resistance to the cyclohexanedione herbicides in addition to the aryloxyphenoxypropionate herbicides is increasing. Therefore, to obtain more accurate information on the occurrence and spread of ACCase-herbicide resistance in *L. rigidum* across southern Australia, the molecular basis for resistance was investigated. To do this, the presence of previously identified nucleic acid changes in the CT domain of the ACCase gene of resistant populations identified from surveys of the mid-north of South Australia (P. Boutsalis unpublished data), a key cropping district covering an area of around 1.5 million hectares, were analysed, and the change in mutation frequency compared over 10 years.

MATERIALS AND METHODS

Plant material Surveys of farmers' fields were conducted through the mid-north of South Australia in 1998, 2003 and 2008 as previously described by Boutsalis *et al.* (2006). Samples of *L. rigidum* were collected from fields and tested for resistance to

diclofop-methyl (500 g a.i. ha⁻¹). The herbicide was applied to 2 leaf plants grown in 10 cm square pots grown outdoors with one population per pot using a laboratory moving boom pesticide applicator. The herbicide was applied in the equivalent of 109 L ha⁻¹ water at a pressure of 250 kPa and a speed of 1 m s⁻¹ using Tee-Jet 001 nozzles. *L. rigidum* populations were identified as being resistant to diclofop-methyl if more than 20% of plants survived this treatment.

Sequencing of ACCase gene Fresh leaf material (~1cm²) was harvested from young leaves of a single resistant plant for each resistant population, snap frozen in liquid nitrogen and stored at -80°C. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Australia) according to the manufacturer's instructions. The concentration of nucleic acids was determined spectrophotometrically on a NanoDrop ND-1000 (Thermo scientific, USA) at 260 nm.

Standard PCR conditions and primers designed against the *Alopecurus myosuroides* (accession number AJ310767) ACCase gene sequence (Table 1) were used to amplify a 1600 bp fragment covering nearly the entire CT domain without any intron. A nested PCR approach was employed with oligo set Acclr9 and Acclr6 (Zhang and Powels 2006) followed by oligo set AccCT 2F and AccCT 2R. PCR reactions of 25 µL contained 20 ng DNA, 1 × PCR Reaction Buffer [20 mM Tris-HCl pH 8.4, 50 mM KCl], 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each specific primer and 1 unit High Fidelity Taq DNA Polymerase (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.

PCR products were visualised on ethidium bromide stained (1 µg mL⁻¹) 1.5% agarose gels. Samples were electrophoresed in 1 × TAE Buffer [40 mM Trizma base, 1 mM Na₂EDTA, pH to 8 with glacial acetic acid] at 100 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 (Australian Genome Research Facility, Australia) using primers CT Mid F and CT Mid R to obtain sequence data covering the full CT domain fragment.

Nucleotide sequences were analysed using the VectorNTi ContigExpress and AlignX software programmes (Invitrogen) and all sequences visually rechecked using the chromatogram files.

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

Primer	Sequence 5' – 3'
Acclr9	ATGGTAGCCTGGATCTTGGACATG
Acclr6	GGAAGTGCATGCAATTCAGCAA
AccCT 2F	CCACTCCTGAATTTCCCAGTGG
AccCT 2R	CGCGATTGAGTGTACAAAGGCTG
AccCT MidF	CCTGAGAATACATGTGATCCTCGTG
AccCT MidR	CCAATTCCTTGGCTGTCAATGCC

RESULTS

The conserved CT domain of the ACCase gene of *L. rigidum* was sequenced from a single plant of each population identified in the random surveys as resistant to diclofop-methyl. Nucleotide sequences from all populations were compared to each other, as well as to the standard susceptible population, VLR1. The presence of nucleotide substitutions in seven previously characterised positions in the CT domain, known to cause resistance (Preston 2009), was analysed. These are amino acid positions 1781, 1999, 2027, 2041, 2078, 2088 and 2096 according to the sequence of *A. myosuroides* ACCase (Délye 2005). Usable sequence of the CT domain of ACCase was obtained from 111 resistant individuals from the 1998 collection, 124 resistant individuals from the 2003 collection and 199 resistant individuals from the 2008 collection.

Of the resistant individuals sampled, 74% of the 1998 collection contained at least one of the known mutations. This increased to almost 90% of resistant individuals from the 2008 collection. About 65% of individuals in all years had only a single mutation in one position in the CT domain, but by 2008, 23% of individuals had a second mutation at a different position in the CT domain (Table 2).

The greatest number of individuals in each year had mutations at amino acid 2041 in the ACCase protein. This was followed by mutations at amino acid 2078 (Table 3). Mutations at amino acids 1999, 2088 and 2096 were not found in the 1998 collection, but were observed in the other two years. Between 1998 and 2008 there was a decrease in the frequency of mutations at amino acids 2027 and 2041 and an increase in the frequency of mutations at amino acids 1781 and 2078.

DISCUSSION

Lolium rigidum populations resistant to ACCase-inhibiting herbicides are widespread in the mid-north of South Australia. Target site mutations were detected in the majority of the resistant individuals surveyed,

Table 2. Percentage of resistant individuals with zero, one or two mutations in each sampling year and the percentage of total alleles carrying mutations.

Year	None	Single	Two	Total R alleles
1998	26%	69%	7%	47%
2003	17%	61%	22%	56%
2008	11%	66%	23%	63%

Table 3. Percentage of mutations in each sampling year at each of seven amino acid positions within ACCase previously characterised as providing resistance to ACCase-inhibiting herbicides.

Year	Ile 1781	Trp 1999	Trp 2027	Ile 2041	Asp 2078	Cys 2088	Gly 2096
1998	9	0	11	50	17	14	0
2003	14	2	9	35	28	11	1
2008	20	2	4	35	23	15	3

ranging from 74% in 1998 to 89% in 2008. In addition to the seven sites within the CT domain of ACCase where amino acid substitutions are known to result in resistance to ACCase-inhibiting herbicides (Preston 2009), amino acid substitutions were observed at a further 12 sites within the CT domain. At present it is not known whether any of these substitutions would result in resistance to herbicides. This survey indicates that target site mutations are a common mechanism providing resistance to ACCase-inhibiting herbicides in *L. rigidum* in the mid-north of South Australia.

While target site mutations are common in *L. rigidum* populations in South Australia, surveys of ACCase-resistant *A. myosuroides* in France found a majority of resistant individuals without a mutation within ACCase (Menchari *et al.* 2006, 2007, Delye *et al.* 2007). It is not clear why this difference occurs, but it may be the result of weed species differences, herbicide usage or selection intensity between the two cropping systems. As would be expected due to the lower frequency of amino acid modifications in ACCase in the *A. myosuroides* from France, there were fewer individuals containing two different ACCase mutations, 0.9% of individuals (Delye *et al.* 2007), than observed in the *L. rigidum* surveys from South Australia (Table 2).

The most common mutation in ACCase found in *L. rigidum* populations in South Australia was at amino acid 2041 with between 35 and 50% of all mutations observed. In contrast, surveys of *A. myosuroides* in France observed more than 50% of mutations in ACCase were at amino acid 1781 (Delye *et al.* 2007). In the latter study, mutations at amino acid 2041 accounted for less than 13% of the mutant alleles detected. Differences in herbicide usage and selection history between France and South Australia

may be the reason for selection of different mutations in ACCase observed.

Between 1998 and 2008 there has been an increase in the frequency of *L. rigidum* populations in South Australia with resistance to the cyclohexanedione herbicides and clethodim in particular (P. Boutsalis unpublished data). Despite this change, there were no dramatic shifts in the frequency of individual amino acid mutations observed (Table 3). The frequency of amino acid substitutions at 2041 and 2079 decreased and the frequency of amino acid substitutions at 1781 increased. Amino acid modification at 2078 is known to provide strong resistance to clethodim, whereas amino acid modifications at the other positions provide weak or no resistance to this herbicide (Delye *et al.* 2008).

There was a large increase in the frequency of individuals containing two amino acid substitutions in resistant *L. rigidum* individuals between 1998 and 2008. It is likely that continued selection of populations with ACCase-inhibiting herbicides has resulted in the accumulation of amino acid modifications within ACCase and this may be contributing to the increase in clethodim resistance in populations.

In conclusion, this research has shown that target site mutations within ACCase are common in populations of *L. rigidum* resistant to ACCase-inhibiting herbicides. Over the 10 year period between 1998 and 2008 there have been some changes in the frequencies of mutations observed. Over the same period there has been a substantial increase in the frequency of individuals carrying multiple mutations in ACCase. Continued selection of these populations with ACCase-inhibiting herbicides will likely result in accumulation of more mutations in ACCase.

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