Amino acid substitutions in ACCase gene of barley grass (*Hordeum glaucum* Steud.) associated with resistance to ACCase-inhibiting herbicides

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Summary The aryloxyphenoxypropionate (APP) herbicides inhibit acetyl-coenzyme A carboxylase (ACCase), the enzyme responsible for fatty acid biosynthesis in plants. ACCase-inhibiting herbicides are widely used to control barley grass (*Hordeum glaucum* Steud.) in dicot crops. However, growers are reporting increasing difficulty in managing barley grass with these herbicides. Dose response experiments were conducted on suspected resistant populations, which identified populations with varying levels of resistance to several ACCase-inhibiting herbicides. These studies showed greater than 30-fold resistance to quizalofop and greater than 15-fold resistance to haloxyfop. Sequencing of the CT domain of the ACCase gene from resistant plants confirmed the presence of previously known mutations at Ile1781Leu and Gly2096Ala. This is the first report to show that these substitutions endow resistance to ACCase-inhibiting herbicides in *H. glaucum*, though resistance in its closely related species *H. leporinum* Link has been previously reported in South Australia and New South Wales.

Keywords Barley grass, herbicide resistance, ACCase-inhibiting, quizalofop, haloxyfop, *Hordeum glaucum*.

INTRODUCTION

Barley grass (*Hordeum glaucum* Steud.) is a problematic weed that has been reported to be increasing in abundance in cropping systems in South Australia. It has been observed that management practices used in cropping systems in South Australia have increased seed dormancy and delayed seedling emergence in this weed species (Fleet and Gill 2010). This change in weed biology tends to reduce the effectiveness of pre-sowing knockdown herbicides applied in late autumn. Therefore, growers need to rely more heavily on post-emergent herbicides for *H. glaucum* control in broadleaf crops. The ACCase-inhibiting herbicides, aryloxyphenoxypropionate (APP) and cyclohexanedione (CHD) are commonly used to control grass weeds in legumes and oilseed crops (Preston 2009). These herbicides inhibit acetyl-coenzyme A carboxylase (ACCase), the enzyme responsible for fatty acid biosynthesis in grass weeds. The increased use of ACCase-inhibiting herbicides has increased the selection pressure for resistance evolution. Resistance to this herbicide group can result from amino acid substitutions in the carboxyl transferase (CT) domain of the ACCase gene (Malone *et al.* 2014).

In the 1980s, resistance to paraquat was reported in *H. glaucum* in SA (Warner and Mackie 1983 and Powles 1986). Since then, paraquat resistance has been the only resistance reported in *H. glaucum* (Hidayat 2004). However, the evolution of resistance to ACCase-inhibiting herbicides has occurred in the closely related species *Hordeum leporinum* Link from South Australia (Matthews *et al.* 2000) and New South Wales (Heap 2014).

Currently, growers are reporting increasing difficulty in managing *H. glaucum* with ACCase-inhibiting herbicides in SA. There is a need to better understand resistance to ACCase-inhibiting herbicides and develop management strategies for the control of resistant populations of this species. Therefore, studies were undertaken to determine the level of resistance and determine the molecular basis of the evolution of resistance to ACCase-inhibiting herbicides in this species.

MATERIALS AND METHODS

Dose response to ACCase-inhibiting herbicides

Two populations collected from South Australian farms were used for this experiment including a known susceptible population (Yaninee) as a control. The seeds were germinated in trays (33 cm × 28 cm × 5 cm) containing standard potting mix. Nine seedlings per pot with three replicates were transplanted into pots (8.5 cm × 9.5 cm × 9.5 cm) containing standard potting mix. The pots were maintained outdoors during the normal growing season (July–August, 2012).

Two most commonly used APP herbicides; quizalofop (Targa®) and haloxyfop (Verdict®) were used in this study. The populations were sprayed at early tillering stage with quizalofop (0, 6.2, 12.4, 24.9, 49.8 and 99.5 g a.i. ha^{-1}) and haloxyfop (0, 9.75, 19.5, 39, 78 and 156 g a.i. ha^{-1}) using a moving-boom laboratory twin nozzle sprayer. However, an additional low rate of quizalofop (3.1 g a.i. ha^{-1}) and haloxyfop (4.9 g a.i. ha^{-1}) were applied to the standard susceptible population. Adjuvants were added to the herbicides as per

label. Survival assessments were recorded at 28 days after treatment (DAT) and plants with new green leaf tissue were recorded as alive. The survival data was analysed with log logistic analysis (GraphPad Prism v6, GraphPad software, San Diego, CA) 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 tissue from resistant and susceptible plants was collected, snap frozen in liquid nitrogen and stored at −20°C. DNeasy Plant Mini Kit (Qiagen, Australia) was used to extract DNA as per manufacturer's instructions. The 1600 bp fragment covering nearly the entire CT domain of ACCase gene without any intron was amplified in standard PCR conditions with the primers designed against *Alopecurus myosuroides* (accession number AJ310767) (Delye 2005) ACCase gene sequence (Table 1). A nested PCR approach was employed with oligo set Acclr9 and Acclr6 (Zhang and Powles 2006b) followed by oligo set AccCT 2F and AccCT 2R. MyFi DNA polymerase kit was used to run a PCR reaction of 25 µL, which contained 5µL of 5xMyFi reaction buffer, 1µL DNA template, 20µM primers each 1µL, 1µL DNA polymerase and 16 µL dH_2O. An automated DNA thermal cycler (Eppendorf Master Cycler® Gradient, Germany) was used for DNA amplification with PCR conditions as follows: 3 min denaturing at 94°C, 39 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 1 × SYBR® Safe DNA stained 1.5% agarose gels. Samples were electrophoresed in 1 × TAE Buffer (40mM Trizma base, 1mM Na_2EDTA, 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 at AGRF, 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 Align X software programmes (Invitrogen) and all sequences visually rechecked using the chromatogram files. The presence of nucleotide substitutions in seven previously characterised positions in the CT domain that cause resistance were analysed (Preston 2009). According to the ACCase sequence of *A. myosuroides*, these amino acid positions are 1781, 1999, 2027, 2041, 2078, 2088 and 2096 (Delye 2005).

RESULTS AND DISCUSSION

Dose response to ACCase-inhibiting herbicides Dose response studies confirmed two *H. glaucum* populations were resistant to the ACCase-inhibiting herbicides, quizalofop and haloxyfop (Table 2). The susceptible *H. glaucum* population 'Yaninee' was successfully controlled by both herbicides (quizalofop and haloxyfop) at the recommended field rates i.e. 24.9 and 39 g a.i. ha^{-1}, respectively (Figure 1). In contrast, the putative resistant populations A1312 and A743.2 survived the recommended field rates of both the herbicides. For quizalofop, the two resistant populations had LD_{50} values of 69 and >99.5 g a.i. ha^{-1} and for haloxyfop 88.7 and 31.6 g a.i. ha^{-1}. The population A1312 was 31-fold resistant to quizalofop and 42-fold resistant to haloxyfop compared to the susceptible population (Table 2). Similarly, population A743.2 was 45-fold resistant to quizalofop and 15-fold resistant to haloxyfop as compared to the susceptible population.

This study confirms the first known case of resistance to the ACCase-inhibiting herbicides in *H. glaucum* in South Australia. The repeated exposure of these populations to ACCase-inhibiting herbicides has provided selection pressure for the evolution of

Table 1. Primer sequences used for amplification of ACCase gene in *Hordeum glaucum* from genomic DNA.

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

Table 2. Estimated LD_{50} (g a.i. ha^{-1}) and resistance index (R/S) values.

	Quizalofop		Haloxyfop	
Population	LD_{50}	R/S	LD_{50}	R/S
A 1312	69.0	31.4	88.7	42.2
A 743.2	>99.5	45.2	31.6	15.0
Yaninee (S)	2.2	–	2.1	–

* LD_{50}, Herbicide required to control 50% of the tested population.
Resistance index (R/S) is the ratio of LD_{50} of resistant population compared with the susceptible population.

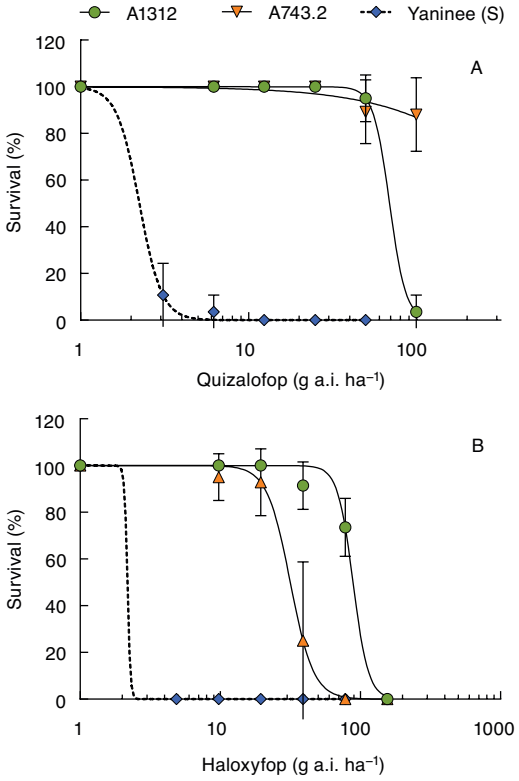

Figure 1. Response of two putative resistant (●, ▲) and one susceptible (◆) populations of *Hordeum glaucum* to quizalofop (A) and haloxyfop (B).

high levels of resistance to APP herbicides. Resistance to ACCase-inhibiting herbicides in grass weeds is quite prevalent in Australia in other grasses, such as *Bromus* spp. (Boutsalis and Preston 2006), *Lolium rigidum* Gaudin (Zhang and Powles 2006b, Boutsalis *et al.* 2012, Broster *et al.* 2012) and *Avena* spp. (Maneechote *et al.* 1994, Owen and Powles 2009). APP herbicide resistance has also been previously reported in *H. leporinum* (Matthews *et al.* 2000).

Sequencing of ACCase gene The CT domain of the ACCase gene was sequenced for resistant and susceptible plants. The sequences obtained were aligned to each other and with the *A. myosuroides* sequence. Total sequence alignment was 1600 bp or 533 amino acids long. The nucleotide sequences for the putative resistant populations differed from that of the susceptible population by a single nucleotide, resulting in a single target-site mutation. In the A1312 population, a codon change from GGC to GCC resulted in an amino acid substitution of Gly2096Ala, whereas, a codon change from ATA to CTA resulted in an amino acid substitution of Ile1781Leu in the A743.2 population.

The glycine to alanine at 2096 position and isoleucine to leucine amino acid substitution at 1781 position in ACCase gene, as identified by sequencing are likely to be the molecular basis of resistance to ACCase-inhibiting herbicides in *H. glaucum* populations. Previously, Gly2096Ala mutations have been reported to be the basis of HR to ACCase-inhibiting herbicides in *Avena fatua* L. (Beckie *et al.* 2012) and *A. myosuiroides* (Delye 2005). The Ile1781Leu substitution is the most predominant substitution known to cause resistance to most APP herbicides. Several other weed species such as *A. fatua* (Christoffers *et al.* 2002 and Beckie *et al.* 2012), *A. myosuroides* (Delye 2005) and *L. rigidum* (Zhang and Powles 2006a) have also been reported to be resistant to ACCase-inhibiting herbicides due to this substitution.

In conclusion, this research has shown presence of two target-site mutations within ACCase gene of *H. glaucum* populations with resistance to ACCase-inhibiting herbicides. These herbicides will not be effective in controlling herbicide resistant *H. glaucum* populations in the field and increasing herbicide dose is unlikely to improve weed control. At present, use of alternative herbicides such as ALS inhibitors should provide control of these ACCase-resistant populations, but these herbicides should only be used as part of an integrated weed management program in order to minimise the risk of rapid evolution of resistance.

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