Next generation testing of herbicide resistance: Rapid detection, multiple herbicides, a single assay

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Summary Annual ryegrass is the major weed in Australian farming systems costing farmers $96 million annually, with control primarily by herbicides. This weed has evolved resistance to many herbicides making control extremely difficult with herbicide-based management programs. Using the genotyping technology DArTseq, a genetic assay has been developed that detects resistance to multiple herbicides in a single test within weeks of field observation. Over 350 individual ryegrass plants were screened with one or more herbicides (diclofop-methyl, chlorsulfuron and glyphosate) using conventional glasshouse based resistance testing procedures. DNA from these samples was then analysed with DArTseq, and herbicide resistance predicted based on the presence/absence of genetic markers. DArTseq analysis predicted herbicide resistance to each of these herbicides with 50–59% accuracy relative to glasshouse testing. This assay surpasses other resistance testing methods which require individual tests, a 9-month timeframe for results, and offers a new tool to combat resistance.

Keywords *Lolium rigidum*, annual ryegrass.

INTRODUCTION

Weeds cost the Australian grains industry an estimated $3.3 billion each year. Annual ryegrass (*Lolium rigidum* Gaudin) is the most costly weed to manage, largely due to the additional costs of controlling resistant populations (Llewellyn *et al*. 2016). These costs can be reduced with the identification of herbicide resistance prior to herbicide application, ensuring that only effective herbicides are used. Currently, commercial detection of herbicide resistance is conducted by either whole plant or germination assays (Boutsalis and Broster 2006). Whole plant testing procedures used for commercial herbicide resistance testing are limited in their ability to provide timely results to identify herbicide resistance in season. Other methods, such as germination, seedling, and plant tissue assays have attempted to reduce the time required for testing, and despite a more rapid delivery of results, these methods are still constrained by seed dormancy or the inability to provide broad testing of herbicides due to the specificity of their assays. In addition to these methods, many technologies have been implemented in identifying different resistance mutations in annual ryegrass using PCR, dCAPS and sequencing (Tan *et al*. 2007, Yu *et al*. 2008, Bostamam *et al*. 2012, Gaines *et al*. 2014, Duhoux *et al*. 2015). These assays are also rapid, precise and can provide detailed information on the mechanism of resistance and are not constrained by seed dormancy. The highly specific nature of these tests has prevented them being implemented in broad detection of herbicide resistance as they are currently limited to screening for well documented mutations, usually in individual assays. Of these, resistance to only one or two modes of action have been detected simultaneously. If a method could be developed using genetic screening, the prospects for the simultaneous testing of multiple forms of herbicide resistance are increased. This could be up-scaled to commercial herbicide resistance testing and it would provide more timely, detailed, and actionable results than current testing methods. As the incidence of herbicide resistance is increasing (Boutsalis *et al*. 2012, Owen *et al*. 2015, Shergill *et al*. 2015) and no new herbicide modes of action commercialised in the last thirty years besides Tirexor™ (expected release date 2020), there is a demonstrated need for better herbicide resistance detection methods to facilitate better resistance management (Duke and Dayan 2015, BASF 2018).

A genetic screening technology yet to be explored for resistance phenotyping is DArTseq. As DArTseq can detect and identify multiple traits simultaneously, it is an ideal technology for resistance phenotyping, as it has the potential to screen for multiple forms of resistance once they are properly characterised. Annual ryegrass is an ideal weed for study as it is genetically diverse, has multiple forms of herbicide resistance and is a widespread weed of economic significance. If DArTseq can be used successfully to identify multiple forms of herbicide resistance in annual ryegrass in a

quick, cheap and accurate assay, the applicability to other less diverse weeds will be demonstrated and a new paradigm in resistance testing will be established. The aim of this study was to identify genetic markers linked to herbicide resistances in populations of annual ryegrass, and to assess the ability of DArTseq to discriminate between herbicide resistant and susceptible phenotypes.

MATERIALS AND METHODS

Seventeen populations were selected with known resistance to diclofop-methyl, glyphosate and chlorsulfuron were selected for study. Seeds from each population were placed within 90 mm plastic Petri dishes lined with filter paper (Advantech No. 2) and covered with 4 mL of deionised water. The Petri dishes were sealed with laboratory film and stored in a growth chamber (12 h 15/25°C day/night). After 6 days germination was complete and seedlings planted into 40 cell plastic containers (8 × 58 × 5 tray, 93 mL cell volume) filled with a 1:3 mix of sand and peat moss and transferred to the glasshouse. Glasshouse conditions were maintained at a day/night temperature cycle of 25/10°C. To enable both herbicide resistance testing and DNA extraction of individual plants, seedlings were cloned via tiller propagation. This resulted in an untreated plant (for DNA extraction) and multiple separate tillers suitable for herbicide application at different rates and herbicides. To encourage tillering, plants were repeatedly cut to a height of approximately 40 mm. Plants were watered and fertilised with commercial fertiliser as required until multiple tillers formed (approximately 9 weeks). Whole plants were then excised from the soil with a scalpel. Tillers were separated by incision between the tillers at the crown. The separated tillers (henceforth clones) were trimmed to 40 mm and 10 mm leaf and root length respectively, measured from crown. Clones were then replanted into new 40 cell plastic containers filled with a 1:3 mix of sand and peat moss. Clones are watered and fertilised as required. As the clones are genetically identical, these can be tested for resistance or used for DNA extraction with confidence that each will have the same resistance mechanism. Multiple clones were taken from each individual plant to allow for dose response determinations to each herbicide. One clone from each seedling was retained for DNA analysis, four were used for glyphosate testing, and two for chlorsulfuron testing. Where an abundance of tillers occurred, one or two additional clones were taken to test for resistance to an additional herbicide, diclofop-methyl. Dose response evaluations were conducted with four concentrations of glyphosate representing 0.5×, 1×, 2× and 4× recommended label rate. Where there were additional tillers for cloning, similar dose response evaluations were conducted for resistance to chlorsulfuron and diclofop-methyl at 1× and 2× recommended herbicide label rates of each herbicide.

To determine herbicide resistance status, seedlings and clones were sprayed with the selected herbicide at the 2–3 leaf stage or one week after propagation. Herbicide treatments, at the recommended label rates and adjuvants (Table 1), were applied using a cabinet sprayer at a 50 cm height with two TeeJet (XR11001) flat fan nozzles at an operating speed of 5 km h^{-1}. Output from the sprayer was calibrated at 85 L ha^{-1} at a pressure of 250 kPa. After treatment, the plants were allowed to dry before being transported to a temperature-controlled glasshouse and commencing 24 hours after treatment, watered as required. To ensure accurate phenotyping, plants were assessed 30 days after treatment to confirm their resistance status. Surviving plants were deemed resistant to the applied herbicide.

DNA extraction was completed by collecting fresh, young, healthy leaf tissue (3–4 leaf stage) from samples and flash freezing them with liquid nitrogen. Samples were then ground into a fine powder with a mortar and pestle before undergoing DNA extraction using the DNeasy Plant Mini Kit (Qiagen). Extracted DNA was tested for quality with gel electrophoresis for quantity and RNA presence with a Nanodrop 1000 (Thermo Scientific, DE, USA). Extracted DNA was submitted to DArT Pty Ltd for DNA complexity reduction and development of SNP markers. A subset of these markers was created by removing non-informative monomorphic markers and selecting high quality markers with call rate >80% and reproducibility >95%.

Table 1. Herbicide and adjuvant application rates for glyphosate, chlorsulfuron and diclofop-methyl.

Herbicide	Application rate (g ai L^{-1})				Adjuvant and rate (% v/v)
Glyphosate*	180	360	720	1440	Wetter TX (0.2%)
Chlorsulfuron	–	20	40	–	BS1000 (0.1%)
Diclofop-methyl	–	375	750	–	BS1000 (0.25%)

* Glyphosate application rate is expressed in g ae L^{-1}.

Frequency of specific markers was compared and correlated with herbicide resistance phenotypes. Markers that did not have a discriminating presence (>30% difference in occurrence between phenotype groups) were discarded. Genome wide association analysis on DArTseq SNP data was performed using ridge regressed best linear unbiased prediction (rrBLUP) using R Studio (Endelman 2011) package rrBLUP. The suitability of rrBLUP phenotype prediction analysis with DArTseq markers have been established by dos Santos *et al*. (2016), Roorkiwal *et al*. (2016) and de Azevedo Peixoto *et al*. (2017). The predictive ability of rrBLUP was validated using a training population to teach marker linkage to phenotype, followed by comparing the prediction of these against the remaining samples (hereafter the validation dataset) and comparing the accuracy of this phenotyping to that of the whole plant analysis. Samples used in the training population are selected at random and removed from the validation dataset to prevent prediction bias. Sampling, training and testing of prediction accuracy was run 1000 times and the average taken. Five training population sizes were trialled to determine the effect on predictive accuracy.

RESULTS

In total, 345 individual plants were tested for glyphosate resistance. Of these, 188 individuals had enough clones for chlorsulfuron testing, and 130 had enough for diclofop testing (Table 2). DNA from plant samples was analysed with DArTseq, producing over 69,000 single nucleotide polymorphism (SNP) markers. Marker association analysis was conducted to link markers to phenotype using rrBLUP. rrBLUP training results increased with accuracy with increasing training population size. Starting training population size was 50 samples, with each additional assessment of training population increasing this number by an additional 50 samples, with the maximum number of samples used being 250. Predictive accuracy from these varying population sizes ranged from 41–50% for glyphosate, 48–50% for diclofop-methyl and 50–59% for chlorsulfuron resistance respectively. Only glyphosate resistance could be tested with a training population of 150+ due to a lack of samples phenotyped with the other herbicides.

DISCUSSION

These results establish the use of DArTseq for herbicide resistance testing for annual ryegrass and proves its ability to detect resistance to multiple herbicides in one assay. This was achieved with greater accuracy than what was achieved with the previous exploratory study using microarray DArT. The predictive power of this assay could be increased with analysis of an inbred F_2 population coupled with greater sample numbers (Peixoto *et al*. 2016). Additionally, specific knowledge of the resistance mutations present in each of the selected samples may have enhanced the accuracy of the resistance phenotype predictions, and allowed for possible sub-categorisation of the different populations. Inference could then be made about the ability of this assay to predict cross-resistance to untested herbicides. The maximum accuracy of 59% for herbicide resistance detection achieved in this study means that a DArTseq assay will predict resistance level with 59% accuracy with the current marker set. Both false negatives and false positives are likely until the many diverse forms of herbicide resistance and the genetic background of *L. rigidum* can be analysed to eliminate spurious marker associations. Resistance prediction may not need to occur with 100% accuracy for a test to be useful, or superior to existing testing methods. Future research could examine the accuracy threshold required of a DArTseq assay to provide meaningful diagnostic information to assist in on-farm agronomic choices.

This proof of concept study establishes DArTseq as a tool for broad-spectrum evaluation of herbicide resistance, having been demonstrated across three

Table 2. Herbicide resistance results for individual annual ryegrass samples at differing herbicide dosage. R indicates the number of resistant samples, S the number of susceptible.

Herbicide	Glyphosate*				Diclofop-methyl		Chlorsulfuron	
Application rate (g ai L^{-1})	180	360	720	1040	375	750	20	40
Phenotype								
R	321	296	321	18	321	13	321	321
S	5	49	153	116	49	18	73	10
Total	326	345	338	134	130	31	188	12

* Glyphosate application rate is expressed in g ae L^{-1}.

herbicide groups at varying doses. This analysis occurred in a three-week time frame, but could be completed in as little as a week in a single assay. This rapid diagnosis allows for early in-season detection of herbicide resistance, remedying the slowness of conventional glasshouse screening for herbicide resistance. Feasibly, early emerging annual ryegrass could be sampled and analysed with DArTseq and its resistance to a range of herbicides and dosages known within a week. In the Australian farming context, weeds emerging with the first March rain could be analysed, and resistance known before sowing in early April. This would facilitate application of an effective herbicide. Alternatively, ryegrass could be sampled and analysed whenever it is found in field, at any growth stage, as long as viable DNA can be extracted from its tissue. Further study is required before this assay will be appropriate for commercial use, to improve the accuracy of phenotyping and identification of key herbicide resistance markers. Once phenotyping accuracy has been enhanced, additional analysis will be required to validate the method across wider Australian ryegrass populations. These results expand this use of genetic assays for the detection of herbicide resistance and contribute to the overall goal of the development of the next generation of resistance screening assays for better weed management and herbicide stewardship.

ACKNOWLEDGMENTS

We wish to thank GRDC for scholarship top-up funding and Dr John Broster for his advice and assistance.

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