Research Article

Received: 30 October 2023 Revised: 26 December 2023 Accepted article published: 28 December 2023 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.7960

# RNA and protein biomarkers for detecting enhanced metabolic resistance to herbicides mesosulfuron-methyl and fenoxaprop-ethyl in black-grass (*Alopecurus myosuroides*)

**Claudia Lowe,[a¶*†] Nawaporn Onkokesung,[b¶‡] Alina Goldberg,[b] Roland Beffa,[c] Paul Neve,[a§] Robert Edwards[b] and David Comont[a¶]**

## Abstract

**BACKGROUND: The evolution of non-target site resistance (NTSR) to herbicides leads to a significant reduction in herbicide control of agricultural weed species. Detecting NTSR in weed populations prior to herbicide treatment would provide valuable information for effective weed control. While not all NTSR mechanisms have been fully identified, enhanced metabolic resistance (EMR) is one of the better studied, conferring tolerance through increased herbicide detoxification. Confirming EMR towards specific herbicides conventionally involves detecting metabolites of the active herbicide molecule *in planta*, but this approach is time-consuming and requires access to well-equipped laboratories.**

**RESULTS: In this study, we explored the potential of using molecular biomarkers to detect EMR before herbicide treatment in black-grass (*Alopecurus myosuroides*). We tested the reliability of selected biomarkers to predict EMR and survival after herbicide treatments in both reference and 27 field-derived black-grass populations collected from sites across the UK. The combined analysis of the constitutive expression of biomarkers and metabolism studies confirmed three proteins, namely, *Am*GSTF1, *Am*GSTU2 and *Am*OPR1, as differential biomarkers of EMR toward the herbicides fenoxaprop-ethyl and mesosulfuron in black-grass.**

**CONCLUSION: Our findings demonstrate that there is potential to use molecular biomarkers to detect EMR toward specific herbicides in black-grass without reference to metabolism analysis. However, biomarker development must include testing at both transcript and protein levels in order to be reliable indicators of resistance. This work is a first step towards more robust resistance biomarker development, which could be expanded into other herbicide chemistries for on-farm testing and monitoring EMR in uncharacterised black-grass populations.**


*Supporting information may be found in the online version of this article.*

**Keywords:** molecular biomarkers; non-target site resistance; enhanced metabolic resistance; herbicide resistance black-grass; fenoxaprop; mesosulfuron

* *Correspondence to: Claudia Lowe, Protecting Crops and the Environment, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK. E-mail: c.lowe@kew.org*

¶ *Joint first author.*

† *Present address: Royal Botanical Gardens Kew, London, UK*

‡ *Present address: Weed Resistance Research, Frankfurt am Main, Germany*

§ *Present address: Department of Plant and Environmental Sciences, Section for Crop Sciences, University of Copenhagen, Taastrup, Denmark*

¶ *Present address: Protecting Crops and the Environment, Rothamsted Research, Harpenden, UK*

a *Protecting Crops and the Environment, Rothamsted Research, Harpenden, UK*

b *Agriculture, School of Natural and Environmental Sciences, Newcastle University, Newcastle Upon Tyne, UK*

c *Senior Scientific Consultant, Liederbach, Germany*

## 1 INTRODUCTION

The evolution of non-target site resistance (NTSR) is one of the major causes of cross-resistance to multiple herbicides with differing modes of action (MoAs) in grass weeds. The widespread evolution of NTSR in wild grass species, including black-grass (*Alopecurus myosuroides*), rigid ryegrass (*Lolium rigidum*), Italian ryegrass (*Lolium multiflorum*), wild oat (*Avena fatua*) and Brome species (*Anisantha* sp.), results in a significant reduction in herbicidal weed control in agricultural fields.[1–4] In the UK, widespread herbicide resistance to several acetolactate synthase (ALS)-inhibitors (HRAC Group 2) and Acetyl-CoA carboxylase (ACCase)-inhibitors (HRAC Group 1) in black-grass populations has been shown to result in considerable economic and yield losses.[5] While rotations and mixtures of different herbicide chemistries have been implemented to constrain the selection of mutations in herbicide target genes, target-site resistance (TSR), this approach may exert increased selective pressure on black-grass to evolve more 'generalist' NTSR mechanisms capable of conferring resistance to multiple MoAs.[1] Recent evidence for reduced glyphosate sensitivity in UK black-grass populations with NTSR to other herbicide MoAs is a further cause of major concern for the prospective total loss of herbicide control in this species.[6] The ability to rapidly detect NTSR, and adapt management strategies accordingly, is therefore now needed to help mitigate against further increased losses from NTSR multiple resistance in this species.

Based on current understanding, NTSR is typically a multigenic trait conferred by diverse biological processes that protect weeds from herbicide toxicity.[7–9] Among these processes, enhanced detoxification of herbicides is one of the predominant mechanisms, commonly referred to as enhanced metabolic resistance (EMR).[10] NTSR and EMR in grass weeds are typically detected through dose-response experiments in glasshouses, followed by analysis of herbicide detoxification via metabolism studies conducted using liquid chromatography and mass spectrometry in laboratories.[11,12] While these approaches allow for the accurate detection of NTSR and EMR, major drawbacks include (i) long processing times and (ii) the necessity for well-equipped glasshouses and laboratories. To begin to address these drawbacks, the concept of molecular biomarkers of NTSR/EMR has been proposed, which could be developed into field diagnostic kits.

Molecular biomarkers (DNA, RNA and protein) are commonly used in healthcare and clinical research to detect multidrug resistance in cancer cells. This information facilitates physicians to develop specific mitigating treatments for patients.[13–15] The main benefits of biomarker applications are the quick turnaround time, low cost and versatility to detect biomarkers from blood and tissue samples. There is now interest in the potential for adopting molecular biomarkers as the point-to-care (POC) diagnosis of NTSR and EMR in grass weeds on-farm or close to the farm. The POC concept is designed to provide information for advisors and farmers on the potential of resistance development and assist in the decision to test samples in the laboratory and greenhouse. These will help to reduce the cost of testing and may allow farmers to develop specific weed management strategies for each field on a farm to reduce both the risk of resistance evolution and the continued use of ineffective herbicide chemistries. For the past decade, many genes/proteins have been identified by transcriptome and proteome in multiple NTSR/EMR black-grass populations.[16,17] The consistent increased expression of these genes/proteins in NTSR/EMR black-grass suggests that these genes/proteins have a potential to be biomarkers.

Several studies have reported that a glutathione transferase phi (*F*) class 1 (*Am*GSTF1) protein is good candidate biomarker for NTSR in black-grass.[1,16–18] However, while a significant elevation at the constitutive level of *Am*GSTF1 protein is a reliable biomarker for NTSR, this protein alone is not sufficient to pinpoint specific resistance to individual herbicide groups.[17,18] In cases of multidrug resistance, a specific combination of biomarkers is commonly used to detect particular resistance mechanisms directed to specific drugs.[13] We therefore rationalised that this approach could also be used to identify transcript and protein biomarkers of NTSR to specific herbicides.

To develop functional and reliable biomarkers for resistance it is necessary to (i) characterise key in-plant targets that are significantly associated with a resistance phenotype, (ii) confirm their applicability across and within a broad range of populations and (iii) identify the most reliable physiological level at which they should be measured (i.e., DNA sequence, transcript expression, protein abundance). Based on accumulated information from various weed species, seven enzyme families, including cytochrome P450 monooxygenases (CYP450s), glutathione transferases (GSTs), uridine diphosphate (UDP)-glucuronosyltransferases (UGTs), malonyl transferases, ATP-binding cassette (ABC) transporters, and multidrug and toxic compound extrusion transporters, are major contributors to EMR[9,10] and could potentially be exploited for biomarker discovery. Several genes within these families have now been implicated in the black-grass NTSR mechanism through transcriptomic analysis of multiple populations.[16,19] Nevertheless, variability in these previously reported markers has been observed across and within NTSR populations from different origins,[16] and their relationship with the metabolism of specific herbicides with differing MoAs remains to be determined. Similarly, while transcriptomic analysis has been the predominant means to study NTSR-related genes, there is growing evidence of pre- and post-transcriptional regulation that influences the utility of such biomarkers when determined at the plant protein level.[20]

This study explores the suitability of molecular biomarkers to detect EMR in black-grass. The detoxification-related genes identified in a previous study[16] were used to test this concept. Enhanced metabolism towards an aryloxyphenoxypropionate (fenoxaprop-ethyl; HRAC Group 1) and a sulfonylurea (mesosulfuron-methyl; HRAC Group 2) was used to test associations between potential EMR biomarkers and detoxification as determined *in planta* of these two herbicides, which differed in their MoAs. Additionally, the predictive ability of candidate biomarkers for EMR was assessed at both the level of the gene transcript and in-plant protein concentration. The outcomes of this study provide important information to support the development of molecular biomarkers for EMR detection and diagnosis in NTSR black-grass.

## 2 MATERIALS AND METHODS

### 2.1 Plant materials and growth conditions

Two widely studied black-grass reference populations were used throughout these analyses to provide individuals with a known herbicide-sensitive (Rothamsted) and broad-ranging non-target site resistance phenotype (Peldon).[16] A further reference population 'Notts', which exhibits ACCase target-site resistance but not NTSR, was used as a secondary herbicide sensitive standard for biomarker verification. Additionally, 27 field-collected black-grass

seed populations were used for wider testing of biomarkers. These populations were collected as seeds from winter wheat fields across the UK arable cropping area between July and August 2014 as detailed by Comont *et al.*[1]

In all cases, to generate seedlings for herbicide screening and tissue sampling, initial propagation involved germination in Petri dishes lined with filter paper (Whatman No. 1) soaked with 2 g $L^{-1}$ $KNO_3$, placed in an incubator (MLR-350, Sanyo, Tokyo, Japan) fitted with fluorescent bulbs (MASTER TL-D 90 De Luxe 36 W/965 1SL/10; Philips, Eindhoven, the Netherlands) for 7 days on a 17 °C 14-h day, 11 °C 10-h night cycle. For herbicide phenotyping, germinated seedlings were transplanted into 9-cm pots filled with a Kettering loam soil supplemented with 2 kg $m^2$ osmocote fertilizer, with six seedlings per pot. Pots were maintained in a glasshouse at 18/12 °C for 14/10 h day/night with supplementary lighting if ambient light levels were low (230 W LED; Kroptek, London, UK). A single pot was used as one biological replicate and three biological replicates (three pots) were used for each herbicide or control treatment. Pots were arranged in a randomised block design and plants were grown until they reached the Biologische Bundesanstalt, Bundessortenamt and Chemical industry (BBCH)11 growth stage (first true leaf)[21] before herbicide treatment.

### 2.2 Biomarker selection

The RNA biomarkers used in this study were identified from a wider set derived from RNA sequencing of the herbicide-sensitive and NTSR black-grass populations.[16] From this set, 24 RNA contigs encoding genes involved in herbicide detoxification were selected (Table 1) and the relative expression of each biomarker was quantified using quantitative real-time PCR (RT-qPCR), see Table S1 for method and primers. Briefly, herbicide-sensitive and NTSR reference populations were grown to BBCH 13–15 before harvesting. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. A total of 15 individual plants were pooled together to make one biological replicate and three biological replicates were used for total RNA isolation and RT-qPCR analysis. All 24 contigs were confirmed to have significantly increased (>2-fold) expression in the NTSR population compared to the herbicide-sensitive population.

To narrow this down further, five genes from amongst this set were chosen based on (i) gene family, (ii)relative expression between herbicide-sensitive and NTSR, and (iii) prior information on their association with NTSR. The chosen genes were a CYP450, two GSTs, a UGT and an ABC transporter (Table 2). In addition, a transcript encoding an oxidoreductase similar to 12-oxophytodienonate reductase-1 (OPR1) was identified. Even though the function of OPR1 in herbicide detoxification is unclear, its significantly increased expression was recently reported in NTSR black-grass.[19] We therefore included AmOPR1 as a further sixth molecular biomarker for EMR. These six genes were used as putative biomarkers throughout the remainder of this study (Table 1).

### 2.3 Herbicide phenotyping of black-grass populations

To provide population-level herbicide resistance phenotypes for analysis, a glasshouse whole-plant resistance assay was used. Black-grass plants (BBCH 11) were sprayed with either 68.75 g ai $ha^{-1}$ of the aryloxyphenoxypropionate herbicide fenoxaprop-p-ethyl (applied as the commercial formulation 'Polecat') or 10.8 g ai $ha^{-1}$ of the sulfonylurea herbicide mesosulfuron (applied as the commercial formulation 'Atlantis', which also contains iodosulfuron). This treatment is referred to from this point on as 'mesosulfuron'. The fenoxaprop dose represents the UK field rate, while the mesosulfuron dose is 75% of the UK field rate. The slightly reduced dose of mesosulfuron was used due to increased herbicide efficacy when spraying this active ingredient in laboratory conditions compared to the field. Plants were sprayed using a laboratory track sprayer fitted with a Teejet 110015VK ceramic nozzle. Plants were maintained for 3 weeks after spraying before assessing survival. The number of surviving plants from each pot was counted and the average survival rate from three biological replicates (three pots with six plants per pot) was used to calculate the percentage survival of each black-grass population.

**Table 1.** The average relative transcript expression of 24 DNA contigs identified by RNA sequences in herbicide sensitive (HS) and NTSR (Peldon) black-grass populations

| Contig | ID | HS | NTSR |
|---|---|---|---|
| R00041432 | CYP450s | 1.14 ± 0.12 | 4.02 ± 0.26 |
| R00030509 | CYP450s | 1.09 ± 0.08 | 3.01 ± 0.19 |
| R00027925 | CYP450s | 1.04 ± 0.08 | 3.43 ± 0.08 |
| R000277289 | Isoflavone hydroxylase | 1.06 ± 0.05 | 2.29 ± 0.12 |
| R002332027 | OPR1 | 0.97 ± 0.04 | 51.49 ± 2.01 |
| R00052495 | OPR1 | 1.30 ± 0.35 | 71.09 ± 7.36 |
| Rm00002116 | OPR1 | 1.15 ± 0.14 | 50.90 ± 2.02 |
| R00029421 | Carboxyl esterase | 1.00 ± 0.18 | 4.41 ± 0.48 |
| R00029215 | Zeatin UGT | 1.21 ± 0.19 | 14.38 ± 1.51 |
| R00007921 | GSTU6-like | 1.04 ± 0.04 | 5.31 ± 0.49 |
| R00030700 | GSTU6-like | 1.01 ± 0.09 | 18.40 ± 0.11 |
| R00005793 | GSTU6-like | 1.15 ± 0.14 | 24.30 ± 0.90 |
| R00096975 | GSTU6-like | 1.00 ± 0.08 | 27.77 ± 1.14 |
| R00029476 | GSTF1 | 1.00 ± 0.08 | 8.30 ± 0.39 |
| R00010869 | Aminotransferase | 1.03 ± 0.03 | 27.94 ± 5.93 |
| R00029959 | Cellulose synthase | 1.39 ± 0.34 | 6.03 ± 0.95 |
| Rm00043661 | ABC transporter | 1.08 ± 0.15 | 1.71 ± 0.10 |
| R00030815 | MATE transporter | 1.31 ± 0.13 | 4.16 ± 0.25 |
| R0000345 | Thiol methyl transferase | 0.93 ± 0.08 | 18.06 ± 0.28 |
| Rm00016513 | Thiol methyl transferase | 0.84 ± 0.14 | 18.30 ± 0.67 |
| Rm00004119 | Thiol methyl transferase | 1.06 ± 0.09 | 14.91 ± 0.56 |
| R00029303 | Pathogenesis related protein | 0.98 ± 0.04 | 1.22 ± 0.15 |
| R00003857 | Pathogenesis related protein | 0.97 ± 0.05 | 16.58 ± 0.31 |
| R00004163 | Gag-pol retrotransposon | 0.85 ± 0.17 | 4.32 ± 0.27 |

ABC, ATP-binding cassette; CYP450s, cytochrome P450 monooxygenases; GSTF1, glutathione transferase phi (F) 1; GSTU6, glutathione transferase tau (U) 6; ID, MATE, multidrug and toxic compound extrusion; OPR1, 12-oxophytodienonate reductase-1; UGT, UDP-glucuronosyltransferases. The relative expressions were quantified in leaf tissues by quantitative real-time PCR (RT-qPCR). The sequence of each contig was identified by comparison with the DNA sequence in the National Center for Biotechnology Information (NCBI) public database (https://www.ncbi.nlm.nih.gov). The average basal expression (mean ± standard deviation, $n = 3$) of each contig was reported.

**Table 2.** The assembled DNA contigs identified by RNA sequencing of HS and NTSR black-grass

| Gene ID | Annotation | Accession number |
|---|---|---|
| *Am*CYP450 | Cytochrome P450 family 72A1 | |
| *Am*OPR1 | *Alopecurus myosuroides* mRNA for 12-oxophytodienoate reductase 1 | KY172653.1 |
| *Am*UGT | Cis-zeatin O-glucosyltransferase 2 | |
| *Am*GSTF1 | *Alopecurus myosuroides* mRNA for glutathione transferase 2c | AJ010453 |
| *Am*GSTU2 | *Alopecurus myosuroides* mRNA for glutathione s-transferase U2 | KY172655 |
| *Am*ABC | Possible ABCI7 *A. tauschii* | |

The contigs were assembled as described in Cummins *et al.*[17] The gene annotations and accession numbers were obtained by comparing assembled sequences with the published database (National Center for Biotechnology Information (NCBI); https://www.ncbi.nlm.nih.gov). (NB: Full sequences for *Am*CYP450, *Am*UGT and *Am*ABC were not available, therefore no accession numbers are listed for these genes).

### 2.4 Herbicide metabolism of field-collected populations (HPLC)

To provide an assessment of herbicide metabolism across the 27 populations, the quantification of radiolabelled herbicides and their metabolites was assessed using Radio-High-Performance Liquid Chromatography (HPLC). Plants were grown to BBCH11, and samples comprising a shoot and leaf were taken from 16 unsprayed plants per population, per herbicide tested. Samples were placed in a 96-deep-well plate filled with 600 µL of water solution containing 400 000 dpm $^{14}$C-radiolabelled fenoxaprop (specific activity 4.02 MBq mg$^{-1}$) or $^{14}$C-radiolabelled mesosulfuron (specific activity 4.02 MBq mg$^{-1}$). Samples were incubated for 16 h at 28 °C under illumination. After 16 h, samples were removed from plates and washed with purified water for mesosulfuron-treated samples or with 80% (v/v) acetone for fenoxaprop-treated samples. All the following steps were then performed in 96-well plates. Leaf samples were extracted twice with 600 µL of methanol and once with 90% (v/v) acetonitrile using a tissue-lyser system (Qiagen, Netherlands). After each extraction, plates were centrifuged at 6000 × g for 10 min. The supernatants from each extraction were collected and combined. The extracts were evaporated to dryness using a vacuum evaporator (TurboVap, Biotage, Sweden) and each resuspended in 200 µL of 90% (v/v) acetonitrile prior to filtration using a MultiScreen Solvinert 96 Well Filter Plate (Merck, Germany). The filtrate was analysed to determine the relative quantities of herbicide and related metabolites after their separation using a JASCO XL-C 3158 AS (Jasco, Japan) fitted with a Phenomenex Kinetex (2.6 µm C18 100A) column (Phenomenex, USA). A total of 80 µL samples were injected and metabolites resolved using a gradient of 0.05% (v/v) formic acid (FA) + $H_2O$ (solvent A) and acetonitrile (ACN) + 0.05% (v/v) FA (solvent B). Further details of the gradient settings for fenoxaprop and mesosulfuron analysis are described in Tables S2 and S3. The radioactivity was measured using a Raytest Miranda detector (Elysia-Raytest, Switzerland). In each case total metabolism was calculated by determining the proportion of parent herbicide remaining (peak area). Examples of the chromatograms for fenoxaprop-ethyl and mesosulfuron metabolism are shown in Figs S1 and S2.

### 2.5 Transcript expression of putative biomarkers

Expression of the six chosen putative biomarkers was assessed from all field and reference populations at the gene transcript level. Plant tissue comprising 2-cm leaf tips from unsprayed plants at BBCH11 were harvested into aluminium foil and flash-frozen in liquid nitrogen, then stored at −80 °C before further processing. Samples were taken from three biological replicates (three pots with 10 plants per pot) per population. The plant tissue was then ground in liquid nitrogen before transferring 100 mg to a chilled 1-mL microcentrifuge tube; any remaining tissue was stored at −80 °C in a second microcentrifuge tube. Total RNA was extracted using the Qiagen RNeasy Plant Mini Kit (Qiagen, Netherlands) following the manufacturer's guidelines. The extracted RNA was then stored at −80 °C. cDNA was synthesized from 0.5 µg of total RNA using Invitrogen superscript IV (Invitrogen, USA) with an Oligo d(T)20 primer according to the manufacturer' guidelines. cDNA samples were stored at −20 °C.

Normalised expression of the six putative biomarker genes (Table 2) was quantified using a 7500 Fast Real-Time PCR System (Appled Biosystems, USA) in 96-well semi-skirted plates (Starlab, UK). Reaction mixes were prepared using Takyon Low Rox SYBR MasterMix dTTP Blue (Eurogentec, Belgium), then 2.5 µL of cDNA template (1:25 dilution) was added to 10 µL of Takyon MasterMix (Eurogentec, Belgium), 2 µL of 100 nM forward primer, 2 µL of 100 nM reverse primer and 3.5 µL of nuclease-free water. qPCR primer sequences and cycling conditions are listed in Tables S4 and S5. Relative normalized gene expression was calculated using the standard curve method. Briefly, a four-point dilution series of the cDNA produced from the Rothamsted (herbicide sensitive) population was used to quantify the efficiency of each primer pair and subsequently the raw threshold cycle (Ct) values were adjusted to account for primer amplification efficiency. The two reference genes used were ubiquitin (*Am*UBQ) and glyceraldehyde 3-phosphate dehydrogenase (*Am*GAPDH).[22]

### 2.6 Protein immunoblots (Western blot)

First, ∼100 mg of pulverized above ground tissue of black-grass (BBCH 13–15) was extracted in 1 mL of extraction buffer (100 mM Tris-HCl, 150 nM NaCl, 5 mM EDTA, 5% glycerol, 2% PVPP, 10 mM DTT and pH 7.5) as described.[1] Approximately 75 µg of protein was then resolved by electrophoresis on a 12% SDS-polyacrylamide gel, with polypeptides transferred onto a polyvinylidene difluoride (PVDF) membrane using a dry blotting system (iBlot system, ThermoFisher Scientific, UK). The PVDF membrane was then processed as previously described[4] using antisera raised to *Am*GSTF1, *Am*GTSU2 and *Am*OPR1 as the primary antibody and anti-rabbit IG as the secondary antibody. The protein signal was developed using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) premixed solution (Sigma Aldrich, USA) and visualised with Chemidoc system (BioRad, USA).

### 2.7 Protein concentration of putative biomarkers (ELISA)

Following transcript analysis (Section 2.6), the three most promising candidate biomarkers, *Am*GSTF1, *Am*GSTU2 and *Am*OPR1, were assessed across all populations at the level of leaf protein concentration. Total protein from foliar tissues was extracted as above. For *Am*GSTF1, the assays were performed as per Comont *et al.*[1] For *Am*GSTU2 and *Am*OPR1, indirect enzyme-linked

immunosorbant assay (ELISA) was used. A total of 50 µg $mL^{-1}$ of antigens (total protein extracted from foliar tissue) was diluted in carbonate bicarbonate buffer (Sigma Aldrich, USA). A total of 100 µL of antigen per well was added to a Microwell-96 well plate (NUNC, Thermo Fisher, USA) and incubated at 4 °C overnight. Plates were washed with Phosphaat-buffer saline (PBS)-Tween (0.05% v/v) buffer using a microplate washer system (Thermo Fisher Scientific, USA). After four washes, plates were blocked in 1% bovine serum albumin (BSA) in PBS buffer for 1 h at 37 °C. After four washes with PBS-Tween buffer, 100 µL of AmGSTU2 or AmOPR1 antisera (1 µg $mL^{-1}$) was added to each well and plates were incubated for 1 h at 37 °C. After four washes, 100 µL of the secondary antibody, anti-rabbit horseradish peroxidase (HPR), was added before incubating for 1 h at 37 °C. After four washes, 200 µL of SIGMAFAST OPD substrate (Sigma Aldrich, USA) was added to each well and plates were incubated in the dark at room temperature for 30 min. The signal was read at an optical density of 450 nm ($OD_{450}$) using a HIDEX-sense microplate reader (Hidex). A standard curve for the *Am*GSTU2 recombinant protein (four-parameter logistic regression) was made using Prism software (Version 10.1.1, Graphpad, USA). *Am*GSTU2 protein concentration in plant samples was calculated from the standard curve. The semiquantitative approach to compare the relative levels of *Am*OPR1 protein among the samples was conducted by determining the $OD_{450}$ signal in a similar concentration of total protein (50 µg) from each sample.

### 2.8 Pyrosequencing of SNPs

The single nucleotide polymorphisms (SNPs) in the ALS and ACCase genes known to confer TSR were quantified using pyrosequencing.[23] A total of 24 plants per population was sequenced ($n = 720$) at two loci in the ALS gene and five loci in the ACCase gene. The PCR amplification and pyrosequencing method was as described in Comont *et al.*,[1] and sequences and cycling conditions are shown in Table S6.

### 2.9 Statistical analysis

The relationship between enhanced metabolism and transcript expression was analysed with linear regression. The measure of enhanced metabolism used for these models was the proportion of parent herbicide remaining (peak area), detected by HPLC following incubation with $^{14}$C-labelled fenoxaprop or mesosulfuron. Enhanced fenoxaprop metabolism and enhanced mesosulfuron metabolism were regressed in separate models against the transcript expression of each of the six biomarkers (*Am*GSTF1, *Am*GSTU2, *Am*CYP450, *Am*OPR1, *Am*UGT and *Am*ABC). Models were fitted in R version 4.2.1.

The relationship between resistance phenotype (proportion of plants surviving herbicides applied at field rate) and transcript expression or protein abundance was analysed with generalised linear regression (GLM) with a logit link function. As the resistance phenotype of a population is likely to result from a combination of TSR and NTSR mechanisms, ACCase TSR frequency was included in

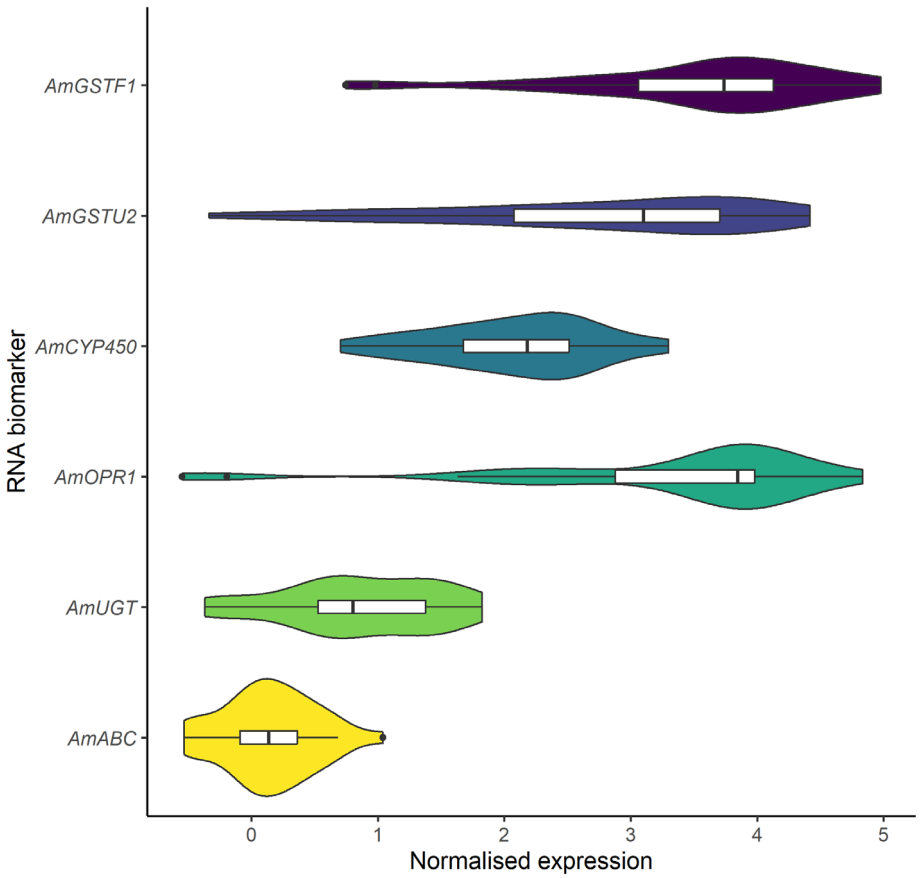


**Figure 1.** Relative expression of six RNA biomarkers across black-grass populations. The distributions of the basal transcript expression of *Am*GSTF1, *Am*GSTU2, *Am*CYP450, *Am*OPR1, *Am*UGT and *Am*ABC from above-ground tissues of BBCH 11 stage black-grass plants from 27 field populations, herbicide-sensitive, non-target site resistance, and the target site resistance populations.




*Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

the fenoxaprop resistance models and ALS TSR frequency was included in the mesosulfuron resistance models. TSR frequency is defined as the proportion of plants with one or more SNPs in the specified gene. Fenoxaprop resistance phenotype was regressed against the transcript expression of each of the six biomarkers (*Am*GSTF1, *Am*GSTU2, *Am*CYP450, *Am*OPR1, *Am*UGT and *Am*ABC), combined with ACCase TSR frequency and against the protein abundance of the three most promising biomarkers (*Am*GSTF1, *Am*GSTU2 and *Am*OPR1) also in combination with ACCase TSR frequency. Mesosulfuron resistance phenotype was regressed against the transcript expression of each of the six biomarkers (*Am*GSTF1, *Am*GSTU2, *Am*CYP450, *Am*OPR1, *Am*UGT and *Am*ABC), combined with ALS TSR frequency and against the protein abundance of the three most promising biomarkers (*Am*GSTF1, *Am*GSTU2 and *Am*OPR1) also in combination with ALS TSR frequency. All GLM models were fitted in R version 4.2.1 with the 'lme4' package.[24]

# 3 RESULTS

## 3.1 Validation of molecular biomarkers to predict EMR in reference black-grass populations

All 24 putative NTSR-associated contigs corresponding to the selected biomarker genes were confirmed to have significantly increased (>2-fold) expression in the reference NTSR population compared to the reference herbicide-sensitive population (Table 1). Additionally, in a recent study, enhanced constitutive expression of these potential biomarker genes corresponded with the increased metabolism in the NTSR (Peldon) populations of fenoxaprop and chlortoluron, which are known to be detoxified by GSTs and cytochrome P450s, respectively.[25] From the 24 DNA-contigs identified as biomarker candidates (Table 1), six corresponding genes were prioritised as potential biomarkers (Table 2) in this study based on previous research and their relative expression in herbicide-sensitive and NTSR samples. These six potential biomarker genes included representatives of the GST and CYP450 families, as well as a UGT, an ABC transporter and OPR1.

## 3.2 Validation of transcriptional biomarkers for detecting EMR in black-grass field populations

To examine the broader reliability of potential EMR biomarkers in field-collected black-grass populations, we assessed the basal expression of the six biomarkers in 27 field-collected populations of black-grass (Fig. 1) displaying herbicide resistance to two classes of herbicide (Fig. 2)[1] as well as three reference populations, namely, Rothamsted (herbicide sensitive), Peldon (NTSR) and Notts (a point mutation acetyl-CoA carboxylase, TSR). Of the six biomarkers, *Am*GSFT1, *Am*GSTU2 and *Am*OPR1 displayed the greatest variation in basal expression across these 30 populations. These expression patterns corresponded to the broad range of herbicide resistance profiles observed in these black-grass populations.

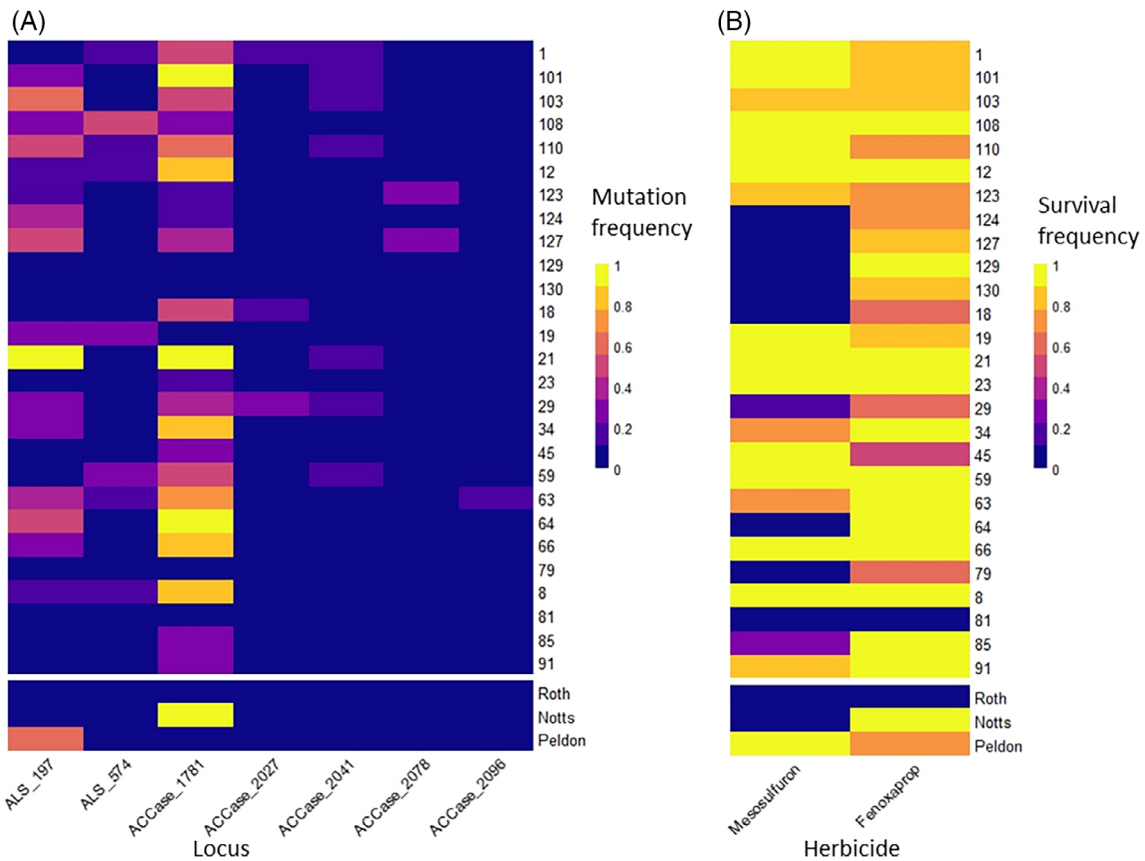


**Figure 2.** (A) Heatmap representing the proportion of mutations present at seven loci measured using pyrosequencing for 30 populations with 24 plants sampled per population ($n = 720$). Each population is represented by a horizontal row across the heatmap. (B) Heatmap representing the proportion of plants surviving sprayed with 9 + 1.8 g ai $ha^{-1}$ mesosulfuron-methyl + iodosulfuron-methyl-sodium (Atlantis) or 68.75 g ai $ha^{-1}$ fenoxaprop-p-ethyl (Polecat). The top 27 rows on each heatmap represent the wild collected populations and the bottom three rows represent the three reference populations.

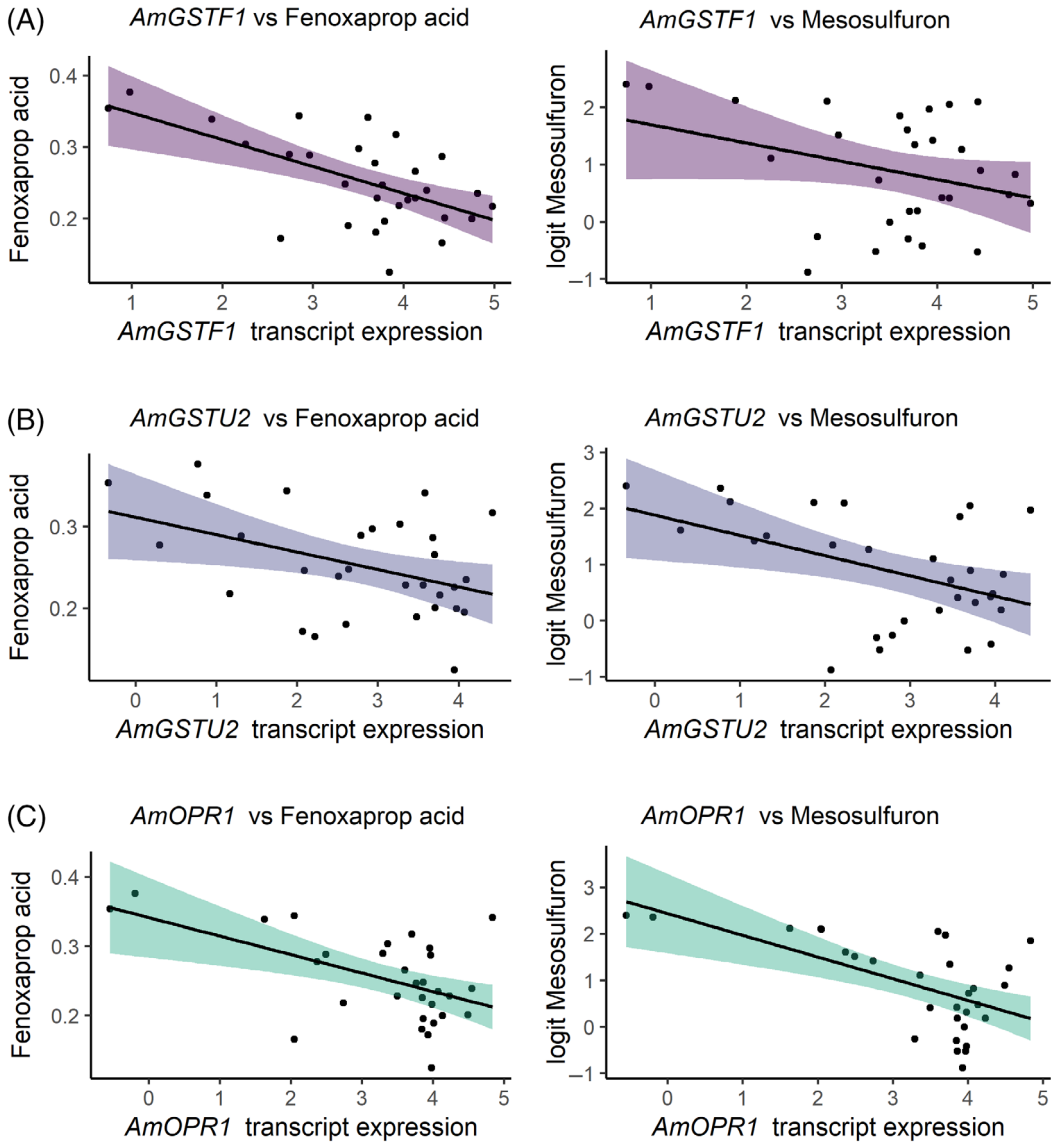


**Figure 3.** The relationships between basal expression of biomarker genes and the level of radiolabelled ($^{14}C$) fenoxaprop or mesosulfuron at 16 h after treatment in black-grass populations. Fitted linear regression of three models with fenoxaprop metabolism (left) or mesosulfuron metabolism (right) predicted by transcript expression of (A) *Am*GSTF1$_{fenoxaprop}$ ($P < 0.001$), *Am*GSTF1$_{mesosulfuron}$ ($P = 0.17$), (B) *Am*GSTU2$_{fenoxaprop}$ ($P < 0.001$), *Am*GSTU2$_{mesosulfuron}$ ($P < 0.001$) and (C) *Am*OPR1$_{fenoxaprop}$ ($P < 0.01$), *Am*OPR1$_{mesosulfuron}$ ($P < 0.01$). Solid lines show the fitted model with shaded regions showing 95% confidence limits and solid points are the original data used to fit the model.

To link the transcript expression of these potential biomarkers with the metabolism of specific herbicides, we analysed the detoxification of fenoxaprop and mesosulfuron in the 30 black-grass populations. For fenoxaprop, the basal expression of the three most promising biomarkers identified above (*Am*GSFT1, *Am*GSTU2, *Am*OPR1) showed significant linear relationships with the relative degree of fenoxaprop metabolism observed (*Am*GSTF1: $F = 16.67$, $P < 0.001$; *Am*GSTU2: $F = 6.24$, $P < 0.001$; *Am*OPR1: $F = 11.24$, $P = 0.002$; Fig. 3 and Table S7). However, despite positive correlations amongst the six biomarkers, the basal expression of *Am*UGT, *Am*CYP450 and *Am*ABC, had no significant linear relationships to the relative degree of fenoxaprop metabolism (Table S7). In contrast to fenoxaprop, only two biomarkers, namely, *Am*GSTU2 and *Am*OPR1, showed significant linear relationships with mesosulfuron metabolism (*Am*GSTU2: $F = 7.64$, $P = 0.010$; *Am*OPR1: $F = 15.79$, $P < 0.001$; Fig. 3 and Table S8). The other four biomarkers, including *Am*GSTF1, showed no significant relationship with mesosulfuron metabolism at the level of the gene transcript (Table S8). Our results therefore demonstrated that of the most promising six biomarkers, only three genes (*Am*GSTU2, *Am*OPR1 and *Am*GSTF1) had the potential to accurately predict EMR towards fenoxaprop or mesosulfuron.

### 3.3 *Am*GSTF1, *Am*GSTU2 and *Am*OPR1 proteins as biomarkers of EMR

As the metabolism of herbicides is affected by enzymes and transporters rather than the respective transcripts, the selected biomarkers were further quantified and analysed at the protein level to determine their relationships to NTSR traits in the black-grass populations. Specific antibodies were raised in rabbits toward *Am*GSTF1, *Am*GSTU2 and *Am*OPR1, and their specificity was tested in total protein extracted from reference herbicide-




*Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

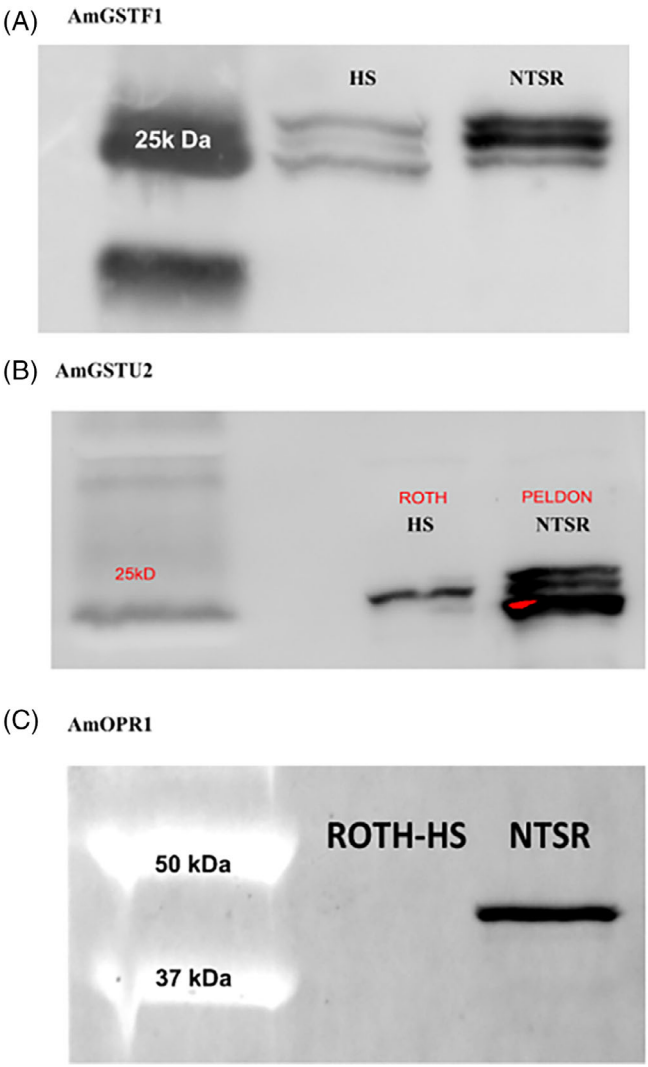


**Figure 4.** *Am*GSTF1, *Am*GSTU2 and *Am*OPR1 antisera detected specific proteins in black-grass. The protein immunoblots (western blot) of (A) *Am*GSTF1, (B) *Am*GSTU2 and (C) *Am*OPR1 from total proteins extracted from three- to five-leaf herbicide sensitive (HS) and non-target site resistance (NTSR) plants. The molecular weight of each protein was calculated using online software (https://web.expasy.org/compute_pi/). The expected molecular weights (MW) were *Am*GSTF1 = 24.93 kDa, *Am*GSTU2 = 24.46 kDa and *Am*OPR1 = 40.03 kDa.

sensitive and NTSR black-grass populations. The antibodies for *Am*GSTF1 and *Am*GSTU2 proteins detected polypeptides of the typical molecular mass of GST subunits, namely, *Am*GSTF1 MW 24.9 kDa and *Am*GSTU2 MW 24.4 kDa in both herbicide-sensitive and NTSR black-grass. The abundance of the immuno-recognized protein bands was clearly greater in the NTSR population in both cases (Fig. 4(A,B)). For *Am*OPR1, although the expression of this transcript was detected in both herbicide-sensitive and NTSR black-grass, no specific band for the *Am*OPR1 protein (approximated MW 40.0 kDa) was detectable in herbicide-sensitive black-grass (Fig. 4(C)). These results confirm the specificity and sensitivity of antibodies that can detect enhanced expression of these three protein biomarkers in black-grass plants. We therefore used these antibodies to quantify the basal expression of the corresponding biomarker proteins in field populations, then examined the link between the respective abundance of the polypeptides and herbicide metabolism.

Across the wider 30 populations, the relative abundances of both *Am*GSTF1 and *Am*GSTU2 polypeptides were significant predictors of both fenoxaprop metabolism (*Am*GSTF1: $F = 10.53$, $P = 0.003$; *Am*GSTU2: $F = 17.15$, $P < 0.001$) and mesosulfuron metabolism (*Am*GSTF1: $F = 11.96$, $P = 0.002$; *Am*GSTU2: $F = 14.31$, $P < 0.001$; Fig. 5 and Tables S9 and S10). In contrast, the basal level of *Am*OPR1 protein was a significant predictor of fenoxaprop, but not mesosulfuron (Fig. 5 and Tables S9 and S10) $AmOPR1_{fenoxaprop}$ ($F = 5.67$, $P = 0.02$), $AmOPR1_{mesosulfuron}$ ($F = 0.02$, $P = 0.89$). These results highlight the potential to detect EMR toward fenoxaprop and mesosulfuron in black-grass populations through protein biomarkers.

### 3.4 Predicting black-grass survival from herbicide treatments through combined analysis of mutation frequency and basal expression of biomarkers

Finally, as both NTSR and TSR mechanisms are known factors that determine plant survival from herbicides treatments, we tested the capacity of using the expression of *Am*GSTF1, *Am*GSTU2 and *Am*OPR1 transcript or protein and TSR mutation frequency as explanatory (predictor) variable to predict survival to fenoxaprop and mesosulfuron. Additionally, we tested the accuracy of using TSR frequency as a sole predictor variable. In the 30 test populations, the transcript and protein expression of *Am*GSTF1 and *Am*GSTU2 in combination with TSR frequency were significant predictors of survival to fenoxaprop and mesosulfuron (Tables 3–7). Importantly, the combination of TSR frequency and biomarkers improved the survival prediction compared to the model that used TSR frequency alone (Table 3–7). While incorporating *Am*GSTF1 protein in the models resulted in improved prediction of mesosulfuron survival, an incorporation of *Am*GSTF1 transcript expression was not a significantly improved prediction of mesosulfuron survival (Table 3–7). In contrast to *Am*GSTF1 and *Am*GSTU2, the prediction of mesosulfuron and fenoxaprop survival was significantly improved when *Am*OPR1 transcript expression and TSR frequency were used as predictors compared to when TSR frequency was used alone (Tables 3–7). However, *Am*OPR1 protein expression combined with TSR frequency was not a significant predictor for mesosulfuron and fenoxaprop survival.

## 4 DISCUSSION

The evolution of herbicide resistance in agricultural weed species causes significant losses in herbicide control, leading to subsequent yield losses.[5] Although laboratory and glasshouse methods accurately identify resistance to specific herbicides, these conventional methods are time-consuming and require expertise in molecular biology and analytical chemistry. Importantly, because these methods require seed or seedling collection and take some time to perform, current resistance diagnostics tests have a limited ability to inform weed control strategies in real time within the crop production season. This may lead to the ineffectual use of post-emergence herbicides and suboptimal weed control. Recently, we developed a first-generation rapid diagnostic test for NTSR in black-grass and other wild grasses. This test uses low-cost and rapid lateral flow immunodetection, based on the relative quantification of the NTSR biomarker *Am*GSTF1.[18] While this diagnostic is an important first step in real-time herbicide resistance diagnostics, it has a limited capacity to influence the usage of specific herbicides to restore control as it is unable to categorise the type of EMR present in wild grass populations. To establish a functioning in-field diagnostic, several factors need to be considered. These factors are (i) the ability of pinpoint

**Table 3.** Survival to fenoxaprop predicted by transcript expression and ACCase mutation frequency (output from quasibinomial generalised linear regression)

| Model | Response variable | Explanatory variable | df | Deviance difference | Residual df | Residual deviance | *P* value |
|---|---|---|---|---|---|---|---|
| 1 | Fenoxaprop survival | *Am*GSTF1 | 1 | 67.17 | 28 | 148.36 | <0.001** |
| | | ACCase | 1 | 54.57 | 27 | 93.97 | <0.001** |
| 2 | Fenoxaprop survival | *Am*GSTU2 | 1 | 68.93 | 28 | 146.60 | <0.001** |
| | | ACCase | 1 | 54.57 | 27 | 122.21 | 0.013* |
| 3 | Fenoxaprop survival | *Am*OPR1 | 1 | 45.24 | 28 | 167.23 | <0.001** |
| | | ACCase | 1 | 54.57 | 27 | 139.26 | <0.001** |

The normalised basal expression of each biomarker and ACCase mutation frequency were used as independent variables to predict survival to spraying with fenoxaprop.
*$p < 0.05$;
** $p < 0.01$.

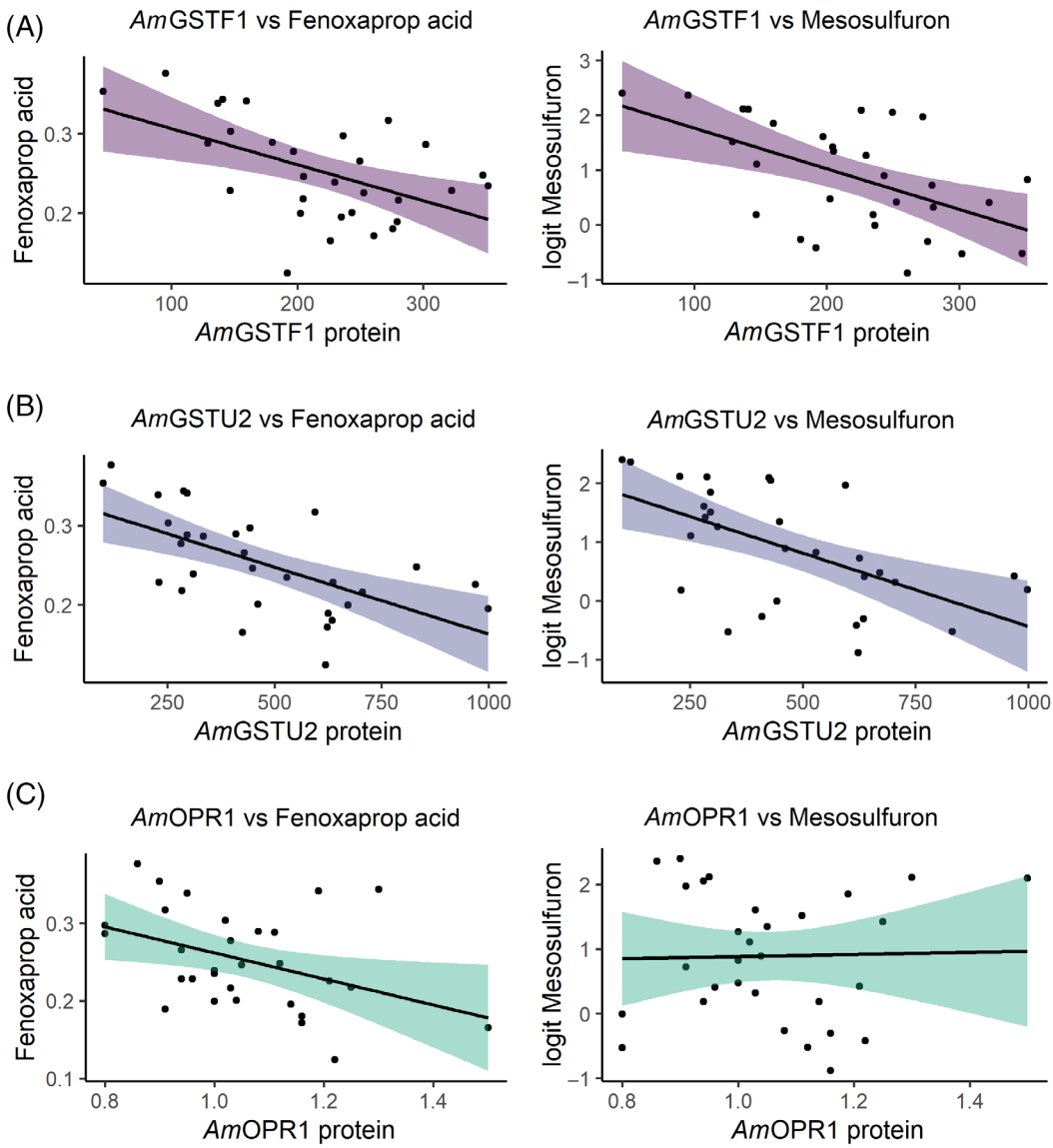


**Figure 5.** Fitted linear regression of three models with fenoxaprop metabolism (left) or mesosulfuron metabolism (right) predicted by protein expression of (A) $AmGSTF1_{fenoxaprop}$ ($P < 0.01$), $AmGSTF1_{mesosulfuron}$ ($P < 0.01$), (B) $AmGSTU2_{fenoxaprop}$ ($P < 0.001$), $AmGSTU2_{mesosulfuron}$ ($P < 0.001$) and (C) $AmOPR1_{fenoxaprop}$ ($P < 0.05$), $AmOPR1_{mesosulfuron}$ ($P = 0.89$). Solid lines show the fitted model with shaded regions showing 95% confidence limits and solid points are the original data used to fit the model.

**Table 4.** Survival to mesosulfuron predicted by transcript expression and ALS mutation frequency

| Model | Response variable | Explanatory variable | df | Deviance difference | Residual df | Residual deviance | *P* value |
|---|---|---|---|---|---|---|---|
| 1 | Mesosulfuron survival | *Am*GSTF1 | 1 | 43.48 | 28 | 492.24 | 0.04* |
| | | ALS | 1 | 233.96 | 27 | 258.28 | <0.001** |
| 2 | Mesosulfuron survival | *Am*GSTU2 | 1 | 186.66 | 28 | 349.06 | <0.001** |
| | | ALS | 1 | 141.91 | 27 | 207.15 | 0.013* |
| 3 | Mesosulfuron survival | *Am*OPR1 | 1 | 181.60 | 28 | 354.12 | <0.001** |
| | | ALS | 1 | 131.60 | 27 | 222.51 | <0.001** |

Output from quasibinomial generalised linear regression. The normalised basal expression of each biomarker and acetolactate synthase (ALS) mutation frequency were used as independent variables to predict survival to spraying with mesosulfuron.
*$p < 0.05$;
** $p < 0.01$.

**Table 5.** Survival to fenoxaprop predicted by protein expression and ACCase mutation frequency

| Model | Response variable | Explanatory variable | df | Deviance difference | Residual df | Residual deviance | *P* value |
|---|---|---|---|---|---|---|---|
| 1 | Fenoxaprop survival | *Am*GSTF1 | 1 | 39.22 | 28 | 176.3 | 0.006** |
| | | ACCase | 1 | 51.1 | 27 | 125.2 | 0.002** |
| 2 | Fenoxaprop survival | *Am*GSTU2 | 1 | 48.62 | 28 | 166.9 | 0.001** |
| | | ACCase | 1 | 34.63 | 27 | 132.3 | 0.006** |
| 3 | Fenoxaprop survival | *Am*OPR1 | 1 | 17.57 | 28 | 198 | 0.056 |
| | | ACCase | 1 | 46.33 | 27 | 151.6 | 0.002** |

Output from quasibinomial generalised linear regression. The normalised basal expression of each biomarker and ACCase mutation frequency were used as independent variables to predict survival to spraying with fenoxaprop.
** Significant differences.

**Table 6.** Survival to mesosulfuron predicted by protein expression and ALS mutation frequency

| Model | Response variable | Explanatory variable | df | Deviance difference | Residual df | Residual deviance | *P* value |
|---|---|---|---|---|---|---|---|
| 1 | Mesosulfuron survival | *Am*GSTF1 | 1 | 114.6 | 28 | 421.1 | <0.001*** |
| | | ALS | 1 | 196.2 | 27 | 225 | <0.001*** |
| 2 | Mesosulfuron survival | *Am*GSTU2 | 1 | 235.2 | 28 | 300.6 | <0.001*** |
| | | ALS | 1 | 180.4 | 27 | 120.2 | <0.001*** |
| 3 | Mesosulfuron survival | *Am*OPR1 | 1 | 13.28 | 28 | 522.4 | 0.289 |
| | | ALS | 1 | 251.5 | 27 | 270.9 | <0.001*** |

Output from quasibinomial generalised linear regression. The normalised basal expression of each biomarker and ALS mutation frequency were used as independent variables to predict survival to spraying with mesosulfuron.
*** Significant differences.

EMR to multiple MOAs, (ii) an understanding of the weed population structure in-field, (iii) a sampling regime that ensures good coverage of that population, (iv) knowledge of the molecular biomarkers of EMR, (v) an effective methodology for extracting and analysing molecular biomarkers in-field, and (vi) to estimate a given population's likelihood to survive herbicide applications, an estimate of TSR frequency. In this study, we began to address the fourth factor by assessing the links between molecular biomarkers (RNA and proteins) and EMR in a set of black-grass (*Alopecurus myosuroides*) populations.

EMR is a primary mechanism underlying cross-resistance to multiple herbicide chemistries[9,10] and is the predominant non-target-site resistance mechanism in black-grass. As such, EMR in black-grass is now known to involve the enhanced detoxification of herbicides catalysed by the concerted action of CYPs, bioconjugating enzymes and active transporters.[9,26–28] As each herbicide chemistry is metabolised by different routes involving differing combinations of CYPs, GSTs and UGTs, we rationalised that an increased expression of specific detoxification enzymes could potentially be used as a set of biomarkers for EMR that linked to a single class of herbicide. Overall, our results confirm that several transcript and protein markers associated with detoxification are good EMR biomarker candidates. We observed significant positive relationships between the basal expression of three biomarkers (*Am*GSTF1, *Am*GSTU2 and *Am*OPR1) and the increased metabolism of the herbicides fenoxaprop and mesosulfuron in black-grass populations collected from fields across the UK. While these results demonstrate the benefits of targeting detoxification genes for biomarker screening, only three genes from the six candidates were robust potential biomarkers of

**Table 7.** Summary of biomarkers which significantly predict survival to mesosulfuron or fenoxaprop in linear models with ALS or ACCase TSR frequency, respectively, and a comparison with the model of survival predicted by TSR frequency alone with *P* values indicated by stars

| Biomarker type | Biomarker | Herbicide | Survival predicted by biomarker + TSR | Better than TSR alone |
|---|---|---|---|---|
| RNA | *Am*GSTF1 | Mesosulfuron | Yes | No |
| Protein | *Am*GSTF1 | Mesosulfuron | Yes | Yes* |
| RNA | *Am*GSTF1 | Fenoxaprop | Yes | Yes*** |
| Protein | *Am*GSTF1 | Fenoxaprop | Yes | Yes** |
| RNA | *Am*GSTU2 | Mesosulfuron | Yes | Yes** |
| Protein | *Am*GSTU2 | Mesosulfuron | Yes | Yes*** |
| RNA | *Am*GSTU2 | Fenoxaprop | Yes | Yes*** |
| Protein | *Am*GSTU2 | Fenoxaprop | Yes | Yes** |
| RNA | *Am*OPR1 | Mesosulfuron | Yes | Yes* |
| Protein | *Am*OPR1 | Mesosulfuron | No | No |
| RNA | *Am*OPR1 | Fenoxaprop | Yes | Yes* |
| Protein | *Am*OPR1 | Fenoxaprop | No | No |

*** $P < 0.001$;
** $P < 0.01$;
* $P < 0.05$.

EMR. These results do not confirm the activity of the three positive biomarkers in EMR, or conversely that the other markers tested in the panel are not biologically active in herbicide detoxification, rather they highlight the need to screen several candidates at both the transcript and protein levels to identify reliable biomarkers. This selection is particularly important based on the size of the gene families encoding proteins involved in detoxification, notably the respective multiplicity of the CYPs, GSTs, UGTs and transporters being discovered in black-grass and other wild grasses as their respective transcriptomes and genomes are sequenced.

The discrepancy between transcript and protein level in biomarker utility observed here highlights the need to understand the relationships between molecular components (transcript and protein) and EMR to specific herbicides according to the functional 'level' at which they are measured. While molecular mechanisms of EMR are commonly studied at the transcript expression level, the translation of these transcripts into functional proteins is poorly understood. The different effectiveness of transcript and protein levels (*Am*OPR1 and *Am*GSTF1) to predict EMR highlights the need to explore the relationships between translational control and transcript expression of genes involving in herbicide resistance. Additionally, the effectiveness of antibodies to detect protein biomarkers might be another contributing factor to the outcome discrepancy. The optimisation of antibody specificity is a complex process. The detection specificity of *Am*GSTF1 and *Am*OPR1 antibodies used in this study was confirmed by western blot (Fig. 4). Nevertheless, the epitope mapping of these antibodies should be done in future to ensure the high specificity of these antibodies. It is noteworthy that the *Am*OPR1 protein level was semiquantified due to the lack of recombinant protein while *Am*GSTF1 and *Am*GSTU2 levels were quantified based on the concentration of respective recombinant proteins. This might affect the quantification of the *Am*OPR1 protein level. The generation of *Am*OPR1 recombinant protein and re-quantified AmOPR1 should be done in the future to address this problem.

Considering that *AmOPR1* is a significant predictor of metabolism and survival toward mesosulfuron and fenoxaprop at the transcript level, little is known about the function of this gene in black-grass. It is noteworthy that the expression of OPR1 (*At*OPR1) and various GST genes in *Arabidopsis* were significantly induced after exposure to the herbicides acetochlor, metolachlor and triazine.[29,30] While *At*GSTs can directly catalyse the detoxification of these herbicides, the role of *At*OPR1 in the metabolism of these herbicides remains unknown.[29] The information from *Arabidopsis* leads to the hypothesis that although *Am*OPR1 might not function directly in fenoxaprop or mesosulfuron detoxification, this protein might function in the regulatory networks of EMR or NTSR, therefore additional experiments to functionally characterise *Am*OPR1 are required to explain the role of this protein in NTSR and EMR.

*Am*GSTF1 was also an effective biomarker of EMR to both fenoxaprop and mesosulfuron at the protein level, but only to fenoxaprop at the transcript level. It is interesting that in a previous study *Am*GSTF1 had no activity towards fenoxaprop and had low glutathione conjugating activity toward other herbicides.[30] However, the accumulated information strongly suggests important roles of *Am*GSTF1 in NTSR linked to detoxification and redox homeostasis, which help protect plants against multiple herbicides.[17] The significant relationship between black-grass survival to herbicide (fenoxaprop and mesosulfuron) spraying and *Am*GSTF1 expression might therefore derive from the role of this protein as a regulator of NTSR.

In contrast to *Am*GSTF1, *Am*GSTU2 was an effective biomarker of both mesosulfuron and fenoxaprop at the transcript and protein level. Based on available information, GSTs are the main enzyme in fenoxaprop detoxification, while CYP450s are required for mesosulfuron metabolism.[31,32] In previous assessments *Am*GSTU2 has been proven to metabolise fenoxaprop,[30] therefore this could explain the significant linear relationship between *Am*GSTU2 and fenoxaprop EMR. The future assessment of *Am*GSTU2 activity towards mesosulfuron is required to establish the link with mesosulfuron detoxification. However, regardless of *Am*GSTU2's detoxifying activity, we have shown it to be an effective biomarker of both mesosulfuron and fenoxaprop EMR.

The prospect of detecting EMR through basal expression of biomarkers without glasshouse experiments provides an alternative approach to detect resistance to specific herbicides in

uncharacterised black-grass populations. It is possible that this method, combining two or three molecular assays, could be implemented as the first-line predictor for the survival of uncharacterised black-grass populations before spraying herbicides. Furthermore, the biomarker detection could be used in stewardship programmes to monitor the extent of herbicide resistance in black-grass populations. As an important caveat, we have observed a discrepancy between transcript and protein expression of biomarkers in predicting EMR and plant survival which might derived from the translational control. As such, future biomarkers will need to be identified and validated at both the RNA transcript and protein expression levels.

## 5 CONCLUSION

The significant reduction in herbicide control of agricultural weeds creates negative impacts on crop production and farm economy. The ability to predict the effectiveness of herbicides prior to application in fields is a desirable step towards improving weed control and minimising losses in crop production and the farming economy. We demonstrate in this study an improved understanding of molecular biomarkers of enhanced metabolic resistance and their relationship with black-grass survival to fenoxaprop and mesosulfuron herbicides. This is a critical step in developing a point-to-care protocol for herbicide resistance that will provide reliable first-line screening results that will assist the decision for further tests in the greenhouse or laboratory setup. Future work includes identification of additional markers for a greater range of herbicides and highly specific detection methods such as epitope mapping of antibodies, as well as studies of biomarker suitability in other weed species. In this initial proof of concept we demonstrate the potential to incorporate diagnostic biomarkers into weed management programmes as a screening test prior to herbicide applications as well as resistance monitoring programmes.

## ACKNOWLEDGEMENTS

The populations of black-grass studied were collected from The BGRI black-grass network, established with funding from the Biotechnology and Biological Sciences Research Council (BBSRC; BB/L001489/1) and the Agriculture and Horticulture Development Board (RD-2012-3807). CL was supported by a BBSRC Industrial CASE studentship (BB/M016420/1) which was funded in part by Bayer, AG. At the time of completing this work, DC was supported at Rothamsted research through the BBSRC 'Growing Health' (BB/X010953/1) Institute Strategic Programme. The authors wish to thank Richard Hull and Laura Crook (Rothamsted Research) for their assistance in the growth and maintenance of plant material throughout this study, and Falco Peter and Julia Unger (Bayer) for their support with the HPLC analyses and pyrosequencing.

## DATA AVAILABILITY STATEMENT

Data will be openly available in a public repository that issues datasets with DOIs (Rothamsted Repository) after successful publication

## CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

## REFERENCES

1 Comont D *et al.*, Evolution of generalist resistance to herbicide mixtures reveals a trade-off in resistance management. *Nat Commun* **11**:3086 (2020).
2 Suzukawa AK, Bobadilla LK, Mallory-Smith C and Brunharo CACG, Non-target-site resistance in *Lolium* spp. Globally: a review. *Front Plant Sci* **11**:2137 (2021).
3 Keith BK, Lehnhoff EA, Burns EE, Menalled FD and Dyer WE, Characterisation of *Avena fatua* populations with resistance to multiple herbicides. *Weed Res* **55**:621–630 (2015).
4 Davies LR, Onkokesung N, Brazier-Hicks M, Edwards R and Moss S, Detection and characterization of resistance to acetolactate synthase inhibiting herbicides in Anisantha and Bromus species in the United Kingdom. *Pest Manag Sci* **76**:2473–2482 (2020).
5 Varah A, Ahodo K, Coutts SR, Hicks HL, Comont D, Crook L *et al.*, The costs of human-induced evolution in an agricultural system. *Nat Sustain* **3**:63–71 (2020).
6 Comont D, Hicks H, Crook L, Hull R, Cocciantelli E, Hadfield J *et al.*, Evolutionary epidemiology predicts the emergence of glyphosate resistance in a major agricultural weed. *New Phytol* **223**:1584–1594 (2019).
7 Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61**:317–347 (2010).
8 Délye C, Duhoux A, Pernin F, Riggins CW and Tranel PJ, Molecular mechanisms of herbicide resistance. *Weed Sci* **63**:91–115 (2015).
9 Gaines TA, Duke SO, Morran S, Rigon CAG, Tranel PJ, Küpper A *et al.*, Mechanisms of evolved herbicide resistance. *J Biol Chem* **295**:10307–10330 (2020).
10 Yu Q and Powles S, Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiol* **166**:1106–1118 (2014).
11 Moss S, Ulber L and den Hoed I, A herbicide resistance risk matrix. *Crop Prot* **115**:13–19 (2019).
12 Panozzo S, Scarabel L, Collavo A and Sattin M, Protocols for robust herbicide resistance testing in different weed species. *J Vis Exp* **2015**:e52923 (2015).
13 Aberuyi N, Rahgozar S, Ghodousi ES and Ghaedi K, Drug resistance biomarkers and their clinical applications in childhood acute lymphoblastic leukemia. *Front Oncol* **9**:1496 (2020).
14 O'Driscoll L and Clynes M, Biomarkers and multiple drug resistance in breast cancer. *Curr Cancer Drug Targets* **6**:365–384 (2006).
15 Van Zyl B, Tang D and Bowden NA, Biomarkers of platinum resistance in ovarian cancer: what can we use to improve treatment. *Endocr Relat Cancer* **25**:R303–R318 (2018). https://doi.org/10.1530/ERC-17-0336.
16 Tétard-Jones C, Sabbadin F, Moss S, Hull R, Neve P and Edwards R, Changes in the proteome of the problem weed black-grass correlating with multiple-herbicide resistance. *Plant J* **94**:709–720 (2018).
17 Cummins I, Wortley DJ, Sabbadin F, He Z, Coxon CR, Straker HE *et al.*, Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. *Proc Natl Acad Sci USA* **110**:5812–5817 (2013).
18 Onkokesung N, Brazier-Hicks M, Tetard-Jones C, Bentham A and Edwards R, Molecular diagnostics for real-time determination of herbicide resistance in wild grasses. *J Biotechnol* **358**:64–66 (2022).
19 Cai L, Comont D, MacGregor D, Lowe C, Beffa R, Neve P *et al.*, The black-grass genome reveals patterns of non-parallel evolution of polygenic herbicide resistance. *New Phytol* **237**:1891–1907 (2023).
20 Vogel C and Marcotte EM, Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**:227–232 (2013).
21 Hess M, Barralis G, Bleiholder H *et al.*, Use of the extended BBCH scale—general for the descriptions of the growth stages of mono; and dicotyledonous weed species. *Weed Res* **37**:433–441 (1997).
22 Duhoux A and Délye C, Reference genes to study herbicide stress response in *Lolium* sp.: up-regulation of P450 genes in plants resistant to acetolactate-synthase inhibitors. *PloS One* **8**:e63576 (2013).

23 Beffa R, Figge A, Lorentz L and Hess M, Weed resistance diagnostic technologies to detect herbicide resistance in cerealgrowing areas. A review. *25th German Conference on Weed Biology and Weed Control* **434**:75–80 (2012).
24 Bates D, Mächler M, Bolker BM and Walker SC, Fitting linear mixed-effects models using lme4. *J Stat Softw* **67**:1–48 (2014).
25 Franco-Ortega S, Goldberg-Cavalleri A, Walker A, Brazier-Hicks M, Onkokesung N and Edwards R, Non-target site herbicide resistance is conferred by two distinct mechanisms in black-grass (*Alopecurus myosuroides*). *Front Plant Sci* **12**:194 (2021).
26 Cummins I, Dixon DP, Freitag-Pohl S, Skipsey M and Edwards R, Multiple roles for plant glutathione transferases in xenobiotic detoxification. *Drug Metab Rev* **43**:266–280 (2011). https://doi.org/10.3109/03602532.2011.552910.
27 Lee C, Raffaghello L and Longo VD, Starvation, detoxification, and multidrug resistance in cancer therapy. *Drug Resist Updat* **15**:114–122 (2012).
28 Sultan I, Rahman S, Jan AT, Siddiqui MT, Mondal AH and Haq QMR, Antibiotics, resistome and resistance mechanisms: a bacterial perspective. *Front Microbiol* **9**:2066 (2018).
29 Mezzari MP, Walters K, Jelínkova M, Shih MC, Just CL and Schnoor JL, Gene expression and microscopic analysis of arabidopsis exposed to chloroacetanilide herbicides and explosive compounds. A phytoremediation approach. *Plant Physiol* **138**:858–869 (2005).
30 Sobajima H, Takeda M, Sugimori M *et al*., Cloning and characterization of a jasmonic acid-responsive gene encoding 12-oxophytodienoic acid reductase in suspension-cultured rice cells. *Planta* **216**:692–698 (2003).
31 Cummins I, Bryant DN and Edwards R, Safener responsiveness and multiple herbicide resistance in the weed black-grass (*Alopecurus myosuroides*). *Plant Biotechnol J* **7**:807–820 (2009).
32 Zhao N, Yan Y, Liu W and Wang J, Cytochrome P450 CYP709C56 metabolizing mesosulfuron-methyl confers herbicide resistance in *Alopecurus aequalis*. *Cell Mol Life Sci* **79**:1–14 (2022).