Glutathione transferase (*Am*GSTF1) as a biomarker of multiple herbicide resistance in *Alopecurus myosuroides* (black-grass)

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Abstract

Modern agriculture manages competitive weed species through the intensive use of herbicides to ensure crop productivity. Resistance to aryloxyphenoxypropionate and sulphonylurea herbicides in the problem weed black-grass (*Alopecurus myosuroides*) is now a major problem in the United Kingdom, affecting approximately 80 % of cropland. Increasing numbers of black-grass populations now exhibit multiple herbicide resistance (MHR), based on an enhanced expression of the biosystem responsible for herbicide detoxification termed the "Xenome". The xenome is composed of cytochrome P450s (CYPs), glutathione transferases (GSTs) and glycosyltransferases (UGTs) and transporter proteins from the ABC and MATE families.

In the current work, specific xenome components as biomarkers of MHR were identified for use as diagnostic markers of metabolic resistance. The identification of potential biomarkers was carried out using a combination of targeted and non-targeted "omic" approaches. *De novo* next generation sequencing (NGS) was carried out on MHR and WTS (wild-type susceptible) black-grass, enabling a virtual transcriptome of xenome and associated genes to be assembled. Eight xenome unigenes were identified as being differentially expressed namely a CYP, GSTU6, GSTF1, an oxophytodienoic acid reductase (OPR1), UGTZ, a thiol methyltransferase (TMT) and ABC and MATE transporters. To examine the potential of these expressed RNAs as biomarkers of resistance, real-time qPCR was used on characterised populations of resistant black-grass from around the UK. The results indicated several sequences as potentially functional transcriptional biomarkers of MHR.

Of the MHR-associated genes identified, GSTF1 was particularly interesting as the respective protein was known previously for constitutive up-regulation in populations showing metabolic resistance. To characterise the role of this protein in greater detail, a polyclonal antiserum was raised to the recombinant GSTF1 from MHR "Peldon". The anti-*Am*GSTF1-serum reacted with three polypeptides of 25kDa, 24kDa and 22kDa in crude extracts of MHR black-grass. In Peldon, the two upper polypeptides were up-regulated relative to WTS plants. When tested blind against a panel of ten populations the antiserum proved diagnostic for these polypeptides in MHR populations using either immunoblotting, or ELISA assay. In contrast, WTS or plants showing target site resistance (TSR) mechanisms did not show elevated GSTF1 expression. The antiserum also potentially identified orthologous polypeptides in MHR *Lolium rigidum* and *Avena fatua* indicating GSTF1 is diagnostic of metabolic resistance

in other grasses. Intriguingly, the relative abundance of the GSTF1 polypeptides of differing molecular mass varied between populations, suggesting the presence of multiple component isoenzymes.

Based on the results of the PhD, a prototype lateral flow device using immunodiagnostic detection of GSTF1 has been developed with industrial partners. It is undergoing field evaluation for the determination of MHR in black-grass populations. The project has also identified the potential use of other xenome genes as DNA biomarkers of MHR for diagnostic applications in managing herbicide resistance in black-grass.

In dedication to the most inspirational Grandad a person could wish for

Forever remembered

Unreservedly cherished

This is for you

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Declaration and Copyright

I declare that this thesis is my own work and that I have correctly acknowledged the work of others. This submission is in accordance with University and School guidance on good academic conduct. I certify that no part of the material offered has been previously submitted by me for a degree or other qualification in this or any other University. Any reference to this work should be acknowledged and permission should be sought from the author prior to the publication of any quotation from this work. I confirm that the word length is within the prescribed range as advised by my school and faculty.

Publications arising from this work

Federico Sabbadin, Rachel Glover, Rebecca Stafford, Zuriñe Rozado-Aguirre, Ian Adams, Neil Boonham, Rick Mumford and Robert Edwards*. (2016) Transcriptome sequencing identifies novel persistent viruses in herbicide resistant wild grasses. *Submitted for review*.

Melissa Brazier-Hicks[1], Rebecca Stafford[1], Catherine Tetard-Jones[1], Federico Sabbadin and Robert Edwards[*]. Functional Biomarkers of Multiple Herbicide Resistance in black-grass (*Alopecurus myosuroides*). *In preparation*

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Chapter 1. Introduction

1.1 Herbicide Resistance - Overview

Modern farming has been revolutionised over the last 65 years due to the reliance on herbicides as the primary method of weed control in agronomic crops, thereby replacing cultural controls including soil pH, salinity or fertility levels, manual labour, animals or mechanical weed control (Heap, 2014). The dramatic increase on the usage of and reliance on chemical control has created a selective pressure, driving the adaptation of herbicide resistant weed populations. Resistance to herbicides was first documented over 40 years ago, with the initial report of resistance to triazines in *Senecio vulgaris* in 1968 (Ryan, 1970). Herbicide resistant weeds are now viewed as the greatest biotic threat to global agriculture, responsible for over 34% of crop yield losses worldwide (Oerke, 2006). Annual economic losses were estimated in the USA at US$20 billion in 2004 and £0.5 billion in the UK (Basu et al., 2004). One of the greatest impacts of herbicide resistance on food production is evidenced in cereal crops, which are responsible for sustaining more than 6 billion people worldwide (Powles and Yu, 2010). In the UK, and many other western European countries, black-grass (*Alopecurus myosuroides*) is the most important grass weed amongst cereal crops. Herbicide resistance in black-grass has been confirmed in 35 counties in England (Moss, 2011). In 2014, the average UK farm wheat yield was 8.6 T/ha (DEFRA, 2014) - the Home Grown Cereals Association expected 22 % of total UK wheat crop land (1.9m) to suffer a yield loss of 5 % in 2015 (HGCA, 2014). Consequently, there is a need to focus research on understanding the mechanisms driving the adaptation of herbicide resistance in weeds of cereal crops mainly the wild grasses annual ryegrass (*Lolium rigidum*) and black-grass.

Herbicides are chemical substances or cultured biological organisms that kill or suppress plant growth by affecting one or more of the processes that are vital to plant survival such as: cell division, respiration and/or enzyme activity (Powles and Yu, 2010).There are several classifications for herbicides according to their use, activity or mechanism of action (MoA). In relation to their use herbicides can be separated based on the timing of application for instance pre-emergence or post-emergence, the latter can be classified as either selective or nonselective. Selective post-emergence herbicides are available for control of annual and perennial broadleaf weeds, grass weeds and sedges, whilst nonselective post-emergence herbicides will severely injure or kill all weeds as well as desirable plants. Herbicides categorised based on their MoA are classified based on the first enzyme, protein or

biochemical step affected in the plant following application. The main mechanisms of action are: Acetyl-CoA carboxylase (ACCase) inhibitors, Acetolactate synthase (ALS) inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors, Photosystem II and Photosystem I inhibitors all of which will be explored in greater detail in this chapter.

Historically the first modern herbicide, 2, 4-D was discovered and synthesised in 1941 following research conducted in both the UK and US during the Second World War. It demonstrated that if the chemical was applied appropriately it would kill certain broad-leaved weeds in cereals without harming the crops, however 2, 4-D was not commercially released until 1946 triggering a worldwide revolution in agricultural output as it became the first successful selective herbicide. Further discoveries included the triazine family introduced in the 1950s which was also a selective herbicide whilst 1974, saw the introduction of the first nonselective weed control compound Glyphosate.

Globally, it is estimated that weeds cause comparable crop losses to those caused by insect pest and crop diseases combined (Oerke, 2006). Whilst there are 302 different herbicides on the global market, between them there are only 18 different MoAs for disrupting the physiology of weeds, broadly covering plant light processes, cell metabolism and growth/ cell division (Heap, 2014, Heap, 2016) (Figure 1 and Table 1). Since the 1940s, the use of herbicides have allowed cost effective control of weed species contributing to increased crop yields (Heap, 2014). The EU National Agrochemical Market in 2010 was estimated to be £5.3 billion with herbicides contributing £2.1 billion (greater than fungicides or insecticides) (Walker, 2015). Currently there is resistance to a broad range of herbicides with varying MoAs, resulting in 470 independent documented cases globally of herbicide resistance, of which 220 weed species are documented to have resistance to one or more herbicide modes of action (Heap, 2014).

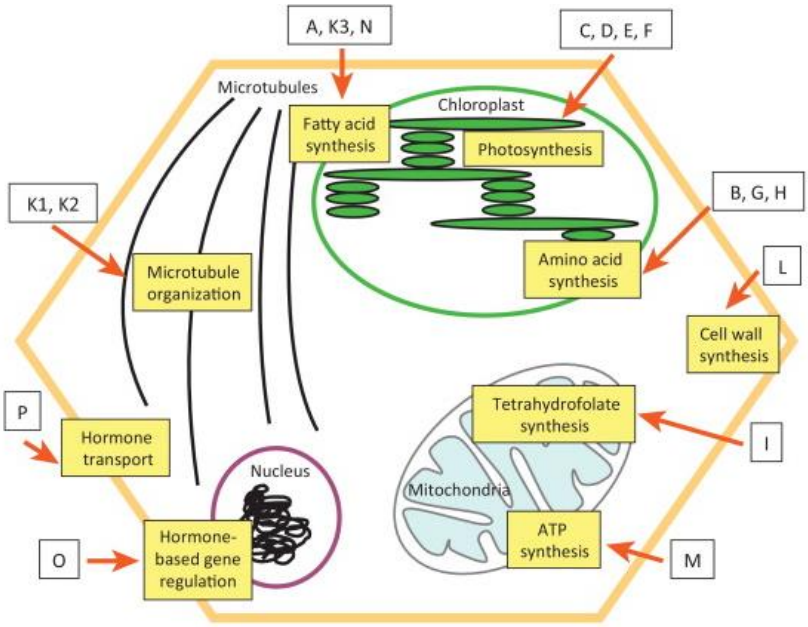


Figure 1 Cellular targets of herbicide action and classification by mode of action.
Herbicide Resistance Action Committee (HRAC) assigned the classification (Delye et al., 2013). Letters denote herbicide classes detailed in Table 1.

Table 1.Herbicide mode of action classification according to the Herbicide Resistance Action Committee (HRAC).
Table reconfigured using information from Weedscience.org. Each letter denotes on of the 18 different MoAs.

HRAC group[a]	Herbicide mode of action	Pathway or process targeted	Example Herbicides
A*	Inhibition of Accetyl coA carboxylase (ACCase)	Fatty acid biosynthesis	Diclofop-methyl
B*	Inhibition of Acetohydroxyacid synthase (AHAS)/ acetolactate synthase (ALS)	Amino acid biosynthesis (Leu, ile, val)	Chlorsulfuron
C	Inhibition of photosystem II protein D1	Photosynthesis (electron transfer)	Atrazine/ chlorotoluron
D	Diversion of electrons transferred by the photosystem 1 ferrodoxin (Fd)	Photosynthesis (electron transfer)	Paraquat
E	Inhibition of protoporphyrinogen oidase (PPO)	Photosynthesis (heme biosynthesis for chlorophyll)	Oxyfluorfen
F	Inhibition of phytoene desaturase (PDS) or 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) or of an unknown protein	Photosynthesis (carotenoid biosynthesis)	Amitrole/ chloro-flurenol
G*	Inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase)	Amino acid biosynthesis (Phe, Trp, Tyr)	Glyphosate
H	Inhibition of glutamine synthase	Amino acid biosynthesis (Gln)	Isoxaflutole
I	Inhibition of dihydropteroate synthases	Tetrahydrofolate biosynthesis	Asulox
K1, K2	Enhancment of tublin depolymerisation	Microtuble polymerisation	Trifluralin
K3	Inhibition of fatty acid synthase (FAS)	Fatty acid biosynthesis	Alachlor
L	Inhibition of cellulose synthase	Cell wall biosynthesis	Dichlobenil
M	Uncoupling of oxidative phosphorylation	ATP biosynthesis	Dinoseb
N	Inhibition of fatty acid elongase	Fatty acid biosynthesis	Butylate
O	Stimulation of transport inhibitor response protein 1 (TIR1)	Regulation of auxin responsive genes	2,4-D
P	Inhibition of auxin transport	Long range hormone signaling	Naptalam
Z	Unknown		

One of the greatest challenges now facing the agri-chemical industry is the development of novel selective herbicides that weeds have not already developed resistance to. There have been no new herbicides brought to the market for over 20 years (Duke, 2012), and with

widespread reliance on successful herbicides there has been limited incentive to develop new herbicides (Mueller, 2005). Although crop protection companies are investigating in new methods for controlling plants (Syngenta, 2015, Bayer Crop Science, 2015), the cost of discovery and development of synthetic pesticides has increased substantially from US $184 million in 2000 to US $256 million in 2008. The increase in costs is partly due to increased regulatory requirements (Walker, 2015). Neve et al. (2009) identified that little had changed in terms of crop yield losses through the attribution of weeds since 1960s. It was suggested therefore, that crop protection companies, crop breeders, farmers and weed biologists are locked in a 'weed management arms race' (Figure 2).

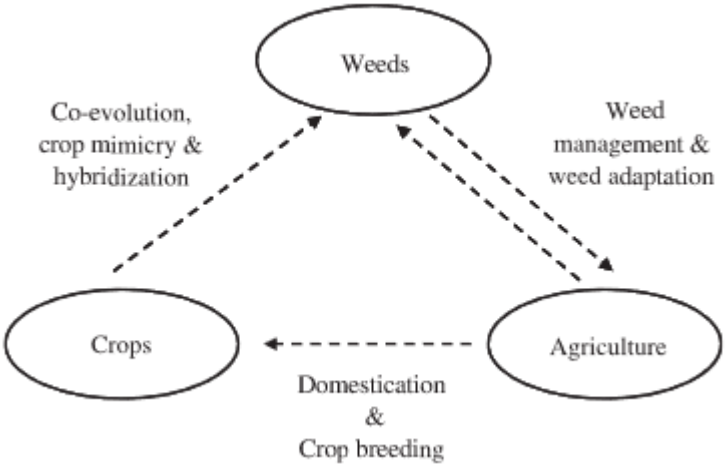


Figure 2. A schematic diagram of the 'Weed management Arms race'.
Showing the co-evolutionary dynamics of interactions between humans, crops and weed populations. (Neve et al., 2009)

Due to the severity of chemical loss of weed control, there is a great interest in understanding the processes and molecular mechanisms that enable plants to tolerate and survive exposure to herbicides. Several factors have been identified to influence the adaptation of herbicide resistance in a weed population: background population genetics, biology of weed species and herbicide management (Neve et al., 2009) (Yuan et al., 2007). Neve hypothesised that the rate at which herbicides are used and reductions in crop rotations can drive the type of resistance that a population presents. High application rates select for individual plants that naturally carry a mutation in the structure of the protein targeted by that herbicide (Target Site Resistance, TSR). Whereas, the (inappropriate) practice of herbicide application at lower than advised field rates causes a sub-lethal effect on plants, and is thought to have driven the adaptation of a range of plant processes that enable a plant to tolerate chemical stress (Non-Target Site Resistance, NTSR). In understanding these processes, and how the use of

herbicides has driven a rise in resistance it may help to identify strategies to overcome and prevent the further spread of resistance. The current understanding in the molecular mechanisms underlying TSR and NTSR common mechanisms are discussed in greater detail in the following sections.

1.2 Target Site Resistance, TSR

Herbicides have chemistries that enable them to effectively enter and be translocated in a plant so that they reach their target site at a lethal dose the majority of which, inhibit specific plant enzymes' active sites resulting in key plant processes responsible for growth, reproduction and survival being disrupted (Powles and Yu, 2010). Currently, the major chemistries used in post-emergence weed control belong to three classes: aryloxypropionates (AOPPs), cyclohexanediones (CHDs) and sulphonylureas which inhibit ACCase and ALS respectively. Target Site Resistance (TSR) arises when there is a mutation that confers an alteration in the amino acid sequence (substitution or deletion) at the target proteins active-site, resulting in the inability of the herbicide to bind and thus inhibit the enzymes normal functional role (Yuan et al., 2007). Notably, substitution mutations for both ALS and ACCase target sites occur at the herbicide binding site, distinct from the enzyme active site. Therefore, mutations can disrupt herbicide binding, whilst maintaining enzymatic activity. However there is a potential to observe associated fitness costs (Tranel and Wright, 2002).

1.2.1 Amino acid substitution at the site of action

The evolution of target-site resistance is linked to alterations at the site of action. The resulting mutations decrease the binding efficiency of the herbicides that target specific enzymes or proteins, and thus target-site resistance is monogenic and involves a point-mutated target enzyme. Resistance due to mutations has been comprehensively reviewed (Powles and Yu, 2010, Gressel, 2009, Devine and Shukla, 2000, Yuan et al., 2007, Delye et al., 2013) therefore this section will focus on the mechanisms which lead to increased diversity in the mechanisms driving weed resistance.

Since the 1950s, the use of chemical control in the agronomic sector has become the norm. Reports in 1970 indicated the first signs of evolved herbicide resistance in the population *Senecio vulgaris* (Groundsel) to simazine (triazine class) (Ryan, 1970), which targets Photosystem II. There was a strong indication that due to the wide adoption of triazine classes of herbicides in maize growing regions in the world and the persistence of the chemical, genetically diverse weed populations had evolved resistance. Subsequently, triazine resistance has globally evolved in 73 weed species. Artzen *et al.* (1982) and Gronwald (1989) identified

that triazine resistance has arisen through a single mutation, which has arisen independently worldwide (www.weedscience.com). The point mutation resides in the maternally inherited chloroplastic *psbA* gene which encodes the D1 protein causing a Ser-264-Gly amino acid substitution in the plastoquinone (PQ) binding site (Goloubinoff P, 1984, Hirschberg J, 1983)

Subsequently, reports of resistance to 1,3,5-triazines were reported commonly in weeds of maize, caused by the high dependency on this selective herbicide (Cummins and Edwards, 2010). It was noted that the presence of a mutation in the D1 protein of Photosystem II complex resulted in decreased binding capacity of the herbicide (Menne, 2007) thereby causing a loss of photosynthetic efficiency, ultimately conferring an associated fitness cost when herbicide selection is removed (Vila-Aiub et al., 2009). Acetyl-CoA carboxylase (ACCase) and acetolactate synthase (ALS) are additional examples of target-site resistance.

ACCase catalyses the first step in the biosynthesis of essential fatty acids (Devine and Shukla, 2000, Delye et al., 2005). Herbicides such as aryloxypropionates (AOPPs) and cyclohexanediones (CHDs), commonly known as 'Fops' and 'Dims' respectively, act by inhibiting the plastidic form of ACCase in monocotyledonous plants, therefore these compounds are widely used in selective control of grass weeds. Target-site resistance to Fops and Dims is now widespread, due to natural selection of individuals carrying mutant forms of ACCase, which confer reduced affinity of herbicide binding compared to wild type ACCase (Hall et al., 1997). In addition to producing this mutant form of ACCase, herbicide resistant plants also have increased ACCase gene expression (Parker et al., 1990a, Parker et al., 1990b). Acetolactate synthase (ALS) is an enzyme that catalyses the first step in the synthesis of the three branched-chain amino acids: leucine, isoleucine and valine. This enzyme is inhibited by several classes of herbicides including: sulfonylureas, sulfonanilides and imidazolinones. Due to the widespread usage of these herbicides, resistance to ALS herbicides has been reported world-wide in several weed species, all demonstrating mutations in the active site that reduce the binding efficiency of the herbicide to ALS. The mutations for each case have been well documented with the level of resistance that they confer against the herbicide classes (Tranel and Wright, 2002).

TSR conferred resistance against both ACCase and ALS has been reported in several independent populations of weed species, notably annual ryegrass (*Lolium rigidium,* (Heap, 1986, Heap, 1990) and black-grass (*Alopecurus myosuroides*, (Moss, 1990) due to both enzymes possessing significant plasticity with relation to sites of mutation; i.e. there are multiple positions in the primary amino acid sequence which enable them to evolve mutations

that generate specific herbicide resistance profiles within weed populations (Delye et al., 2005, Zhang and Powles, 2006a, Zhang and Powles, 2006b, Delye et al., 2007).

The global database of mutations indicates that ALS resistance has arisen from a large pool of mutations, 21 have been detected to date across weed species (Tranel and Wright, 2002). In order to gain a clearer understanding of the molecular mechanism conferring ALS mutations, ALS derived from *Arabidopsis thaliana* (*At*ALS) was co-crystallised with various ALS-targeting herbicides (sulfonylureas and imidazolinones) and demonstrated that the ALS-binding herbicides occupied a site found on the surface of the catalytic subunit of ALS. The study also indicated that both sulfonylureas and imidazolinones have partially overlapping binding sites and are capable of inhibiting the enzyme by binding within and obscuring the channel leading to the active site (McCourt et al., 2006),providing a rational explanation for the different modes of binding between herbicide classes and ALS. This leads to the different and specific resistant profiles which are recorded for ALS mutations.

Target-site resistance towards ACCase has arisen at a slower rate than ALS primarily because ACCase herbicides are selectively lethal to grass weed species whilst the initial introduction of ALS inhibiting herbicides had high persistence in the soil leading to increasing selective pressures of generating a resistant seed-bank (Gressel, 2002). ACCase-inhibiting herbicides proved to be an important tool in the farmers' arsenal as they had the capacity to control grass weeds by targeting the plastidic homomeric ACCase isoform found only in grass species (Konishi and Sasaki, 1994). Resistance to ACCase herbicides is highly complex as there are a multitude of variables to consider: the specific resistant allele(s), the homo/heterozygous status of plants for the specific resistant allele(s), and the combinations of different resistant alleles. As observed with ALS herbicide resistance, specific substitution mutations of the ACCase enzyme result in specific resistance profiles to the different ACCase herbicide classes (Powles and Yu, 2010). An example of a point mutation observed in annual rye-grass, Cys2088Arg or Asp2078Gly conferred resistance to all ACCase herbicide classes tested (Yu et al., 2007, Kaundun et al., 2012). In contrast, black grass has only acquired ACCase resistance to herbicides belonging to AOPP class and no others based on the point mutation Gly2096Ala (Petit et al., 2010). ACCase-herbicide binding interactions in plants was investigated through the expression of carboxyltransferase (CT) domain in yeast. This was bound to three commercial classes of ACCase herbicides which provided an insight into the ACCase-herbicide binding site interactions in plants (Zhang et al., 2004, Yu et al., 2010). The active site of the CT domain indicated that AOPPs were bound close to the active site, thereby

indicating that the herbicides are able to compete with the acetyl-CoA substrate for access to the active site.

1.2.2 Amino acid deletion at the site of action

An extremely rare type of mutation is an amino acid deletion in the codon, to date this has only been observed in the case of *Amaranthus tuberculatus* (waterhemp) resistance to protoporphyrinogen oxidase (PPO)-inhibiting herbicides (Patzoldt et al., 2006) and has not been identified in resistance arising through antibiotics or pesticides (Gressel, 2009). PPO inhibiting herbicides are widely used to control weeds in a variety of crops, specifically by disrupting the biosynthesis of chlorophyll and heme (Beale and Weinstein, 1990). This disruption leads to an accumulation of its substrate protogen IX, which is exported from the organelles into the cytoplasm where herbicide-insensitive peroxidase-like enzymes in the plasma membrane convert it to proto IX (Lee and Duke, 1994). The accumulation of proto IX in the cytoplasm in the presence of light induces the formation of singlet oxygen which is damaging to cell membranes (Patzoldt et al., 2006, Duke et al., 1991). PPO inhibitors have two herbicide target sites in plants. However, resistant *A. tuberculatus* is thought to have overcome this problem through the presence of *PPX2L*, which contains a codon deletion (loss of Gly210) and is predicted to encode both plastid and mitochondria-targeted PPO isoforms. Studies investigated *Escherichia coli* PPO mutant strains transformed with *PPX2L* and ΔG210 *PPX2L*. The results confirmed that the Gly210 deletion conferred resistance to a multitude of chemical families of PPO inhibitors (Patzoldt et al., 2006). Further studies have indicated that PPO-inhibitor-resistant populations are likely to have evolved independently given the distinct locations which have resistant biotypes present (Shoup et al., 2003, Li et al., 2004, Patzoldt et al., 2005, Beale and Weinstein, 1990). This is likely the result of the species obligatory nature to outcross and that resistance can be transmitted both maternally and paternally.

1.3 Non-Target Site Resistance

Non-target site resistance (NTSR) is a complex, polygenic quantitative trait which generally refers to all mechanisms of resistance that are not conferred by changes in the amino acid sequence of the target protein. Due to its complexity, the mechanisms underlying NTSR are less understood compared to TSR. NTSR can include both exclusion based resistance and metabolic based resistance. Herbicide metabolism is associated with the co-ordinated up-regulation of xenobiotic detoxifying enzymes and transporters (Yuan et al., 2007, Cummins and Edwards, 2010). These enzymes are responsible for the enhanced metabolism of detoxification and/or the sequestration of herbicides thereby limiting the bioavailability in the

plant, to ensure that the herbicide cannot bind to the site of action at a sufficient dosage to disrupt the plants normal functional processes. Collectively, this metabolic pathway system in plants has been termed the 'xenome' (Edwards et al., 2005).

1.3.1 The role of the plant xenome in NTSR

The ability of plants to detoxify, transport and metabolise xenobiotics (i.e. the xenome) encompasses a four phase schema, Figure 3 (Yuan et al., 2007).

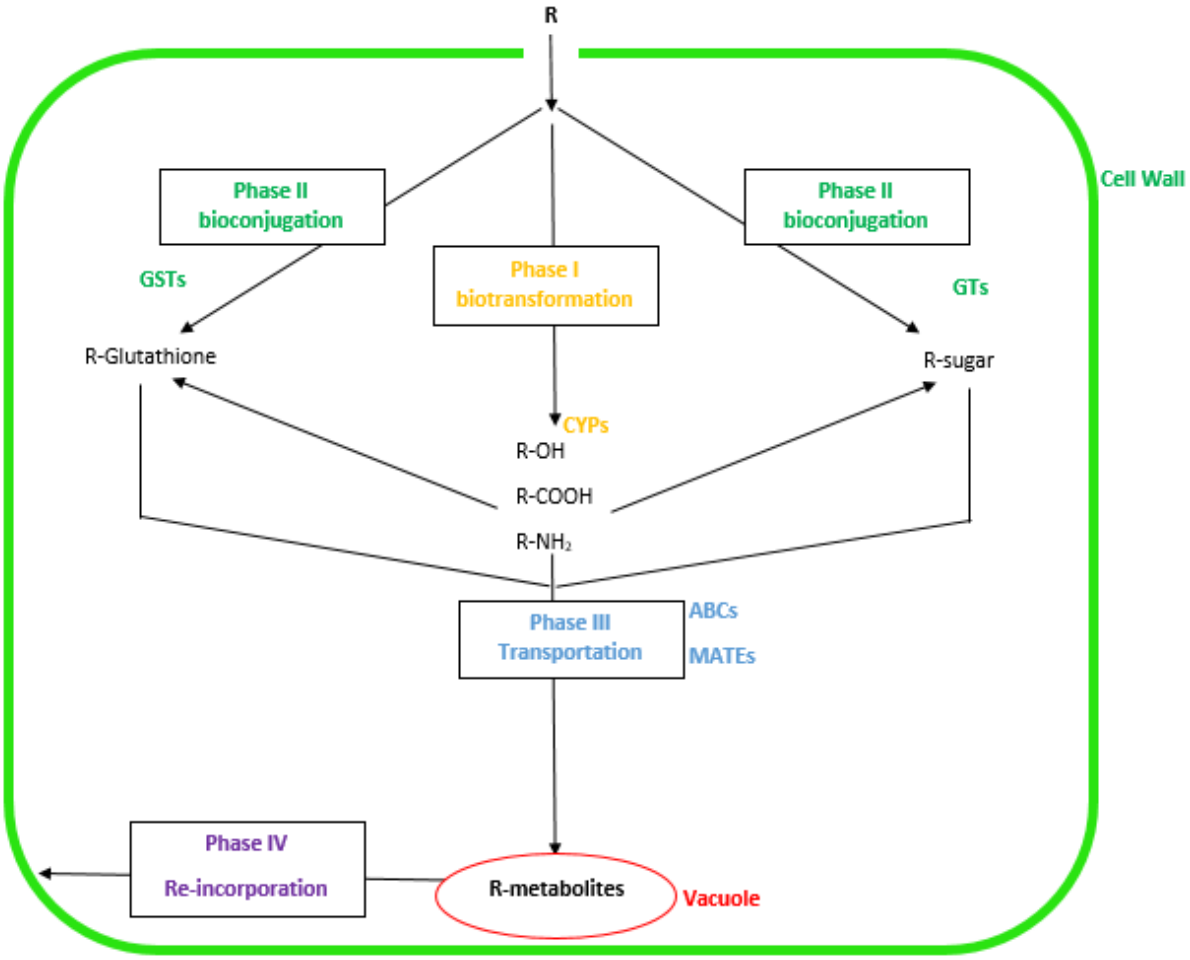


Figure 3 Generalised schema of the plant 'Xenome' indicating the four phases of xenobiotic detoxification.
R; xenobiotic, CYPs; cytochrome P450, GSTs; glutathione transferases, GT; glycosyltransferases, ABC; adenosine triphosphate binding cassette transporters, MATE; multidrug and Toxic compound Extrusion protein.

Phase I of the xenome involves the biotransformation of xenobiotics via dealkylation or oxidative reactions catalysed by cytochrome P450 mixed function oxidase (CYPs) (Van Eerd, 2003). As a result a reactive functional group is revealed or introduced into the xenobiotic structure facilitating further processing in phase II. Phase I detoxification pathway is a common route of metabolism for xenobiotic compounds but as Figure 3 depicts this isn't

always necessary if the molecule already contains a suitably reactive centre. Phase II xenobiotic detoxification involves the conjugation of the xenobiotic with the tripeptide glutathione (GSH) or with sugar moieties catalysed by glutathione transferases (GSTs) and glycosyltransferases (GTs) respectively. The resulting conjugated molecules have increased solubility and eliminates the reactive centres (rendering the conjugate non-phytotoxic in most cases), thereby facilitating the entry of the xenobiotic-conjugate into Phase III metabolism (Bowles et al., 2005, Cummins et al., 2011, Cummins et al., 2013). Phase III is denoted as the active transportation of the xenobiotic-conjugate from the cytosol to the vacuole through two mechanisms: transport of glutathionylated xenobiotics across the membrane relies on ATP-binding cassette transporters, subfamily C (ABCs) whilst the transport of glucosylated xenobiotics across the vacuolar membrane has been shown to utilise ATP-dependent transporters (Rea, 2007). Finally, Phase IV metabolism involves the compartmentalisation of the xenobiotic-derived metabolites into the plant cell wall. Xenobiotic residues have also been noted to be recycled by re-entering the detoxification pathway (Brazier-Hicks et al., 2008a). Studies have also indicated that a single compound can simultaneously be detoxified through different phases; the herbicide Atrazine simultaneously undergoes N-dealkylation (Phase I) and S-glutathionylation (Phase II) within single cells (Edwards and Owen, 1986).

The role of the plant xenome shares similarities to the biotransformation steps in the animal xenome, although there are a couple of fundamental differences. Firstly, the mammalian xenome has evolved the capacity to detoxify foreign compounds, whilst plants contain a greater number of potential xenobiotic detoxifying enzymes as a result of their capacity to synthesise secondary metabolites. This stark contrast is reflected in the xenome gene familes, for instance: in *Arabidopsis thaliana* vs mammals there are (P450s:273 vs 57; Family 1 glycosyltransferases:107 vs 27; soluble GSTs: 54 vs 17 and ABC transporters: 120 vs 50) (Edwards et al., 2011). It is therefore believed that some xenobiotics are classically detoxified by xenome enzymes upon entering the plant cell, there is an assumption that others are identified as secondary metabolites and are subsequently biotransformed by enzymes involved in endogenous biosynthesis (Neuefeind et al., 1996). This indicates that there is a potential for plant xenome enzymes to self-regulate their own natural products as well as those arising as a result of the external environment. Secondly, a key difference between xenobiotic detoxification in plants and animals is associated with how the inert products are subsequently handled. Animal detoxified compounds such as pesticides, drugs and pollutants are normally exported from cells for final extrusion in bile or urine, whereas in plants the inert compounds are stored in the vacuole.

By far the best studied enzymes involved in the xenome are the CYP and GST families, although the large gene family sizes have made it difficult for researchers to understand the exact roles of individual genes in xenobiotic detoxification. With respect to xenobiotics the study of CYPs primarily focused on their role in herbicide metabolism. The involvement of the plant xenome was initially noted in the 1960's where Shimabukuro *et al*. (1966) reported the metabolism of atrazine in pea plants (*Pisum sativum*). Metabolism occurred via oxidative dealkylation of the 4-ethylamino group to the respective free amine (Shimabukuro, 1966). Despite no enzyme being identified for the catalysed reaction, the involvement of CYPs was inferred. Subsequently, CYPs have been investigated to a greater extent in maize (*Zea mays*), where cultivars indicated tolerance to atrazine through the formation of GST-catalysed glutathione conjugation, and could therefore detoxify the herbicide more rapidly than susceptible cultivars (Frear and Swanson, 1970). Further investigations demonstrated that the same associated up-regulation of xenome components was observed in herbicide resistant weed species including black-grass (Christopher et al., 1991, Anderson and Gronwald, 1991).

The wild grass *Alopecurus myosuroides* (black-grass) was initially identified in 1984 in Peldon, Essex as possessing the capacity to resist toxicity to herbicides from four chemical classes across three sites of action, thereby discrediting the possibility of TSR mechanisms (Moss, 1990, Hall et al., 1997). The population was therefore termed multiple herbicide resistant (MHR), as it indicated an enhanced capacity to metabolise multiple herbicides through the associated up-regulation of CYPs and GSTs which had previously been termed enhanced metabolic resistance (Hall et al., 1997, Hyde et al., 1996). Subsequently, this mechanism of evolved herbicide resistance to multiple herbicides through enhanced metabolism was also recorded in annual rye-grass (Christopher et al., 1991, Preston and Malone, 2015). These increasing incidents of MHR are becoming of greater concern as weeds are more frequently showing herbicide resistance, irrespective of their MoA due to the stimulated rate at which the compounds are effectively metabolised. It has been suggested that the resistant weed biotypes mimic the pathways and mechanisms utilised in crop species in order to tolerate lethal doses of graminicides (Hyde et al., 1996, Christopher et al., 1991). As a result of increased study there is now a greater awareness of the role and impact that the xenome gene families (CYPs, GSTs GTs and ABCs) have on Multiple Herbicide Resistance (MHR), which are discussed in the following sections.

1.3.2 Cytochrome P450 and herbicide resistance

Cytochrome P450s (CYPs) are membrane associated, haem-containing proteins which utilise reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen to

catalyse the insertion of an oxygen atom into a substrate molecule. CYPs are responsible for carrying out multiple types of bio-transformations mediated through hydroxylations, oxidations, demethylation, dealkylations and desaturations of the substrate (Werck-Reichhart et al., 2000, Guengerich, 2001). The number of CYP family members in plants is much larger than animals with 273 CYP genes reported to be encoded by the genome Arabidopsis and more than 450 in rice.

Plants depend on CYPs for a large proportion of their functional processes. In several sequenced angiosperms, CYP genes constitute up to 1% of the protein coding genes (Nelson et al., 2008). In crop tissues, CYP-mediated degradation of herbicides has been demonstrated for at least 25 different compounds across 8 chemical classes (Siminszky, 2006). This capacity has been noted in wheat and maize, and is seen as an inherent capacity for CYP-mediated herbicide metabolism in weed populations (e.g. *Lolium rigidium, Alopecurus myosuroides, Echinochloa phyllopogon*) that have evolved metabolic resistance to ALS-, ACCase- and PSII inhibiting herbicides (Powles and Yu, 2010). P450 inhibitors suppress *in vivo* herbicide metabolism and can subsequently reverse resistance, indicating that P450s are involved in herbicide metabolic resistance (Hall et al., 1995, Hall et al., 1997, Preston et al., 1996). The roles of CYPs in herbicide metabolism have been the focus in recent studies for remediating chemically contaminated land by harnessing the CYP enzyme (Morant et al., 2003). CYPs are important enzymes catalysing oxidation and reduction reactions with xenobiotics. Furthermore, there are now known to be multiple routes of CYP-mediated degradation of herbicides which can exist within a single plant. For example, chlorotoluron, a member of the phenylurea class of herbicides and an inhibitor of photosystem II, can be metabolised via oxidation of a methyl substituent on the phenyl ring or, via successive N-dealkylation reactions (Figure 4). In both cases the ring-methyl oxidation product (2) and di-N-dealkylation product (4) are non-phytotoxic, however the mono-N-dealkylation product (3) is not (Ryan et al., 1981).

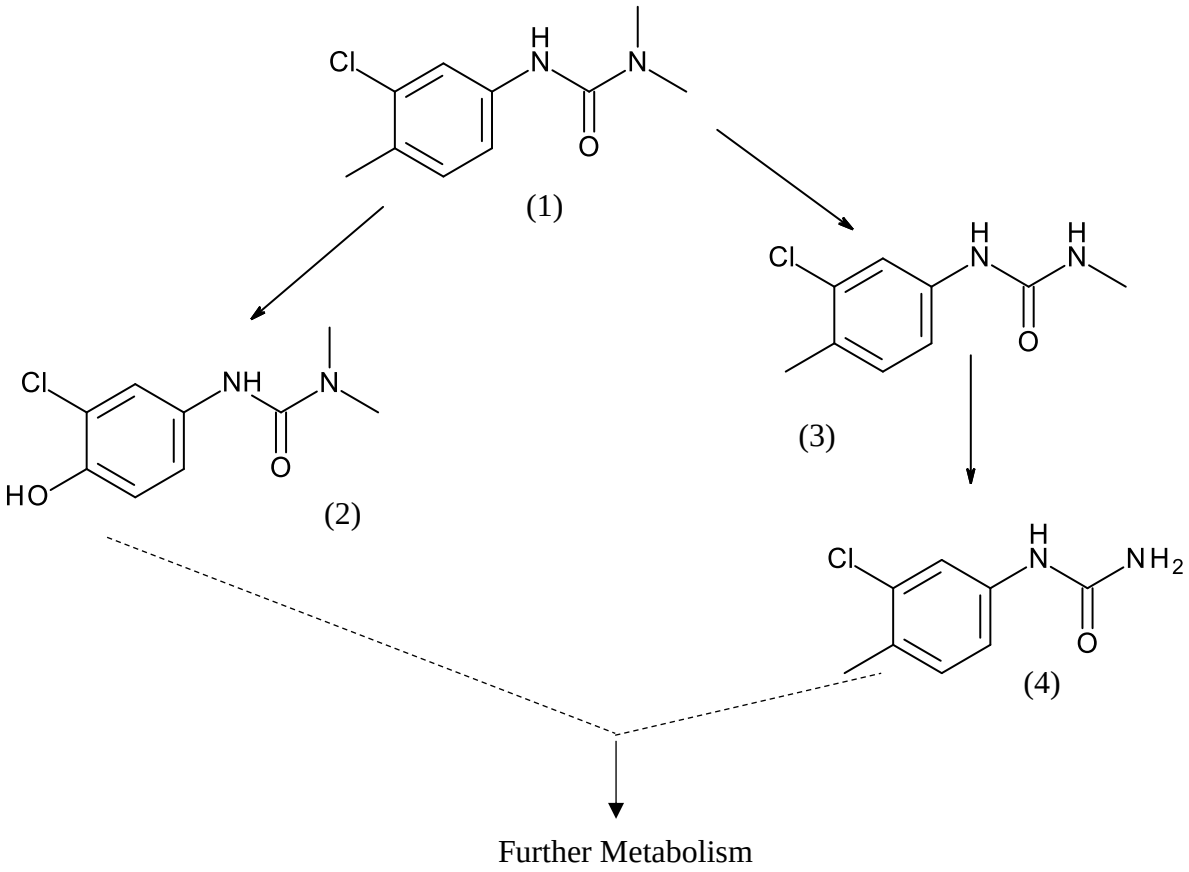


Figure 4 Alternative routes of chlorotoluron metabolism in plants.
Chlorotoluron (1) can be metabolised to the non-phytotoxic ring-methyl oxidation product (2) or it can undergo the removal of the N-methyl groups to further yield the phytotoxic mono- (3) and non-phytotoxic di-N-dealkylation (4) products (Ryan et al., 1981)

Initial studies using wheat (*Triticum aestivum*) and herbicide-susceptible black-grass (*A. myosuroides*) exposed to chlorotoluron demonstrated tolerance in the crop, which correlated well with the route and speed of chlorotoluron metabolism. During the study, chlorotoluron metabolites (2) – (4) were accumulated in both species, indicating that two routes of herbicide metabolism were functioning in both species. Interestingly, herbicide-susceptible black-grass retained higher levels of chlorotoluron in the leaves and accumulated the phytotoxic mono-N-dealkylation product (Ryan et al., 1981). During the 1990's, further exploration into the metabolism of chlorotoluron in MHR black-grass biotype was undertaken due to its implementation of NTSR mechanisms (Moss, 1990). The study revealed that the MHR biotype had modified xenome components which behaved in a similar manner to that of wheat. The MHR biotype preferentially accumulated the non-phytotoxic ring-methyl oxidation product (Hyde et al., 1996). A similar result was observed in MHR annual rye-grass which was resistant to chlorosulfuron, a member of the sulfonylurea class of herbicides which inhibit ALS (Sweetser et al., 1982).

Chromatography results indicate that there was a significantly greater abundance of the detoxified metabolite (glucosylated derivative) in the MHR biotype than chlorosulfuron-treated weed extracts (Christopher et al., 1991). These studies of CYP-mediated herbicide metabolism in MHR weed species provide evidence that components of the resistant weed xenome are up-regulated (by an unknown mechanisms) in MHR biotypes, rather than being due to the expression of novel xenome detoxification genes. To date CYP genes/ proteins responsible for herbicide detoxification have not been isolated from resistant weed species. There are multiple factors which are likely to contribute to a lack of identification of weed CYPs such as the many homologous CYP genes per weed genome and the difficulty in purifying membrane-associated proteins, however there are strategies of quantifying the rates and products of herbicide metabolism. The use of chemical CYP inhibitors such as: Piperonyl butoxide, Malathion, Tetcyclasis and I-aminobenzotriazole have aided the identification of 10 weed species with populations demonstrating herbicide resistance due in part to CYP-mediated herbicide degradation (Cocker et al., 2001, Preston et al., 1996, Yun et al., 2005, Yuan et al., 2007, Hyde et al., 1996), but CYPs involved in non-xenobiotic metabolism have also been identified.

A barrier to understanding how the plant xenome works is the lack of genomic information for weed species. However, this has been circumvented by the development of Next Generation Sequencing (NGS) technologies. NGS technologies have been successfully used to sequence many complex crop genomes including barley and wheat (Mayer KF, 2012, Brenchley et al., 2012) and subsequently the first efforts have been made to explore weed species using this technique, although the strategy remains in its infancy (Peng et al., 2010, Riggins et al., 2010). Gaines et al. (2014) utilised RNA-Seq transcriptome analysis to find candidate genes that confer metabolic resistance to the herbicide diclofop in a diclofop-resistant population (R) of the grass-weed *Lolium rigidium* (annual ryegrass). This followed an initial global gene expression analysis using Illumina 454 NGS on an untreated control, adjuvant-only control, and diclofop treatment of R and Susceptible (S) populations. A reference cDNA transcriptome was assembled and putative annotations were assigned. Contigs that demonstrated constitutive expression differences between untreated R and untreated S were selected for further analysis. This resulted in the identification of four contigs of significant interest: two CYPs (class CYP72A), nitronate monooxygenase and glucosyltransferase. These four contigs were consistently highly expressed in nine *L. rigidium* field populations adapted towards diclofop, suggesting that they have a major role in resistance. The results of this NGS analysis indicated the potential use of these data as a

means of understanding and potentially exploiting the pathways contributing to herbicide resistance through metabolism. CYP72A genes are prevalent across angiosperms including black-grass although the number of genes within each genome varies greatly. Sequence comparisons have indicated that CYP72As are involved in species-specific metabolic functions in some plants while there is also likely to be functional conservation between closely related species (Prall et al., 2016). However, the evolutionary relationships between CYPs in plant genomes are an essential component in understanding the evolutionary biochemical diversity of plants. CYPs from non-weedy plant species have been isolated, cloned and expressed in transgenic host plants and have been shown to confer herbicide-resistant phenotype. In addition, other CYP families have been implicated in herbicide resistance. CYP71A10 was isolated from soybean, and demonstrated *in vitro* to be involved in the metabolism of both chlorotoluron and a related phenylurea herbicide, via N-demethylation and formation of the ring methyl oxidation product from chlorotoluron. CYP71A10 was subsequently expressed in tobacco (*Nicotiana tabacum*), endowing resistance to both chlorotoluron and linuron (Siminszky et al., 1999). CYP76B1, isolated from Jerusalem artichoke (*Helianthus tuberosus*) was expressed in both Arabidopsis and tobacco resulting in a 10-fold increase in resistance to the herbicide chlorotoluron (Didierjean et al., 2002). The CYP76B1 was found to catalyse the N-dealkylation of chlorotoluron to the nonphytotoxic di-N-diealkylation product (Figure 4). Interestingly, these studies have confirmed the role of plant CYPs in conferring herbicide resistance via enhanced herbicide metabolism. A startling example of the role of CYP-mediated herbicide resistance was brought to the forefront when examining the parallels between human and plant CYPs. The human gene for CYP2B6 was introduced into transgenic rice and subsequently endowed the host plant tolerance to 13 out of 17 herbicides from 8 chemical classes differing in their modes of action and chemical structures (Hirose et al., 2005). What made this result more interesting was the broad spectrum of activity that CYP2B6 demonstrated despite having no exposure to these chemicals *in vivo*. This revealed the potential power of CYP-mediated herbicide detoxification, which can be used to develop herbicide tolerant rice.

1.3.3 Glutathione Transferase and herbicide detoxification

Glutathione transferases (GSTs) are enzymes found in mammals, fungi, insects and plants. GSTs were originally discovered for their ability to catalyse the detoxification of xenobiotics, via conjugation with the tri-peptide γ-Glu-Cys-Gly (glutathione; GSH). They are now known to catalyse a variety of GSH-dependent reactions (Marrs, 1996, Hayes et al., 2005). Within the plant kingdom, GSTs are a large family of enzymes divided into 7 classes with members

of each related by similarity in amino acid sequence, immunodetection and enzyme activity (Edwards and Dixon, 2000b). For example, 55 and 79 GST genes were identified in *Arabidopsis* and *Oryza* (rice) respectively, divided into phi, tau, theta, zeta, lambda, dehydroascorbate reductase (DHAR) and TCHQD classes (Dixon and Edwards, 2010, Jain et al., 2010). Different members from the plant GST classes catalyse a wide variety of GSH-dependent reactions, including hydroperoxide reduction, thiol exchange and bond isomerisations. Some of these activities including GSH-conjugation and hydroperoxide reduction are catalysed by distinct GSTs across multiple classes whilst some activities are class-specific, for example dehydroascorbate reduction (DHAR class) (Dixon and Edwards, 2010). As well as catalytic activities some plant GSTs have ligand-binding functions, whereby the plant has the capacity to bind to multiple plant secondary metabolites including anthocyanins, flavonoids and fatty acids for transport into the vacuole (Mueller et al., 2000, Cummins et al., 2003b, Dixon et al., 2011, Dixon et al., 2008, Dixon and Edwards, 2009).

The populous phi (F) and tau class GSTs (U) tend to be highly stress inducible, and occur in several transcriptomic and proteomic studies. Furthermore, they are predominantly active in xenobiotic detoxification in plants (Cummins et al., 2011). In 1970, the first published study of GST activity in plants reported that maize is capable of detoxifying the herbicide atrazine (Frear and Swanson, 1970). The maize enzyme was purified and displayed the capacity to detoxify the herbicide by nucleophilic displacement of the aryl chlorine atom with GSH. Further studies demonstrated a positive correlation between displacement and the level of atrazine tolerance in different maize cultivars (Frear and Swanson, 1970, Shimabukuro, 1966, Shimabukuro et al., 1971). After the initial discovery of GST-mediated herbicide metabolism in 1970, studies in crop species have identified that GSTs from the GSTF and GSTU classes are capable of detoxifying compounds from multiple herbicide classes with different MoAs and chemical structures via conjugation with GSH. Conjugation typically occurs via addition, whereby GSH is added to an unsaturated bond, or substitution, whereby GSH displaces a group on the herbicide substrate (Figure 5) (Edwards et al., 2011). Substitution reactions tend to be the most common reaction, which can produce a halide group, or displace a halogen atom for example in the metabolism of atrazine and alachlor (Frear and Swanson, 1970, Mozer et al., 1983). Alternatively, the herbicide can be separated into two distinct moieties, as is the case with fenoxaprop detoxification in wheat (Tal et al., 1993)(Figure 5). In many studies, plant species with different herbicidal tolerances have been used to demonstrate that increased tolerance correlated with an increase in the conjugation of the herbicide with GSH. An interesting study by Hatton *et al.* (1999) demonstrated that GSTs in *Setaria faberi*

detoxified herbicides with similar efficiencies to corresponding GST isoenzymes in maize. However, their levels of expression were 20-fold lower than those reported in the crop (Hatton et al., 1999). This study also suggested that some GSTs are clearly conserved in grain crops and weeds whilst others were quite different. Mozer *et al.* originally isolated the GSTs responsible for alachlor detoxification in maize and identified two distinct active fractions during enzyme purification, with each enzyme composed of two GST subunits (Mozer et al., 1983). One of the fractions appeared to contain a homodimer which was composed of two 29kDa subunits, whilst the other was a heterodimer with a 29kDa and a 27kDa subunit. This proved that GSTFs and GSTUs are assembled as dimers, which is also known for the majority of mammalian GSTs (Armstrong, 1997). Most interestingly, Mozer's study indicated that GSTs involved in herbicide detoxification could assemble and operate as both homo- and heterodimers. Dixon *et al.* demonstrated that only subunits within the same GST class were capable of forming dimers (Dixon et al., 1999) and subunit compositions play a key role in determining the catalytic activity of the dimer towards different xenobiotic substrates including herbicides (Dixon et al., 1997, Dixon et al., 1998, Sommer and Böger, 1999).

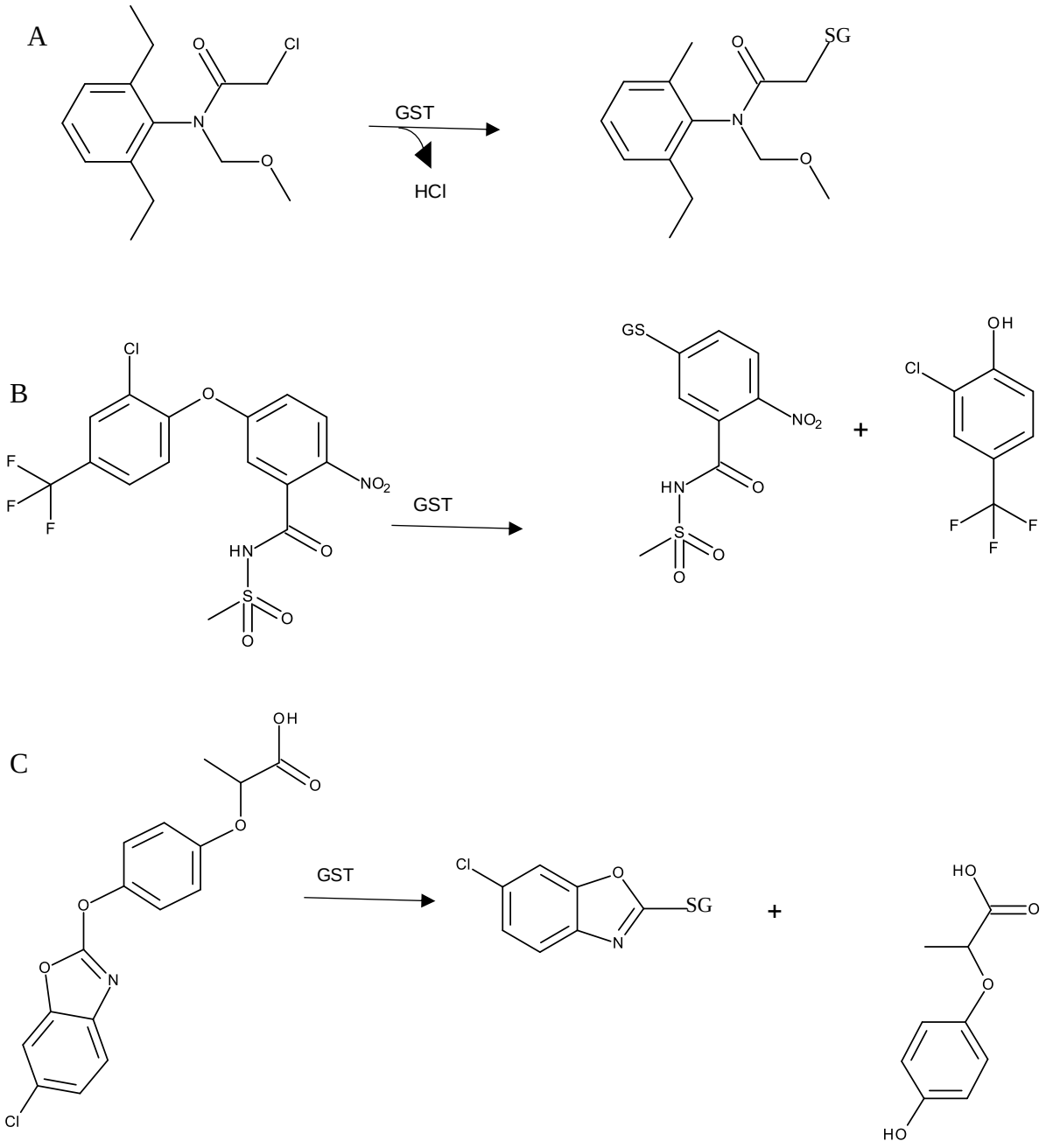


Figure 5 Detoxification of multiple herbicide classes by GST-catalysed glutathione conjugation in crops.
A) Alachlor detoxification in maize, B) fomesafen detoxification in soybean and C) fenoxaprop detoxification in wheat, glutathione conjugation (Mozer et al., 1983, Tal et al., 1993)

Further studies in maize have identified more herbicide-detoxifying GSTs homo- and heterodimers. *Zm*GSTF1-2, a maize heterodimer GST detoxified a hydroperoxide (cumene hydroperoxide) through the use of GSH which was measured using the glutathione peroxidase activity (GPOX). This result proved interesting as it opened up the possibility towards a unique understanding at the time that the primary mode of action of some herbicides was

simply to generate a reactive oxygen species. This would result in extensive damage to cellular membranes. Although maize has been used as the primary example, very similar findings of GST- mediated herbicide metabolism by multiple GST homo- and heterodimers have been demonstrated in other major crops such as wheat and soybean (Cummins et al., 1997, Cummins et al., 2003b, Andrews et al., 2005). Each of the studies have identified distinct catalytic profiles with some possessing GPOX activity. To date, only structures which are GSTF or GSTU homodimers have been reported for herbicide detoxifying activity, 7 out of 10 are from crop species (Neuefeind et al., 1997a, Neuefeind et al., 1997b, Thom et al., 2002, Dixon et al., 2003). GST structural studies demonstrated that despite divergence in amino acid sequence, the tertiary structures of the GSTs are well conserved. All the GSTs contain a GSH binding site with a somewhat variable hydrophobic binding domain for xenobiotics, providing a rationale towards the broad substrate specificities of the different GST subunits.

In crop plants, GST subunits tend not to be constitutively expressed, but instead are inducible by specific chemical and/ or environmental triggers (McGonigle et al., 2000). The best known GST inducers are chemical treatments, particularly with compounds known as herbicide safeners. This is an important agronomic family of compounds that are applied to crops to enhance their ability to metabolise and thus tolerate herbicides. A plethora of studies have identified that safeners induce the gene expression and enzyme activity of all major phases of the crop xenome (I-IV) and therefore speed up the rate of herbicide detoxification, without similarly inducing the weed xenome (Hatzios and Nilda, 2004). However, more recently this concept has been challenged by Cummins *et al.* who demonstrated that safeners can also induce the weed xenome (Cummins et al., 2009). Further analysis of safener chemistry and the MoA have been explored and the plant GST subunits associated with herbicide detoxification are expressed following safener application. However, the exact mechanism that enables safeners to induce GSTs or other xenome enzymes remains unknown.

Studies of the expression of crop GSTFs and GSTUs have been carried out in transgenic host plants, providing further evidence that these enzymes confer herbicide-resistant phenotypes. *Zm*GSTF1 and *Zm*GSTF2 have both been expressed in tobacco and subsequently the host plants conferred resistance to alachlor via the conjugation of GSH with the herbicide. Additionally, *Zm*GSTF2 expression in wheat endows plants with tolerance towards the thiocarbamate herbicide class (Milligan et al., 2001) (Figure 6).

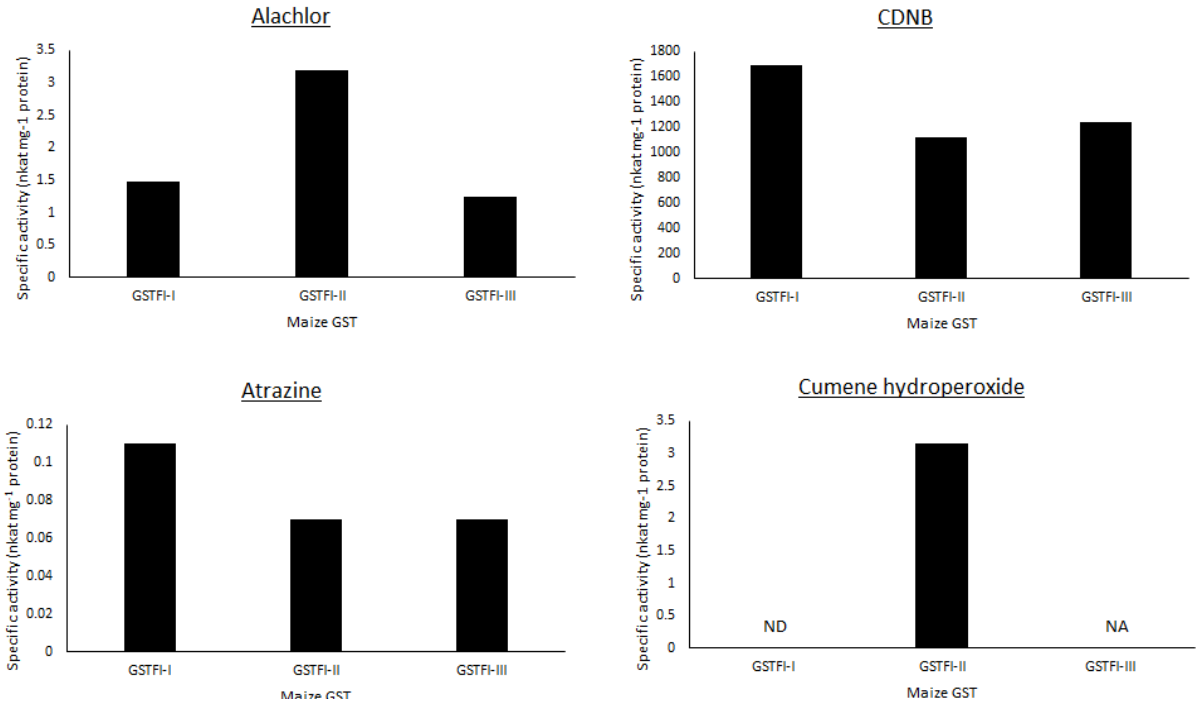


Figure 6 GST subunit composition determines substrate specificities in *Zea mays* (maize). Purified *Zm*GSTFI-I homodimer, *Zm*GSTFI-II heterodimer and *Zm*GSTFI-III heterodimer from Dichlormid-treated maize roots were tested for activity toward a range of substrates: 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide and the herbicides alachlor (chloroactenilide class), atrazine (triazine class). Specific activities are expressed as nkats mg^{-1} protein. ND-not detected. NA-not assayed. Data replotted from (Dixon et al., 1997).

In comparison to the relatively well-studied GSTs associated with herbicide detoxification in crops, GSTs which carry out a similar role in weed species remain relatively obscure. This is largely due to the absence of reference genomes of weed species. The initial observation of herbicide resistance in weeds was reported in an atrazine-resistant biotype of the weed species velvetleaf (*Abutilon theophrasti*) (Gronwald et al., 1989). The resistant biotype demonstrated an increased ability to accumulate the atrazine-GSH conjugate previously described in maize, whilst there was no difference in herbicide uptake, nor in target-site sensitivity when comparing resistant biotypes with sensitive. However, the resistant biotype had increased levels of GST activity toward atrazine (Anderson and Gronwald, 1991). GST-mediated herbicide detoxification via GSH-conjugation has additionally been observed in foxtail *(Setaria spp.)* biotypes resistant to atrazine (Giménez-Espinosa et al., 1996), and in multiple MHR biotypes of black-grass resistant to fenoxaprop-p-ethyl (Hall et al., 1997, Cummins et al., 1997). Furthermore, increased levels of constitutive GST gene expression has been reported in several major grass weeds that are resistant to multiple herbicides, annual ryegrass (*Lolium rigidum*) and black-grass (*Alopecurus myosuroides*) (Gaines et al., 2014, Yu and Powles, 2014, Gardin et al., 2015).

Black-grass is one of the best characterised weed species with respect to the molecular basis of GST-mediated herbicide detoxification. Two biotypes in particular originating from wheat field sites in Essex ("Peldon") and Lincolnshire ("Lincs E1") are resistant to multiple herbicides, which bear constitutively increased levels of CYP and GST expression compared to herbicide sensitive biotypes (Moss, 1990) (Hyde et al., 1996). Interestingly, their ability to detoxify fenoxaprop-p-ethyl appears to be independent of CYP expression, since the use of CYP inhibitors that had no effect on detoxification (Hall et al., 1997). In a parallel study, protein extracts were used to establish that the same two MHR black-grass biotypes expressed GSTs capable of detoxifying fenoxaprop-p-ethyl via its conjugation with GSH, no GST activity was detected in the herbicide susceptible biotypes. Both MHR biotypes were probed with an antibody raised against a herbicide-detoxifying GSTU enzyme from wheat which resulted in two novel GSTs being expressed, this correlated to the activity towards fenoxaprop-p-ethyl. Further analysis of these two biotypes indicated that despite the rates of fenoxaprop-p-ethyl being similar, 'Lincs E' plants were 10-fold more resistant in whole-plant spray trials. This indicated that potentially there were additional mechanisms of herbicide tolerance in at least the 'Lincs E' biotype (Cummins et al., 1997) . Subsequently, a different antibody to the herbicide-detoxifying maize *Zm*GSTF1-2 enzyme, helped to identify further GSTs, this time belonging to the phi class. These GSTs were constitutively expressed in protein extracts from both MHR biotypes but absent in both a herbicide-sensitive biotype and an ACCase target-site resistant biotype (Cummins et al., 1999, Cummins et al., 1997). Cummins *et al.* extracted RNA from MHR biotype 'Peldon', reverse-transcribed this to the complementary cDNA and subsequently a cDNA library was constructed and expressed in bacteria which allowed the colonies to be screened for plant GSTU and GSTF enzyme isoforms through antibody detection. Three clones encoding polypeptides which were recognised by the anti-GSTU-serum and four clones encoding polypeptides that were recognised by the anti-GSTF-serum were identified by Cummins *et al.* (Figure 7).

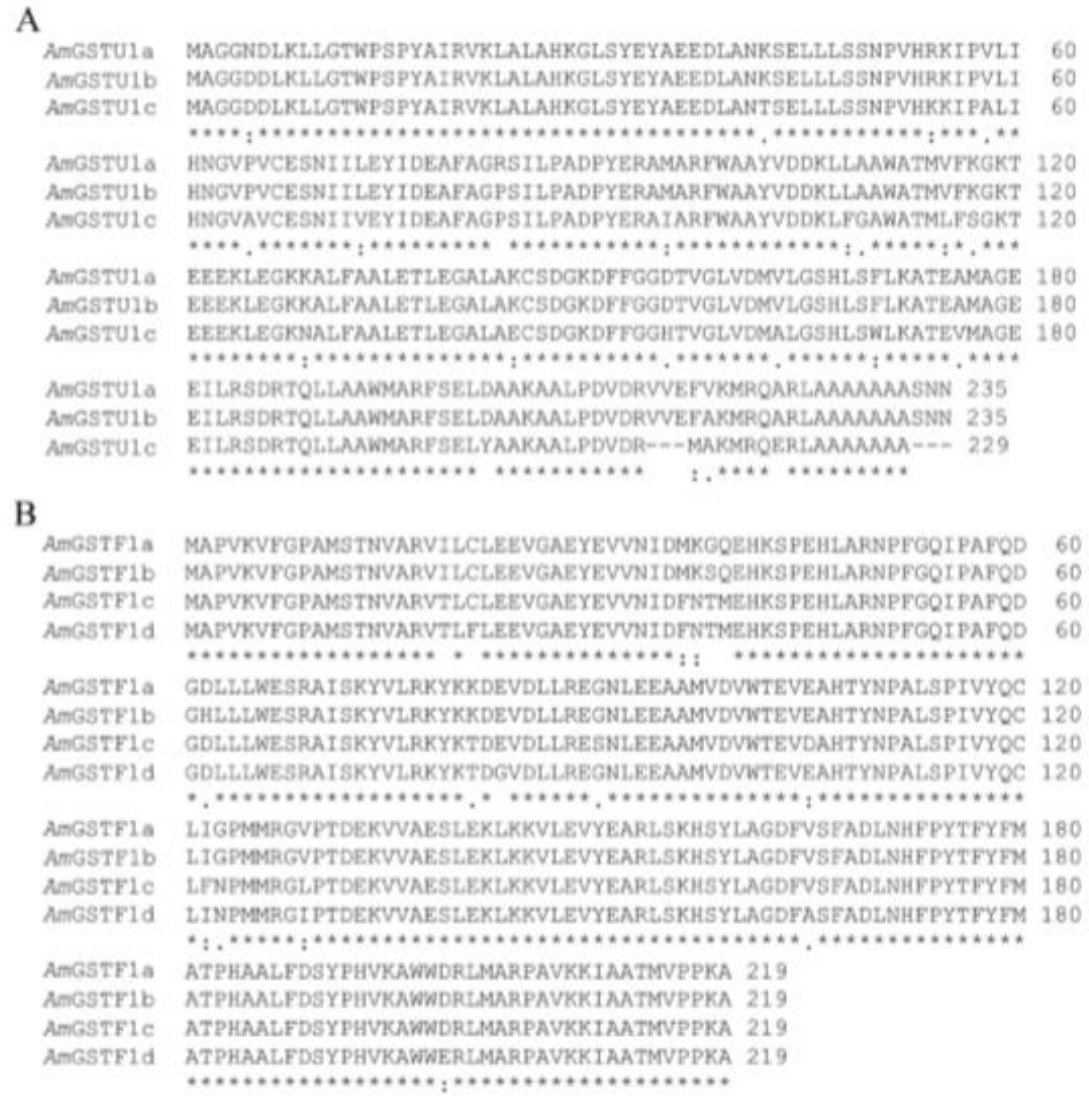


Figure 7. Black-grass GST polypeptide sequences from the recombinant expression of an MHR black-grass cDNA library.
(A) polypeptide sequences of three clones (a-c) recognised by antibodies raised against a herbicide-detoxifying GSTU homodimer from wheat. (B) Polypeptide sequence of four clones (a-d) recognised by antibodies raised to a herbicide-detoxifying GSTF heterodimer from maize reproduced from (Cummins et al., 1999).

Figure 7 indicates that within each class (U/F), the polypeptides were highly similar in amino acid sequence. Recombinant expression and purification of proteins indicated that the proteins co-migrated with the immunodetected bands present in MHR protein extracts. Therefore, it was understandable that the initial interpretation indicated that there was expression of one novel GST isoform, however some protein extracts may actually be a composition of highly similar polypeptides. The *Am*GSTF enzymes had little activity towards the herbicide substrate fenoxaprop-p-ethyl but were 4 times more active towards a long-chain fatty acid hydroperoxide substrate in comparison with cumene hydroperoxide (Cummins et al., 1999). Further studies carried out by Cummins *et al.* demonstrated that when MHR black-grass was

treated with a range of herbicides irrespective of the MoAs, the hydroperoxide content was maintained at a basal level, whilst in stark contrast the susceptible black-grass merely accumulated hydroperoxides. This resulted in the understanding that MHR plants had evolved tolerance to herbicides irrespective of the target-site through the constitutive expression of GPOX-active *Am*GSTF1 enzymes (Cummins et al., 1999). *Am*GSTF1 expression was induced in herbicide-sensitive plants in response to herbicide safeners, dehydration and chemical treatments imposing oxidative stress. Meanwhile, the mechanism underlying constative up-regulation of GST expression in herbicide resistant plants remains unverified. Analysis of genomic deoxyribonucleic acid (gDNA) did not indicate that the gene had been amplified, whereas total RNA extracted from untreated herbicide sensitive and MHR plants produced *Am*GSTF1 transcripts in MHR plants which were absent in the sensitive plants (Cummins et al., 1999).

To date, there have been similar studies utilised to identify GSTs responsible for herbicide detoxification in other weed species, namely Italian rye-grass (*Lolium multiflorum*). Italian rye-grass has elevated GST activity towards herbicides, however this was not investigated in greater depth as little was known about responses in relation to other biotypes with varying herbicide susceptibilities (Del Buono and Ioli, 2011). Recent next generation sequencing transcriptomic analysis of diclofop resistant Italian rye-grass, has enabled identification of GSTs from both phi and tau class. Following a physiological validation experiment in which 2,4-D pre-treatment induced diclofop protection in susceptible individuals, none of the GSTs proved to be strongly associated with the resistance phenotype. This level of analysis raises awareness towards the potential for understanding the molecular mechanisms that enable a GST-mediated herbicide resistance trait in weeds.

1.3.4 Glycosyltransferases and Herbicide Resistance

Glycosyltransferasees (GTs) are found in plants and are a large group of enzymes responsible for the conjugation of acceptor molecules such as: flavonoids, xenobiotics and hormones, to an activated UDP sugar moiety (Bowles et al., 2005). Glycosylation is an important aspect of metabolism of herbicides including chlorosulfuron and diclofop-methyl in crops (Shimabukuro et al., 1979, Sweetser et al., 1982). Glycosylation occurs when the newly-introduced hydroxyl group is presented following the initial hydroxylation of the herbicide by CYP enzymes. On application to weeds, the method of herbicide glycosylation that elicited a herbicide resistant trait was unclear. In black-grass, GT activity increased in correlation to CYP levels, indicating that CYP-mediated hydroxylation of the herbicide is a key prerequisite

for glycosylation enabling herbicide resistance (Christopher et al., 1991, Ahmad-Hamdani et al., 2013).

1.3.5 Transporter Proteins and Herbicide Resistance

ATP-binding cassette (ABC) transporter proteins transport herbicide conjugates into the vacuole for further degradation. The ABC transporter family consists of 131 coding sequences identified in Arabidopsis and 121 in rice (Sánchez-Fernández et al., 2001). Eukaryotic ABC proteins are subsequently divided into 8 major subfamilies regardless of the species origin, instead they are based on protein size, orientation, and sequence similarity (Sánchez-Fernández, 2001, Rea, 2007, Verrier, 2008). ABC transporter proteins are effective at transporting both glycolsylated and glutathionylated herbicides into the vacuole, including hydroxyprimisulfuron-glucoside which is transported into barley vacuoles (Rea, 2007, Martinoia et al., 1993). In Arabidopsis, *At*MRP1 and *At*MRP2, localised in the vacuolar membrane play a role in the transport of glutathione conjugated herbicides (Geisler et al., 2004). These proteins closely resemble transporter proteins which are commonly found in mammals and are associated with multi-drug resistance (Lu et al., 1997, Lu et al., 1998). These transporters may have evolved to fulfil transport roles which extended well beyond those identified in these early studies. In weeds, the characterisation of transporter proteins utilised in herbicide metabolism have not yet been reported, although there is an understanding that there is an emerging mechanism of resistance through the altered translocation and/ or sequestration in the plant. For example, glyphosate translocation has been significantly reduced in both multiple resistant *Lolium* and *Conyza* biotypes, potentially as a result of being the world's most commercially- exploited herbicide (Powles and Yu, 2010).

1.4 Herbicide Resistant black-grass

Alopecurus myosuroide (black-grass) is an annual diploid obligatory out-crossing species with herbicide resistant populations prevalent across the UK and Europe (Moss et al., 2007). The presence of herbicide resistant black-grass is predominantly in the South-East of England and is said to affect an estimated 80% of arable farmland, and contributes to £0.5million loses annually (Moss, 2011). Black-grass populations have evolved both TSR and NTSR resistance mechanisms following exposure to herbicides irrelevant of geographical locations. There are several known point mutations conferring TSR: 2 and 12 for the enzymes ACCase and ALS respectively (Tranel and Wright, 2002, Jang et al., 2013). As technologies continue to develop and a greater understanding at the genome level is achieved further point mutations are still being identified (Kaundun et al., 2013a, Kaundun et al., 2013b). Most worryingly, black-grass

has evolved NTSR mechanisms endowing resistance towards multiple herbicides with varying modes of action and chemical structures (Moss, 1990, Délye et al., 2011). These mechanisms involve the CYPs and GSTs in herbicide metabolism as well as the expression of GSTs which function as GPOXs (Hyde et al., 1996, Hall et al., 1997, Brazier et al., 2002, Cummins et al., 1999, Cummins et al., 1997). Furthermore, NTSR in black-grass has caused resistance to herbicides to which the biotypes have not previously been exposed, for example the commercial herbicide pinoxaden (Délye et al., 2011). This finding highlighted that understanding the molecular mechanisms leading to NTSR is essential to developing a proactive strategy to combat herbicide resistance as opposed to the reactive methods currently used: namely tank-mixes, post-emergence herbicides or cropping rotations. Most recently, work carried out by the Edwards group has identified similarities between the over-expression of the plant phi class GST and the mammalian over-expression of phi 1, which has a known role in multiple drug resistance (MDR) in human tumour cells (Cummins et al., 2013). Cummins identified that there is potential for future mechanisms of control to be explored using similar strategies as multiple drug resistance in human tumour cells. The study identified that MDR-inhibiting pharmacophore 4-chloro-7-nitro-benzoxadiazole also inhibits *Am*GSTF1 activity leading to restoration of herbicidal control of MHR black-grass, confirming the central role that specific GSTFs in play in MHR black-grass. Importantly, these phi class GSTs are constitutively expressed in protein extracts of MHR biotypes but absent in herbicide-sensitive and ACCase target-site resistant biotypes (Cummins et al., 1999). The GST antibody utilised in the study was raised to the herbicide-detoxifying maize *Zm*GSTF1-2, which meant that there could be potentially further novel GSTs with improved diagnostic potential for MHR in black-grass if a more specific antibody was produced.

1.5 Diagnostic tools

As agriculture struggles to support the rapidly growing global population, plant disease and resistance to herbicides in weeds has reduced the production and quality of food. As a result, early information on crop health and disease detection can facilitate the control through proper management strategies such as vector control through pesticide applications or specific chemical application; can improve productivity.

Currently the agricultural industry utilises either field diagnostics or laboratory diagnostics, for monitoring herbicide resistance it is the latter, which is the most widely used mechanism. This is an expensive, labour-intensive and a time-consuming process. Common strategies for laboratory diagnosis include: Rothamsted rapid resistance test (seed testing) and the Syngenta Quick Test (whole plant testing) both rely on the expertise of scientists and the results can

take 6- 12 weeks respectively, each with its own limitations (discussed in Chapter 3). The key reason farmers test weeds for herbicide resistance include:

- Avoiding potentially wasteful use of herbicides- save money and prevent unnecessary use
- Help assess the threat posed by resistance on newly farmed blocks of land
- Monitor the success (or otherwise) of long-term resistance management strategies

Therefore, for this project the field diagnostic techniques needed to be rapid to enable fast decision making, simple to use, enabling access by non-specialist users and inexpensive to enable broad deployment. In plant health field diagnostics have been used since the early 1980's where latex agglutination tests were used to test high grade seed potatoes (Fribourg, 1984). The most successful format to date being the lateral flow device (LFD), which given good binding reagents can be used generically for a large range of different pathogens.

Pocket Diagnostics® uses an LFD which is similar technology to a pregnancy test (point of care (POC) test), for the rapid detection of commercially damaging plant diseases in plants and crops caused by fungi, bacteria and viruses (Figure 8). Pocket Diagnostics® continues to be a market leader in plant disease testing with devices optimised for the detection of: *Erwinia amylovora* (fireblight), *Ralstonia solanacearum, Phytophthora* and Potato virus Y (PVY) all of which market for approximately £20 for 4 tests (Abingdon Health, 2017). The advantages of these in field-diagnostics include: simple to use, no training required, rapid results (less than 10 minutes), cost effective alternative to laboratory testing, portable and removes subjectivity.

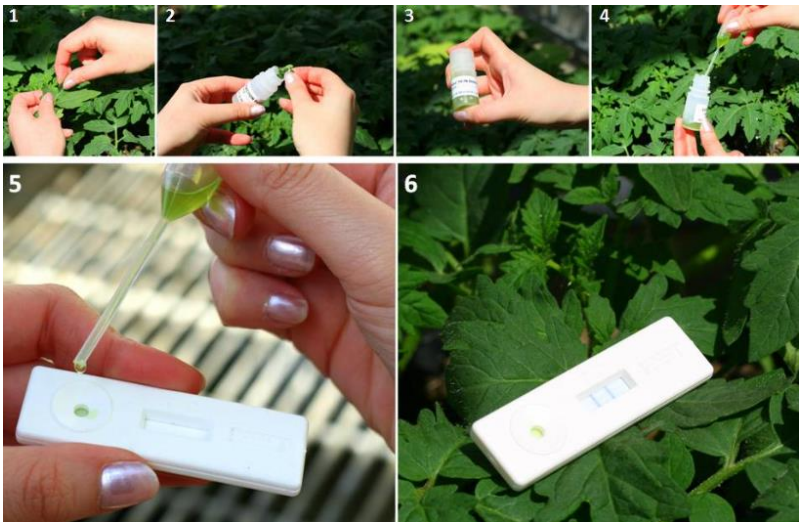


Figure 8 Pocket Diagnostic (R) visual representation of testing plant diseases
(https://www.pocketdiagnostic.com/products/pocket-diagnostic/how-to-use/)

More recently though, research has focused in two key areas: the development of sensitive DNA diagnostics for field use and the use of cutting-edge genomics to identify unknown pathogens associated with new diseases. Although LFDs are the ultimate simple-to-use field diagnostic there are limitations for instance they can lack sensitivity to detect low concentration pathogens. To overcome some of these disadvantages, The Food and Environment Research Agency (FERA) (Sand Hutton, UK) developed a DNA-based field detection method which includes 'loop-mediated isothermal amplification' (LAMP), a sensitive and specific PCR method that doesn't require multiple cycles of heating and cooling to amplify the target sequence. This technique is currently deployed at Heathrow airport to support import inspections. Previous techniques based on PCR, even with elaborate platform development have largely failed to make impact due to the expense of equipment, complexity of the tests and speed to result. Isothermal amplification techniques on the other hand largely solve these issues and several platforms are gaining traction.

Further to this, the application of genomics in the rapid identification of novel pathogens has shown significant progress. The use of next generation sequencing (NGS), combines advances in sequencing technology and DNA data analysis. By taking samples from diseased and healthy plants, sequencing and comparing the DNA in them, it is possible to identify viruses and other pathogens that are the potential cause of disease. This approach is non-targeted and is an excellent way of identifying 'unknown pathogens' which is difficult to do using traditional methods. Therefore, these recent innovations suggest that this method may prove to be the ultimate in generic technologies for the identification of any biological target even on site.

For this study based on the existing technologies it was hypothesised that the initial format for a herbicide resistance black-grass diagnostic would be an LFD. It was anticipated that following a NGS analysis of black-grass a more targeted approach based on differences in DNA sequences could be used in a Clondiag array or another innovative platform which might even be coupled to smartphone technology.

1.6 Background and aim of the thesis

The research described in this thesis builds on the discovery by Cummins et al (1999), of the increased expression of *Am*GSTF1 in the multiple herbicide resistant weed (*Alopecurus myosuroides*). Within my research, I explored the identification and suitability of biomarkers for MHR in the weed black-grass, by investigating the role of the xenome in MHR and

methods of detecting associated resistance in the field. Biomarker identification was carried out using several “omic” approaches that have the potential to be developed into molecular diagnostics to diagnose black-grass populations allowing informed weed management to potentially counteract MHR.

Modern agriculture persists in managing competitive weed species through the intensive use of herbicides to ensure crop productivity. Resistance to aryloxyphenoxypropionate and sulphonylurea herbicides in the problem weed black-grass is now a major problem for wheat production in the UK, affecting much of the best arable land in the south and east of the country. In addition to target site resistance (TSR), increasing numbers of black-grass populations also exhibit multiple herbicide resistance (MHR), based on an enhanced capacity to detoxify herbicides, irrespective of their chemistry. The detoxification process follows a four-phase schema which relies on oxidoreductases most notably cytochrome P450s (CYPs), transferases including glutathione transferases (GSTs) and glycosyltansferases (UGTs) and transporter poteins from the ABC and MATE families. MHR is associated with the up-regulation of this array of detoxification enzymes (Yuan, Tranel et al. 2007)., which is termed the “xenome”.

Thus, the research presented in this thesis is focused on the identification of biomarkers for MHR in the weed black-grass, by investigating the role of the xenome in MHR and proactive methods to detect associated resistance in the field. The identification of potential biomarkers was carried out using various “omic” approaches which had the potential to be developed into molecular diagnostics to study and counteract the phenomena as part of the national black-grass resistance initiative (BGRI).

1.6.1 Aims of the project

The main aim of this project focused on characterising xenome components for their use as diagnostic biomarkers of metabolic based multiple herbicide resistance (MHR). To do this the following questions were explored:

- The methods of characterising herbicide resistance in wild grasses.
- The validity of *Am*GSTF1 as an existing marker for herbicide resistance specifically in black-grass.
- The identification of novel xenome DNA biomarkers associated with herbicide resistance following a next generation sequencing analysis of black-grass.

To address the initial question several black-grass populations (sourced from wheat fields) were characterised for their level of resistance towards contrasting herbicide chemistries compared to a standard susceptible biotype (Chapter 3).

To assess the validity of *Am*GSTF1 as a marker for herbicide resistance, a recombinant GSTF1 from the MHR "Peldon" black-grass population was expressed in *E. coli* and used to raise a polyclonal antiserum in rabbits. The anti-*Am*GSTF1-serum reacted with three polypeptides of 25kDa, 24kDa and 22kDa in the crude extracts of previously characterised MHR black-grass. When tested blind against a panel of ten populations the antiserum proved highly diagnostic for these polypeptides in MHR populations using both immunoblotting and ELISA assay. The antiserum also identified orthologous polypeptides in MHR *Lolium rigidum* and *Avena fatua* indicating that GSTF1 is diagnostic of metabolic resistance in other grass weeds (Chapter 4).

Given the suspected highly polygenic and complex nature of the xenome in NTSR, the project further explored the potential utility of using other xenome genes as DNA biomarkers of MHR for diagnostic applications in managing herbicide resistance in black-grass. De Novo next generation sequencing (NGS) was carried out on MHR and WTS (wild-type susceptible) black-grass, enabling a virtual transcriptome of xenome and associated genes to be assembled. Eight xenome genes were identified as being differently expressed namely a *CYP*, *GSTU6*, *GSTF1*, an oxophytodienoic acid reductase (*OPR1*), *UGTZ*, a thiol methyltransferase (*TMT*) and *ABC* and *MATE* transporters. To examine the potential of these biomarkers of resistance, real-time qPCR was used on populations of black-grass of characterised resistance. The

results indicated several sequences as potentially functional transcriptional biomarkers of MHR. In addition, the expression of these eight biomarkers was examined in herbicide susceptible black-grass, to test whether an incorrect diagnosis of resistance could occur if biomarkers were stress inducible (Chapter 5).

The conclusions from these studies and the development of a prototype lateral flow device using immunodiagnostic detection of GSTF1 for the determination of MHR in black-grass populations in the field is then discussed (Chapter 6).

2. Materials and Methods

2.1 Materials

All molecular biology reagents were purchased from New England BioLabs Inc. (Hitchin, Hertfordshire, UK), Promega (Southampton, Hampshire, UK), Qiagen Ltd (Manchester, Lancashire, UK) or Thermo Scientific (Waltham, Massachusetts, USA). Oligonucleotides were synthesised by and purchased from Eurofins MWG Operon LLC A Eurofins Genomics Company (Ebersberg, Germany). All buffers were generated using ultrapure water. All growth media were autoclaved prior to use.

2.2 Instrumentation

Protein purification, antibody purification and desalting were carried out on an AKTA-FPLC system (GE Healthcare; Amersham, Buckinghamshire, UK). Spectrophometric assays were measured on a Shimadzu UV-1800 UV spectrophotometer (Shimadzu Corporation, Japan). Polymerase chain reactions were performed on a Mastercycler® gradient thermal cycler (Eppendorf; Hamburg, Germany). Real-time Polymerase chain reactions were carried out at the University of York (York, Yorkshire, UK) using the StepOne™Real-Time PCR System (Thermo Scientific; Waltham, Massachusetts, USA), or at Newcastle University (Newcastle-upon Tyne, Northumberland, UK) using the Roche Lightcycler (Roche Diagnostics Ltd; Burgess Hill, West Sussex, UK). Plant material was grown at Newcastle University in Panasonic MLR-352series growth cabinets (Panasonic Biomedical; Loughborough, Leicester, UK) or at the University of York glasshouses or Fera (York, Yorkshire, UK) glasshouses.

2.3 Assay Methods

2.3.1 Plant Studies

Black grass (*Alopecurus myosuroides Huds.*) seeds including multiple herbicide-resistant (MHR-Peldon), wild-type sensitive (WTS- Rothamsted) and other populations of varying resistance were obtained from Rothamsted Research, Hertfordshire, UK. Seeds were incubated at 30^{o}C for 3 weeks prior to sowing in John Innes no. 2 compost, where they were grown for 14 days at 17 ^{o}C with a 14 h photoperiod and a light intensity of 125-150 μmol·m-2·s-1 Photosynthetic photon flux density (PPFD). All above ground material was harvested, where needed this was divided into meristem and leaf tissue, and frozen in liquid nitrogen prior to storage at -80 ^{o}C until required.

2.3.2 Protein Extraction

Tissue was ground under liquid nitrogen using a pestle and mortar. All further steps were carried out on ice. Protein was extracted with 3x (w/v) buffer (50 mM Tris-HCl, 2 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dithiothreitol) pH 7.5) and 50 g L^{-1} polyvinylpolypyrolidone (PVPP). The resulting extract was filtered through a double layer of Miracloth (22-25 µm pore size) (Merk Millipore; Watford, Hertfordshire, UK). The filtrate was transferred to Oakridge centrifuge tubes and centrifuged at 10,000 g for 15 min at 4 ^{o}C. The protein in the supernatant was precipitated through the addition of ammonium sulphate to 80% saturation and recovered following further centrifugation at 4000 g for 20 min at 4 ^{o}C. Pellets were then stored at -20 ^{o}C and desalted prior to use on a Sephadex G-25 column (Sigma-Aldrich; Gillingham, Dorset, UK).

2.3.3 GST assay using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate

CDNB assays were carried out in triplicate, based on the method described by (Edwards, 1996). The assay buffer (0.1 M potassium phosphate, pH 6.5) was warmed to 30 ^{o}C and a mixture was prepared containing 5 mM GSH, 375 nM recombinant enzyme and 1 mM CDNB (prepared as a 40mM stock solution in ethanol), in a total assay volume of 1 mL. The sample was subsequently mixed, and the increase in absorbance was measured for 30 s at 30 ^{o}C by UV-vis spectrophotometry at 340nm. The chemical rate of reaction was measured by replacing the recombinant enzyme with an appropriate non-enzyme substitute. The GST-catalysed reactions were corrected for non-enzymatic contributions. Corrected enzymatic rate and expressed as nmol of glutathionylated product formed per second per mg of recombinant protein, were calculated using the molar extinction coefficient of the product (ε = 9.6 mM-1 cm-1).

2.3.4 Protein concentration determination

Protein concentration was determined using the Thermo Scientific ™ Pierce ™ BCA Protein Assay Kit with minor modifications from the test-tube procedure. The working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A (500 mL solution containing: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide), with 1 part of BCA Reagent B (25 solution containing 4 % cupric sulfate). Each standard contained a total WR volume of 1 mL (50:1, Reagent A: B) and 50 µL of the protein bovine serum albumin (BSA) and were used to generate a standard curve ranging from 2,000 µg/mL to 0 µg/mL. The unknown(s) were assayed alongside, in order to determine their concentration. The samples were measured at 562 nm following an incubation period of 30

mins at 37 ºC. The generated standard curve was plotted once the "blank" (background interference) value was removed, to establish the amount of protein in the samples in µg/mL.

2.3.5 *Am*GSTF1 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay was performed in a 96-well plate format to detect and quantify the presence of GSTF1 in crude plant protein extracts. The technique was based on the principle of indirect plate trapped antigens (PTA). Plant material (approx. 0.2 -1 g) was ground in extraction buffer (2% Polyvinlypyrrolidone K15 (PVP) in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 1 mg/ml. The concentration of the crude protein in the extract was determined using the BCA protein assay. Samples of crude protein were loaded onto a Nunc 96 well ELISA plate at a starting concentration of 200 µg/mL down to 50 µg/mL in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to a final volume per well of 100 µL. Each plate contained purified recombinant *Am*GSTF1 protein loaded at 200 µg/mL and diluted 1:2 serial dilution to 0.78 µg/ml, enabling a standard curve to be generated. Each plate also contained a minimum of 3 blank wells to enable the background to be subtracted. Once coated, the plates were wrapped in cling film and incubated at 2-8 ºC overnight or alternatively, incubated for 2 h at 33 ºC. Subsequently, the plates were washed three times in PBST (20 mM sodium phosphate, 150 mM sodium chloride pH 7.4, and 0.05% Tween20), and then dried. 200 µL of freshly prepared blocking buffer (PBST-20 mM sodium phosphate, 150 mM sodium chloride pH 7.4, containing 0.05% Tween20 +1% BSA) was added to each well and the plates were then covered and incubated for 1 h at 33 ºC, after which the wash step was repeated as above. The primary antibody (Anti-*Am*GSTF1 serum) was diluted 1:500 in dilution buffer (PBST + 0.2% BSA) and added at 100 µL per well. The incubation period of 1 h at 33 ºC was repeated along with a further wash step. The penultimate incubation step utilised the anti-species polyclonal antibody: anti-rabbit IgG (whole molecule)-Alkaline Phosphatase antibody produced in goat (Sigma-Aldrich, USA). A 1:4000 dilution of this antiserum was prepared in dilution buffer and 100 µL added per well, incubated for 1 h at 33 ºC followed by a final wash step. Finally the substrate pNPP (p-Nitrophenyl Phosphate, Sigma-Aldrich, USA) was added at 100 µL per well and incubated in the dark for 1 h at room temperature. The plate's absorbance was read at 405 nm, and *Am*GSTF1 was quantified using a standard curve.

2.4 Characterising herbicide resistance

2.4.1 Herbicide Resistance Assays

The Rothamsted Rapid Resistance test (Moss, 1999a) was used as a guide to investigate ALS and ACCase resistance in populations of *Alopecurus myosuroides*. Prior to carrying out the assays, seeds were pre-treated to break dormancy using heat treatment at 30 ^{o}C for a minimum of 14 days prior to being included in the test. Petri dishes with a diameter of 9 cm containing three cellulose filter papers (Whatman no1) and one glass fibre filter paper (Whatman GF/A) were assembled as illustrated in Figure 9. Potassium nitrate (nil treatment) was added in 7 mL aliquots at a concentration of 2 g/L, and one of three herbicides: LASER® and STOMP® Aqua (BASF, Germnay) or Atlantis WG® (Bayer CropScience, UK) were applied at varying concentrations. The herbicide treatments were applied at the same volume as the nil however the herbicides were diluted in potassium phosphate at concentrations of 5 ppm, 1 ppm or 0.1 ppm. Each population of *Alopecurus myosuroides* had two petri-dishes which contained 50 seeds each. The percentage germination before and after herbicidal treatment was therefore calculated and indicated the statistical significance of the assay. The seeds were incubated in a Panasonic (MLR-352) growth cabinet with the following day/night cycle: 17 ^{o}C for 14 h (lights on) and 11 ^{o}C for 10 h (lights off). After a period of two weeks the number of seeds per plate which had germinated (< 1 cm growth) were counted and a percentage germination established.

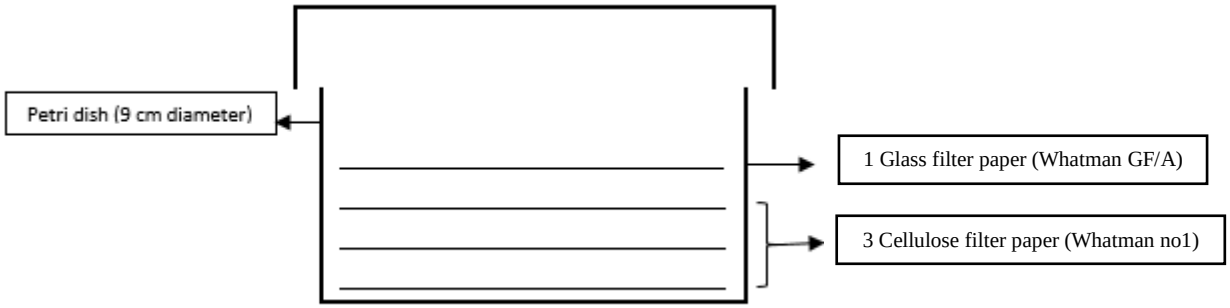


Figure 9 Petri-dish set up for Herbicide Resistance Assays.
Petri-dishes of 9cm diameter containing 3 cellulose filter papers (Whatman no1) and 1 glass filter paper (GF/A) were coated in 7 mL of either potassium phosphate (nil treatment) or the herbicide of choice at the required concentrations in PPM. 50 seeds were placed on the surface of the glass filter paper and incubated in the growth cabinet with the lid on.

2.4.2 *Herbicide spray trials*

Utilising commercially available herbicides Atlantis WG® and Cheetah®Gold (Bayer CropScience, UK) spray trials were performed at the FERA institute, York. In total, 390 (4

inch) pots containing 10 seeds post-heat treatment (30 ^{o}C for 14 days) were sown in John Innes no. 2 compost. Each population of *Alopecurus myosuroides* was sown in triplicate and treated with five different concentrations of the two commercial herbicides. All herbicide treatments were applied by foliar spray using a knapsack. Atlantis WG® was applied at: 0,100,400,800 and 1600 g/Ha with normal field rate at 400 g/Ha. Cheetah® Gold was applied at: 0, 0.625, 2.5, 10 and 20 l/Ha with normal field rate at 1.25 l/Ha. The two herbicide treatments were housed in two independent glasshouses to ensure that the herbicides were contained and cross-contamination was minimised. The pots were assembled in a randomised order and assessments on the damage were made via visual scoring mechanisms which relied on the comparison of the nil replicates to those under herbicidal treatment. The damage was measured 7, 14 and 21 days after treatment (DAT).

2.5 Purification of recombinant protein

2.5.1 Protein Expression in *E.coli*

*Am*GSTF1, which had previously been sub-cloned into the pET24a vector, was transformed into Tunetta cells an *E.coli* strain. A single colony was selected and grown overnight with shaking at 37 ^{o}C at 180 rpm in 50 mL autoclaved LB medium (Tryptone 10 g, Yeast extract 5 g, NaCl 10 g in 1 L water) containing 100 µg mL^{-1} kanamycin and 35 µg mL^{-1} chloramphenicol (35 mg mL^{-1} stock solution in ethanol). The culture was then decanted into 12.5mL aliquots in 4 x 1 L of LB medium containing the same concentrations of kanamycin and chloramphenicol. The cultures were subsequently incubated at 37 ^{o}C shaking at 180 rpm until the optical density reached 0.6-0.8 at an absorbance wavelength of 600 nm (determined against an LB blank). The culture was equilibrated at 20 ^{0}C for 30 mins, and then the expression of the recombinant protein was induced by the addition of 0.1 mM Isopropyl β-D-thiogalactopyranoside (IPTG). The culture was shaken at 180 rpm for 16-20 h at 20 ^{0}C . Cells were then collected via centrifugation (10,000 xg, 15 min at 4 ^{0}C) and the resulting pellets were stored at -20 ^{o}C until required.

2.5.2 Protein purification using S-Hexylglutathione (S-Hexyl GSH) affinity chromatography

Frozen bacterial pellets as prepared in section 2.5.1 were thawed to room temperature and resuspended in 10 mL of 0.1 M Potassium phosphate buffer (pH 7.5), and v/v 1 mM DTT. The samples were placed on ice and then subjected to a total of 5 mins sonication (15 s sonication, 15 s ice) to lyse the cells. The lysate was clarified by centrifugation (10,000 xg, 15 min at 4 ^{0}C), and the supernatant transferred to sterile 15 ml tube and kept on ice. A 10 mL S-Hexylglutathione agarose column (Sigma-Aldrich, USA.), was pre-equilibrated with the 0.1

M Potassium phosphate buffer (pH 7.5) at a flow rate of 0.5 mL/min. Once equilibrated, the cell lysate was loaded onto the column at the same flow rate. The unbound protein was eluted using an elution buffer (5 mM S-hexylglutathione) at a rate of 0.5 mL/min. The absorbances of the individual eluted protein fractions were measured at 280 nm and peak fractions were then later combined for storage at 4 ^{o}C. The column was regenerated by washing with two column volumes of ultrapure water followed by two column volumes of 20% ethanol at a rate of 1 mL/min prior to storage at 4 ^{o}C.

2.5.3 Quantification of partially purified *Am*GSTF1 protein extracts

The concentration of purified *Am*GSTF1 protein was determined by two methods. The initial assessment was carried out using a UV Spectrometer set at A 350-200 nm, and a volume of 100 µL was measured in a quartz cuvette and the A 280 nm identified (determined using elution buffer as a blank). The theoretical absorbance of *Am*GSTF1 at 280 nm was calculated from the amino acid sequence using Richard's protein calculator (Grant, 2005). The protein calculator utilised the protein sequence of *Am*GSTF1 (Figure 10), the A280nm reading and the associated dilution factor. The samples were combined and stored at 4 ^{0}C. The partially purified protein was assessed using the Nanodrop ND1000 spectrometer prior to use to ascertain whether the protein had degraded (Thermo Scientific, USA).

Sequence of AmGSTF1c

MAPVKVFGPAMSTNVARVTLCLEEVGAEYEVVNIDFNTMEHKSPEHLARNPFGQIPAFQDGDLLLWESRAISKY
VLRKYKTDEVDLLRESNLEEAAMVDVWTEVDAHTYNPALSPIVYQCLFNPMMRGLPTDEKVVAESLEKLKKVLEV
YEARLSKHSYLAGDFVSFADLNHFPYTFYFMATPHAALFDSYPHVKAWWDRLMARPAVKKIAATMVPPKA

Sequence ID Accession number AJ010453.1

Figure 10 Sequence of *Am*GSTF1c.
(Sequence ID no. AJ010453.1 Accessed from GenBank. The translated protein sequence of *Am*GSTF1c)

2.5.4 Dialysis of partially purified *Am*GSTF1 protein

Proteins eluted from affinity chromatography were dialysed overnight in 5 L of dialysis buffer (50 mM potassium phosphate buffer, 1.5 M $(NH_4)_2SO_4$, pH 7) which was in accordance to the buffer required for hydrophobic interaction column. The protein fractions were combined and placed into dialysis tubing and sealed ensuring it maintained capacity to float. The dialysis tubing was then submerged into 5 L of dialysis buffer and left stirring for 16 h at 4 ^{o}C. Samples with volumes greater than 1 mL were concentrated using vivaspun 20 columns (membrane size 10,000 MWCO PES) (GE Healthcare,UK)

2.5.5 Hydrophobic interaction column (HIC)

Hydrophobic interaction chromatography was implemented as it separated molecules based on hydrophobicity. This was a useful separation technique for purifying proteins while maintaining their biological activity. A 1 mL GE Healthcare Resource PHE R10 column was equilibrated with start buffer (50 mM potassium phosphate, pH 7.0 and 1.5 M ammonium sulphate) at a flow rate of 0.5 mL/min until five column volumes had passed through the column. 1 mL of start buffer was loaded on to the column followed by 1 mL of partially purified *Am*GSTF1 protein in a high-salt buffer. The flow through was collected for later binding efficiency assessment by SDS-PAGE (2.6.1). The protein sample was injected onto the column at a rate of 0.5 ml/ min. The more hydrophobic the molecule, the less salt is needed to promote binding. Therefore, a decreasing salt gradient was set using an elution buffer (50 mM potassium phosphate, pH 7.0) at a rate of 0.5 ml/ min to reach 100 % after 50 min to elute samples from the column in order of increasing hydrophobicity. The relevant fractions containing the eluted protein were measured at 280 nm.

2.6 Protein Analysis

2.6.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE investigations were carried out using a 12 % discontinuous gel system with a Mini-Protean Tetra cell apparatus (Bio-Rad, USA). The resolving gel was composed of 2.5 mL resolving buffer (1.5 M Tris-HCl, 0.4 % (v/v) tetramethylethylenediamine (TEMED), 0.4 % (w/v) SDS, pH 9), 4.2 mL of H_2O and 3.2 mL 40 % acrylaminde/bis-acrylaminde. The gel solution was de-gassed and 0.1 mL of ammonium persulphate (APS) was added to the solution (10 % w/v), inducing polymerisation of the acrylamide. The solution was immediately transferred to a pre-assembled gel apparatus and, a layer of water saturated butanol was added and allowed to solidify. The stacking gel composed of 4.5 mL stacking buffer (0.14 M Tris-HCl, 0.11 % (v/v) TEMED, 0.11 % (w/v) SDS, pH 6.8) and 0.5 mL 40 % acrylamide/bisacrylamide. The solution was de-gassed and 0.05 mL APS was added, after which the solution was immediately added to the top of the resolving gel, the well-comb was added and the gel was allowed to solidify. The gel tank apparatus was assembled in accordance with manufacturer's instructions and filled with running buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3). Protein samples were prepared using an appropriate volume of 2 x SDS loading buffer (100mM Tris-HCl, 20 % (v/v) glycerol, 4 % (w/v) SDS, 0.2M DTT, 0.2 % (w/v) bromophenol blue, pH 6.8) and boiled for 5 mins at 95 ^{o}C. Samples were run alongside 10 µL pre-stained broad range protein markers to allow the estimation of the molecular weights of unknown proteins. After samples were loaded, gels were run at 100

V until proteins had travelled through the stacking gel, after which it was increased to 200V thereafter until the marker dye-front eluted from the gel. To visualise proteins gels were washed twice for 15min in H_2O and then stained using Coomassie (60 mg Coomassie Brilliant blue G-250 was dissolved in 50 mL ethanol, and then filtered into 100 mL phosphoric acid and the total volume was made to 1 L with water).

2.6.2 Western blotting and immunodetection

Proteins were separated by SDS-PAG using 15 µL sample, which contained protein ranging from 64 ng to 1 ng. Once electrophoresis was completed, the resolving gel was immersed into H_2O and the proteins transferred to iBlot®2 transfer stack polyvinylidene difluoride (PVDF) membrane (0.2 µm pore size, low fluorescence and protein binding capacity of 240 µg/ cm^2 (Thermo Fisher Scientific, USA) using an iBlot® Semi-dry blotting system (Thermo Fisher Scientific, USA), according to the manufacturers instruction. Once transferred, the membrane was immersed in blocking buffer, (10 mM Tris, 150 mM NaCl, 3 % (w/v) skimmed milk powder, pH 7.4) for 1 h at room temperature. The membrane was then incubated in blocking buffer with primary antibody Anti-*Am*GSTF1 serum at 1:500 dilution overnight at 4 ^oC. The membrane was washed twice with 1x TBS (10 x Tris buffered saline: Tris 12.1 g, NaCl 87.7 g in 1 L of water pH 7.4) and 0.05 % (v/v) Tween-20 for a total of 10 min, followed by a wash with TBS buffer for 5 min. The membrane was incubated with blocking buffer along with 0.05% (v/v) anti-rabbit IgG (whole molecule)-alkaline phosphatase antibody (Sigma-Aldrich, USA). After 1 h, the membrane was washed twice with 1 x TBS and Tween-20 buffer for 10 min, followed by washing with 1 x TBS buffer for 5 min. The membrane was then washed with 20 mL staining buffer (100 mM Tris-HCl, pH9.5) for 2 min, prior to incubating with 20 mL staining buffer containing 33 µL 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (prepared as 50 mg/mL^{-1} in 100% dimethyl formamide) and 33 µL nitro blue tetrzolium (NBT) (prepared as 100 mg/mL^{-1}in 70 % (v/v) dimethyl formamide). Once the proteins were visualised the membrane was immersed in approx. 300 mL of H_2O and then air dried.

2.7 Production and affinity purification of Anti-*Am*GSTF1 serum

2.7.1 Production of Anti-*Am*GSTF1 serum

The Polyclonal antibodies were raised to the recombinant protein *Am*GSTF1. The new antibody was raised by Covalab (Covalab UK ltd, Cambridge, UK) following a 67-day immunisation protocol. This involved a pre-bleed of 4-5 mL taken at day 0 after which the rabbit (XY- New Zealand white) was injected with 0. 5 mL antigen (*Am*GSTF1) and 0.5 mL complete Freund's adjuvant. On days 14 and 28 the rabbit was injected with a further 1 mL containing 0.5 mL antigen and 0.5 mL of incomplete Freund's adjuvant. The first test bleed

was taken at day 39 (4-5 mL) and at day 42 a final injection of 1 mL was given with a second test bleed taken at day 53 (10-15 mL). The final bleed was harvested at day 67 and was subjected to immunoassay analysis enzyme-linked immunosorbent assay (ELISA). This was carried out on a 96-microtiter plate with serum dilutions from 1:500 to 1:64,000 (8 dilutions). The results of the ELISA indicated a very good immunoreactivity of the rabbits' sera using *Am*GSTF1 protein as the antigen at > 64,000 dilution.

2.7.2 Ammonium sulphate precipitation of Anti-*Am*GSTF1 serum

20 mL of *Am*GSTF1 antibody serum was filtered into a sterile 50 mL centrifuge tube using a 0.45 µM spin filter column at 4 ºC, 1 mL of Tris-HCl pH 8.0 was then added. The ammonium sulphate saturated solution was prepared (100 g ammonium sulphate to 100 mL of water) and added drop by drop to the serum whilst stirring at 4 ºC. Once 50 % saturation of the *Am*GSTF1 antibody serum was reached, approximately after 10 mL of saturated ammonium sulphate was added the solution was left stirring for 1 h. The sample was then centrifuged at 10,000 xg at 4 ºC for 20 mins, then the supernatant was discarded, the pellet was re-suspended in solution B (50% saturation ammonium sulphate 10 g in 30 mL). Centrifugation steps were repeated. The pellet was dissolved in a small volume of the start buffer (500 mL: 20 mM Tris-HCl pH 7.5, and 150 mM NaCl). The sample was then left to dialyse in binding buffer (same as start buffer) overnight in 5 L at 4 ºC.

2.7.3 Affinity purification of Anti-*Am*GSTF1 serum

2.7.3.1 Protein immobilisation at pH10

AminoLink® Plus immobilisation kit (Thermo Scientific; Illinois, USA) was equilibrated to room temperature. The AminoLink® Plus resin was suspended by end-over-end mixing, followed by the removal of storage buffer through centrifugation (1000 xg for 1 min using a 15 mL collection tube). The coupling buffer (BupH™ citrate-carbonate pack, dissolved in 500 mL of ultrapure water yields 0.1 M sodium citrate, 0.05 M sodium carbonate; pH 10.0) pH10 was added to the column in 2 mL aliquots and subsequently centrifuged (1000 xg for 1 min using a 15 mL collection tube), this was repeated until 4 mL of coupling buffer had passed through the column. The desalted protein was diluted 4-fold in coupling buffer. Then the protein sample was added at a volume of 2-3 mL to the column ensuring the bottom cap was replaced. 0.1 mL of the prepared sample was stored at 4 ºC for subsequent determination of coupling efficiency. The column was mixed by gentle rocking or end-over-end mixing at room temperature for 4 h. After 4 h the column was placed into a 15 mL collection tube to remove non-bound protein through centrifugation (1000 xg for 1 min). The eluted non-bound protein was used to determine coupling efficiency by comparing the protein concentrations of

the non-bound fraction to the starting sample. To block the column, 2 mL of pH 7.2 coupling buffer (PBS) (BupH ^{TM} Phosphate Buffered Saline, 0.1 M phosphate, 0.15 M NaCl; pH 7.2 in 500 mL of water) was added to the column, centrifuged and repeated once (1000 xg for 1 min). In a fume hood, 2 mL of pH 7.2 coupling buffer (PBS) was added to 40 µl of sodium cyanoborohydride solution (5 M) (0.5 ml, $NaCNBH_3$ dissolved in 1 M NaOH) and then added to the column providing a final column concentration of 50 mM $NaCNBH_3$. The column was mixed overnight at 4 °C.

2.7.3.2 Blocking the remaining active sites

To remove the coupling buffer, the column was placed into a new 15 mL collection tube after removing the top cap and bottom caps and subsequently centrifuged (1000 xg 1min). 2 mL of quenching buffer (15 mL, 1 M Tris-HCl, 0.05% NaN_3, pH 7.4) was added to the column, centrifuged and the process repeated. In a fume hood, 2 mL of quenching buffer was added to 40 µl of sodium cyanoborohydride solution (5 M), and added to the column the resulting solution on the column was 50 mM $NaCNBH_3$. The column was mixed gently for 30 minutes by end-over-end rocking.

2.7.3.3 Wash column

To remove the quenching buffer, the column was placed into a new 15 mL collection tube after removing the top and bottom caps and subsequently centrifuged (1000 xg for 1min). The reactants and non-coupled proteins were washed away with 2 mL of wash solution (50 mL, 1 M NaCl, 0.05% NaN_3) and centrifuged (1000xg for 1min using a 15mL collection tube). This step was repeated five times. The wash was monitored for the presence of non-coupled protein, samples of the wash flow through were retained for analysis in an SDS-PAGE (2.6.1). For storage 2 mL of storage buffer was added (phosphate-buffered saline (PBS; same as pH 7.2 coupling buffer) containing 0.05% sodium azide (NaN_3)) and centrifuged, repeated twice. The column was stored with 2 mL of storage buffer on the top of the resin bed and stored upright at 4 °C.

2.7.4 Affinity purification of Anti-*Am*GSTF1 serum using *Am*GSTF1 AminoLink column

The prepared affinity column was equilibrated at room temperature (see section 2.7.3 for column). The storage solution was removed by centrifugation (1000 xg for 1 min). The column was then equilibrated with 6 mL of binding buffer (20 mM Tris-HCl, pH 7.5 and 150 mM NaCl) (3 x 2 mL additions with centrifugation each time). The protein sample (≤ 2 mL) was added to the column in binding buffer (as before). The samples were then timed for 5

mins to allow the protein to enter the resin bed. Then 0.2 mL of binding buffer was added and the top cap was replaced, the column was incubated at room temperature while rocking to allow binding to occur (e.g., 15-60 mins). For samples > 2 ml, add volumes in succession, a total of 6 mL was added to the column, over 15 min. 1 mL of binding buffer added, placed column into tube and centrifuged (1000 xg, 1 min). Flow through retained for subsequent analysis of binding efficiency and capacity. The resin was washed by adding 2 mL of wash buffer (20 mM Tris-HCl, pH 7.5 and 1 M NaCl) added to column resin and then centrifuged. This was repeated four times. The protein was eluted with 2 mL of elution buffer pH 4 (100 mM Tris Acetate, 150mM NaCl), pH 3 (100 mM Tris Acetate, 150 mM NaCl) and pH 2.5 (100 mM Glycine-HCl, 150 mM NaCl). The eluted protein was collected in a centrifuge tube containing 100 µl of neutralization buffer (1 M Tris-HCl at pH 8.5-9.0), then centrifuged (1000 xg, 1 min). The eluted sample was saved and neutralized and then this step was repeated three times. The sample was stored on ice or at 4 ^{o}C until required.

2.8. Transcript analysis using Real-time qPCR

Black-grass (Alopecurus myosuroides) seeds including multiple herbicide-resistant (MHR-Peldon), wild-type sensitive (WTS-Rothamsted) and other populations of varying resistance were obtained from Rothamsted Research, Hertfordshire, UK. Seeds were incubated at 30^{o}C to break their dormancy for two weeks, and then sown in 10cm pots containing compost (John Innes No. 2) with 20 seeds per pot. Plants were subsequently grown in a growth room at 20°C, 16:8 h light: dark photoperiod for two weeks before harvesting shoot tissue and flash freezing it in liquid nitrogen. Due to black-grass seed samples originating from wild populations there wasn't a genetically uniform crop. Therefore, to generate a sample for genetic analysis a pooled sample of 15 individual plants per pot was taken, this was done in technical triplicate. Subsequently, the pooled samples were then used to produce three biological replicates for RNA extraction from each black-grass population. QPCR was performed on ten separate cDNA biological replicates, with technical triplicates of each and fold-changes were measured in comparison to the internal standard glyceraldehyde 3 phosphate dehydrogenase (G3PD).

2.8.1 Extraction of total RNA from Plant Tissue Samples

Approximately 1-3 g of above ground tissue (leaf and meristem) was ground under liquid nitrogen. Less than 100 mg of material was transferred to a pre-chilled microtube and the Qiagen Rneasy plant mini kit (Qiagen, UK) was used in accordance with the manufacturers guidelines. Treated samples with RNase-free DNase I as an additional step (Qiagen, UK).

Samples were quantified using Nanodrop ND1000 spectrometer (Thermo Scientific, USA). The samples were stored at -80 ^{o}C until required.

2.8.2 Synthesis of cDNA from Plant Tissue Samples

An initial 20 µL reaction mixture containing 1 µg of total RNA, 1 µL of Oligo (dT) (10 µM), 1 µL dNTPs (10 mM) and 12 µL H_2O was prepared in a nuclease-free microcentrifuge tube. The solution was incubated for 5 min at 65 ^{o}C followed by 5 mins incubation on ice and samples were briefly centrifuged to ensure the contents of the tubes were collected. 4 µL of 5 x First-Strand buffer (Invitrogen ^{TM} SuperScript ^{TM} II Reverse Transcriptase kit) and 2 µL of 0.1 M DTT was added to the tubes whilst on ice, and then incubated at 42 ^{o}C for 2mins. Following the initial incubation period the samples were held at 42 ^{o}C, to enable the addition of 1 µL of SuperScript™ II reverse transcriptase. The samples were incubated at 42 ^{o}C for a further 50 mins and the reaction was then inactivated by heating the samples at 70 ^{o}C for 15mins. Subsequently, the samples were cleaned up using the Wizard®SV Gel and PCR Clean-up system as per the manufacturer's guidelines (Promega, UK). The eluted 50 µL sample of DNA was quantified using a Nanodrop ND1000 spectrometer (Thermo Scientific, USA). The samples stored at -20 ^{o}C until required.

2.8.3 Real-Time Quantitative Polymerase Chain Reaction (qPCR) using a OneStep ABI instrument

A reaction mixture contained 10 µL 2 x Fast SYBR® Green Master Mix (Thermo Scientific, USA), 2 µL template DNA (1:10 dilution), 900 nM forward primer, 900 nM reverse primer and 6 µL of H_2O in a total volume of 20 µL was prepared. Typically samples were denatured at 95 ^{o}C for 20 s and then subjected to forty heat cycles composed of 95 ^{o}C for 3 s and 60 ^{o}C for 30 s. The sequences of the primers are found in Table 2. The relative level of expression was calculated using comparative Ct method ($\Delta\Delta C_T$). This method involves comparing the Ct values of the samples of interest with a control or calibrated sample. The Ct values of both the calibrator and the samples of interest were normalised to the appropriate endogenous housekeeping gene, glyceraldehyde 3 phosphate dehydrogenase (G3PD).

2.8.4 Real-Time qPCR using a Roche LightCycler® 480 instrument

A 20 µL reaction contained 10 µL 2x LightCycler® 480 SYBR Green 1 Master mix (Roche Diagnostics, UK), 5 µL template DNA (1:25 dilution), 2 µL of 0.5 µM forward primer, 0.5 µM reverse primer (final concentration 1 µM) and 3 µL of H_2O was prepared. As the polymerase requires hot start to activate the samples were pre-incubated for 600 s with a ramp of 4.4 ^{o}C until it reached 95 ^{o}C. After which, a two-step amplification was undertaken: 95 ^{o}C

for 10 s (ramp 4.4 ºC) followed by 60 ºC for 30 s (ramp 2.2 ºC) this was repeated for forty-five cycles. The primers utilised across the screens are found in Table 3 . The relative level of expression was calculated using $\Delta\Delta C_T$.

2.9 Identification of Viral biomarkers

2.9.1 Polymerase Chain reaction (PCR) using Phusion® High-fidelity DNA polymerase

Standard PCR reactions were performed in a total volume of 20 µL. All the reaction components were mixed and centrifuged before use and assembled on ice, before being quickly transferred to a thermocycler. The standard reaction followed the manufacturer's guidelines to generate a 20 µL reaction using 1 µL (< 250 ng) of template DNA, 4 µL of 5x Phusion GC buffer, 0.4 µL dNTPS (200 µM), 1 µL of forward and reverse primers (0.5 µM), 0.6 µL of DMSO (3% conc.) and 0.2 µL of Phusion DNA polymerase (New England Biolabs Ltd, UK). Following denaturation at 98 ºC for 30 s the samples were subjected to 20-35 cycles at 98 ºC for 10 s, 50-65 ºC for 30 s and 72 ºC for 30 s to 1 min. The products were stored at -20 ºC until required.

2.10 Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the effect of STOMP® and LASER® on the germination percentage in characterized populations of black-grass, to determine the effect of Atlantis® WG and Cheetah® Gold on the percentage damage following spray trials, and to determine the expression of GSTF1 in an ELISA. Statistical tests were conducted on populations which were in technical triplicate, containing twenty plants per replicate. Statistical significance was determined at the $p < 0.05$ level and significantly different values distinguished using Tukey's test. All statistical analyses were completed using Minitab, version 17 Statistical software.

Table 2 Real-Time qPCR primers used in screens of *Alopecurus myosuroides*.
24 primers used preliminary qPCR screen against three known populations of *Alopecurus myosuroides* on ABI OneStep. Xenome specific primers taken forward indicated with * symbol. Highlighted indicate the sequences which were not compatible with the Roche Lightcycler instrument.

Phase in Xenome	Description	Contig	Length	Forward	Reverse Primer
Phase 1					
	Cytochrome P450	R00041432	665	AAGCACCCCAATGCCTTGT	TCGCGAAGTTCTGCCCTATG
	Cytochrome P450	R00030509	632	AACCGCGGACGTGATCTC	AGACCTTTTTCCCTTCCGTGTAG
	Cytochrome P450*	R00027925	317	TGATTCTTCAGAGGTTCTCCTTCTC	GGGCCGCAGCGTGAT
	Cytochrome P450	R00027289	831	ATGGCTGCATCCACCATGT	AGAGTGACGATGATGGCAAGTTC
	12-oxophytodienoate reductase*	R00232027	331	GGTACCTCATCGAGCAGTTCCT	TTTTCAAGGCTACCACCGTACTC
	12-oxophytodienoate reductase*	R00052459	579	GGGCCGCAGATTAGTTTTGA	GTCATCGACTATCCCGGGAAT
	12-oxophytodienoate reductase	Rm00002116	572	ATCCGTCTCTCCCCCTTCAC	TCGTTGAGCACGGTAGACATG
Phase 2					
	Glucosyltransferases* (ZUGT)	R00029215	1037	GCAGCAAGCAGAGGTTCATCT	TCGCCGGACTCTGCAAAT
	Glutathione transferase (GSTU6-like)	R00007921	539	GCCAACAAGAGCGAGCTTCT	TGGATGAGCACCGGTATCTTC
	Glutathione transferase (GSTU6-like)	R00030700	938	ACTCCCTCGGGTATCTCGATCT	GACCGTCATGCCGAACATC
	Glutathione transferase (GSTU6-like)	R00005793	624	TTTGTCAGCAGGGTGAAACTTG	TTGTGCACCGGGTTGGA
	Glutathione transferase* (GSTU6-like)	R00096975	322	TCCCTGGTCATCGTGCAGTA	GGGTCGGAGGAAAGCAATG
	Carboxylesterase	R00029421	578	GGCGAGCTCGAGTTCTACGA	AGCTCCTTGGCGGCATTC
	Cellulose synthase (Glucosyltransferases)	R00029959	1151	ACGTGGATTGCGACATGTTC	CAGCAGCAGGCACATAGCAT
	Aminotransferase	R00010869	1998	GCATTGCAACCGTTTGTTGT	TGTATCTTCTTTGCCTGGTTGACT
	GSTF1*	GSTF1	238	AGCATAAGAGCCCCGAGCAC	CCGTCCTGGAAAGCAGGGATTTG
Phase 3					
	ABC transporters*	Rm00043661	756	TGTGGTGCAGGAAATGGTATTTT	TGGTCTGCTGCCCTGCAT
	MATE efflux family protein*	R00030815	854	CCTTCACCATCCTCCTCAACA	GCCATCCTGACCCAATCG
Phase 4					
	Thiol methyl transferase*	R00000345	1110	ACCCTCATGTACCTGCCTCAA	TCGAGCACCGTGGTGTTGT
	Thiol methyl transferase	Rm00004119	734	CCCTCATGTACCTGCCTCAAG	CATAGTCGAGCACTGTGGTGTTG
	Thiol methyl transferase	Rm00016513	428	ACCCTCATGTACCTGCCTCAA	TCGAGCACCGTGGTGTTGT
Miscellaneous					
	Pathogenesis related protein	R00029303	701	ACAGTCTCATCAACGAAGTCTTAGCTA	GTGGCGTGTCGAAGTGGAA
	Pathogenesis related protein*	R00003857	1318	GCTTCGCCATCGAGGTGAT	GTACCCCCAGTGACGGAACTT
	Gag-pol retrotransposon	R00004163	659	AGATCGTCGAGTATCAACCGTATG	TTTGACGTTCCGCCTTAAGAG

Table 3 Real-Time qPCR primers used on the Roche Lightcycler 480 instrument for a xenome screen of *Alopecurus myosuroides*.
Ten primer sets optimised for screening populations of black-grass and stress populations of black-grass. Populations with * indicate those which had overlapping coding regions. Highlighted sequences indicate the primer set sequences which are different from table 1.

Phase in Xenome	Description	Contig	Length	Forward	Reverse Primer
Phase 1					
	Cytochrome P450	R00027925	317	TCCCCTAAGTACGTCCATGC	CTACATCTCCGGGCTCTTCA
	12-oxophytodienoate reductase*	R00232027	331	GGTACCTCATCGAGCAGTTCCT	TTTTCAAGGCTACCACCGTACTC
	12-oxophytodienoate reductase*	R00052459	579	GGGCCGCAGATTAGTTTTGA	GTCATCGACTATCCCGGGAAT
Phase 2					
	Glucosyltransferases (ZUGT)	R00029215	1037	GCAGCAAGCAGAGGTTCATCT	TCGCCGGACTCTGCAAAT
	Glutathione transferase (GSTU6-like)	R00096975	322	TCCCTGGTCATCGTGCAGTA	GGGTCGGAGGAAAGCAATG
	GSTF1	GSTF1	238	AGCATAAGAGCCCCGAGCAC	CCGTCCTGGAAAGCAGGGATTTG
Phase 3					
	ABC transporters	Rm00043661	756	TGTGGTGCAGGAAATGGTATTTT	TGGTCTGCTGCCCTGCAT
	MATE efflux family protein	R00030815	854	TCCACAACCTCTCTGTGCTG	TGGGAACTCCGACCAAGTAG
Phase 4					
	Thiol methyl transferase	R00000345	1110	ACCCTCATGTACCTGCCTCAA	TCGAGCACCGTGGTGTTGT
Miscellaneous					
	Pathogenesis related protein	R00003857	1318	GCTTCGCCATCGAGGTGAT	GTACCCCCAGTGACGGAACTT

Chapter 3. Characterisation of Herbicide Resistance

3.1 Classical Characterisation of Herbicide Resistance in Grasses

3.1.1 Introduction

As established in Chapter one there are various mechanisms by which resistance can arise in populations of wild grasses such as *Alopecurus myosuroides* (black-grass). As a pre-requisite to this project it was essential to ascertain the best method for identifying resistance in populations of black-grass and other wild grasses. The techniques and methodologies utilised were those which are commonly used by researchers in the agronomic sector. The techniques were trialled in both seedlings and plants to establish a standard protocol for germination and detection of resistance. Initially, three characterised populations of black-grass were used to establish a baseline for future phenotypic comparisons of resistance. The populations were: Peldon 07, Notts 05 and Broadbalk 07, which have been characterised as MHR (multiple herbicide resistant), TSR (target-site resistant) and WTS (wild-type susceptible) respectively (Brown et al., 2002, Hall et al., 1997). Subsequently, the protocols developed were implemented in larger "blind studies" of black-grass populations, as well as screenings of other wild grasses, namely *Avena fatua* (Wild-oats) and *Lolium rigidium* (Rye grass). Through these techniques a general protocol for defining resistance was achieved, however it did not explore the mechanisms and pathways which provided the resistant traits. These were explored further through an "omics" approach (Chapter 4 and 5). As a final method of MHR screening, the detection of GSTF1 was used as a marker for herbicide resistance through screening by immunoblotting an anti-*Zm*GSTF1 antiserum (Cummins et al., 1999). The over-expression of the plant specific phi class protein GSTF1 is known to be related to the constitutive expression of metabolically evolved herbicide resistance.

3.2 Rothamsted Rapid Resistance Test

3.2.1 Introduction

A Petri-dish technique was introduced in 1999 for the identification of herbicide resistance to arloxyphenoxypropionate "FOPs" and cyclohexanedione "Dims" in grass weeds black-grass, wild oats and rye grass (Moss, 1999b). The Rothamsted Rapid Resistance test was the quickest and simplest test which addressed the issue of breaking dormancy, a common limiting factor on the growth of wild grasses in time, light and temperature controlled environments. This test was limited however, to herbicides with modes of actions (MoAs) from Groups 1 and 3 (HRAC, 2005, WSSA, 2004).

The method was adapted to test for resistance in three known populations of black-grass sourced from Rothamsted (Hertfordshire, UK): Peldon 07 (MHR), Broadbalk 07 (WTS), Notts 05 (TSR) and ten unknown populations (Table 4). The ten populations were investigated in a blind study" to ascertain their levels of resistance in comparison to the three known populations of black-grass. The populations were exposed to STOMP® Aqua and LASER® at a concentration of 5ppm. In each case, to improve the uniformity of germination the seeds were treated for two weeks at 30 ºC to break dormancy. The commercial herbicides selected had different MoAs and were specifically selected for their growth inhibiting effect. STOMP® Aqua was implemented as it's the most concentrated straight pendimethalin containing product (455 g/L). First introduced to the market in the 1970s, STOMP® Aqua was commonly used for pre and post-emergence weed control in a wide range of crops. It has the capacity to inhibit root growth through the prevention of cell elongation and division, specifically microtubule assembly. The inclusion of this MoA provided a good general metabolism assessment of the plant's capacity to survive under a less targeted approach than that of LASER®. The inclusion of LASER® enabled the populations of black-grass to be exposed to cycloxydim, a known inhibitor of Acetyl-CoA Carboxylase (ACCase). This MoA belongs to Group 1 which includes the chemical families Cyclohexanedione ('Dims') and Arloxyphenoxy-propionate ('Fops'). These chemicals are responsible for the inhibition of meristematic growth through the prevention of the first step in fatty acid biosynthesis. For the identification of TSR the ACCase inhibiting herbicide would prove invaluable.

3.2.2 Screen optimisation using- three known populations of black-grass

The primary focus of the resistance screen was to utilise three characterised populations of black-grass originating from different locations in the UK, with varying degrees of resistance, to confirm the detection capacity of the assay. Brown et al.(2002) and Moss et al.(2003) identified a novel ACCase codon alteration at position 1781 (isoleucine to leucine) enabling the population Notts 05 to survive treatment with actives from the chemical family

cyclohexanedione “Dims” (Délye et al., 2002). Subsequently, this population was used as an indicator for target-site resistant (TSR) population. At the upper limit of resistance the population referred to as Peldon (07) was used, as it is characterised as multiple herbicide resistant (MHR). The population was identified as MHR in 1982 (Moss, 1992) and was used as a key marker for enhanced metabolism of xenobiotics, with the coupled capacity to resist damage to ALS herbicides. Population Broadbalk (07) is a known standard for susceptibility, as it has never been treated with herbicides thereby making it a suitable reference population for comparative purposes as a marker for wild-type susceptibility (WTS) (Marshall and Moss, 2008).

The detection techniques aimed to identify two of the principal biochemical mechanisms conferring resistance, an alteration in the target site enzymes (ACCase or ALS) reducing sensitivity to the herbicides and, an increase in the rate of herbicide detoxification (enhanced metabolism).

3.2.2.1 *Results of three population screen*

The initial test focused on utilising the three known populations to ensure the method was optimised and could be used in comparable tests. Figure 11 provided a good indication that the three known populations of black-grass behaved in accordance with previous literature findings. Notts 05 was the only population to show no significant decrease in germination between treatment with LASER® and control. This therefore confirmed that Notts 05 could be used as an indicator of TSR as it was insensitive to treatment with the ACCase inhibiting herbicide. Notts 05 was significantly different (p value 0.000) to the populations, Broadbalk 07 and Peldon 07 when exposed to LASER®. This result confirmed that Notts 05 possessed a genetic mutation enabling it to resist cycloxydim. In contrast, Peldon 07 and Broadbalk 07 germination percentage decreased significantly (p value =0.005) following treatment with LASER®. This indicated that the two populations did not contain the same mutation as Notts 05, or have the enhanced capacity to metabolise the xenobiotic.

Following exposure to STOMP® Aqua, Peldon 07 and Broadbalk 07 were not significantly affected in comparison to control treatment. There was a significant difference when comparing the treatment of LASER® and STOMP® Aqua in Notts 05, as germination decreased significantly following treatment with STOMP® Aqua. The inclusion of pendimethalin is associated with its use as a selective pre- and post-emergence herbicide. The occurrence of resistance to dinitroanilines remains relatively uncommon, however it has been identified in *Eleusine indica (Goosegrass) and Echinochloa crusgalli (Barnyardgrass). The*

resistance is a result of a point mutation in major microtubule cytoskeletal protein, α2-tubulin (Anthony and Hussey, 1999, Anthony et al., 1998). From the results of this assay it is possible to suggest that Peldon 07 may possess a mutation which enables it to survive treatment more successfully than Notts 05. Further studies in *Setaria* have suggested that the mutation conferring resistance to dinitroanilines has associated fitness costs. *Setaria* are less adapted to grow with crop canopies resulting in receiving less light. Seed weight is also lighter making them less suitable for development in adverse conditions (Darmency et al., 2011). Therefore, there is a possibility that Peldon's incapacity to metabolise LASER® is linked to an associated fitness cost, as microtubule polymerisation and cell division are likely to impact growth and reproduction. Although, currently there is no evidence of genetic studies in black-grass and its associated mechanism of detoxifying dinitroanilines.

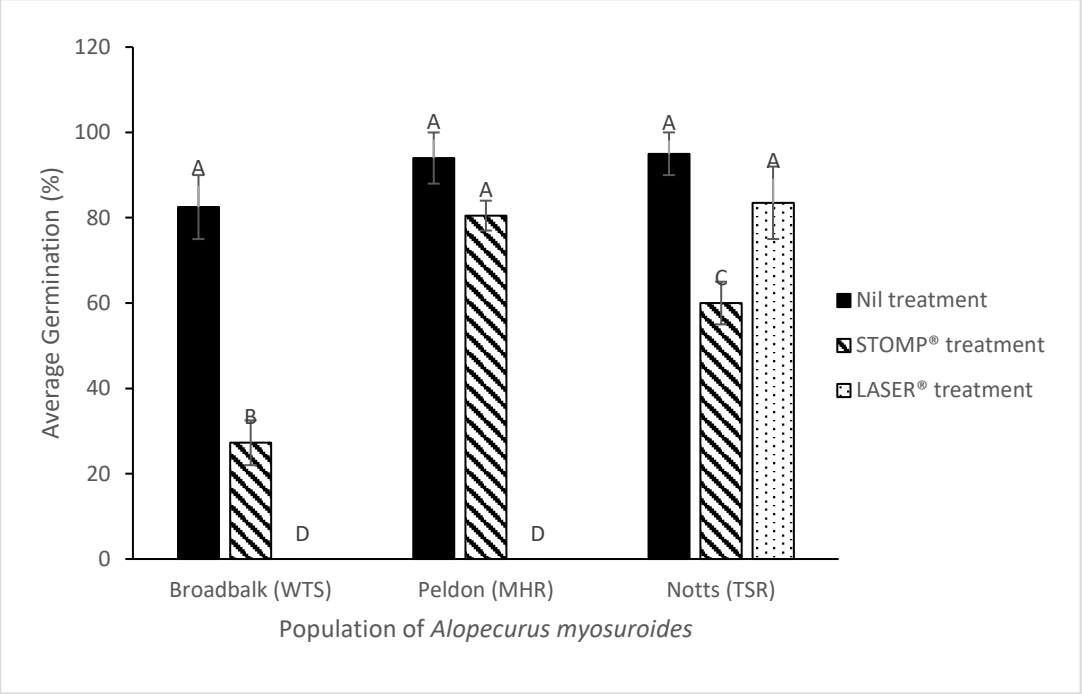


Figure 11 Bar chart of percentage germination in nil treatment vs. treatment with LASER® and STOMP® in three *A.myosuroides*.
Nil treatment ((potassium nitrate (2g/L)) vs. LASER®and STOMP® Aqua (5ppm) two weeks after treatment. Three populations: Peldon (MHR), Broadbalk (WTS) and Notts (TSR). (n = 3 replicates per population each replicate containing 20 plants). ANOVA analysis was performed, bars with different letters indicates significant difference at ($P < 0.05$).

Population Broadbalk (07) demonstrated a 55 % loss in germination following exposure to STOMP® Aqua and total loss in the presence of LASER®. These results show characteristics associated with susceptible populations, hence its annotation as WTS. The results from this initial screen confirmed the resistance traits in all three populations as either: MHR, TSR or WTS.

3.2.3 *Screening of unknown populations of black-grass*

The subsequent experiment focused on assessing the levels of resistance in a "blind study" containing ten populations of black-grass. The populations were sources from Rothamsted Research, UK, the study was carried out under the same conditions as that of the original three population screen. The intention was to compare the unknown populations with the three standard populations: MHR, WTS and TSR and assign a resistant trait.

3.2.3.1 *Results of ten unknown populations screening of black-grass*

Figure 12 indicates that across the ten populations there is a large level of variation in resistance which is illustrated by the differing relationships seen within and between populations following the treatment with herbicides. In Figure 12, Roth 09 is significantly (P value=0.000) decreased following treatment with STOMP® which is denoted by the association of a different letter (A-Roth 09) to the control/ nil treatment (D). This result confirms that it is a WTS biotype of black-grass. Figure 12 illustrates the two populations Peldon 05 ss (MHR) and Suffolk 09 labelled C, are significantly different to their respective control (D) following treatment with STOMP®, although not to one another. The two populations despite being significantly different to their controls, retained approximately 71% germination after treatment with STOMP®. Interestingly, they are also significantly different to Roth 09 (WTS), Kent1 02, and Notts 05 (TSR), as illustrated by not containing C in their associated label in Figure 12.

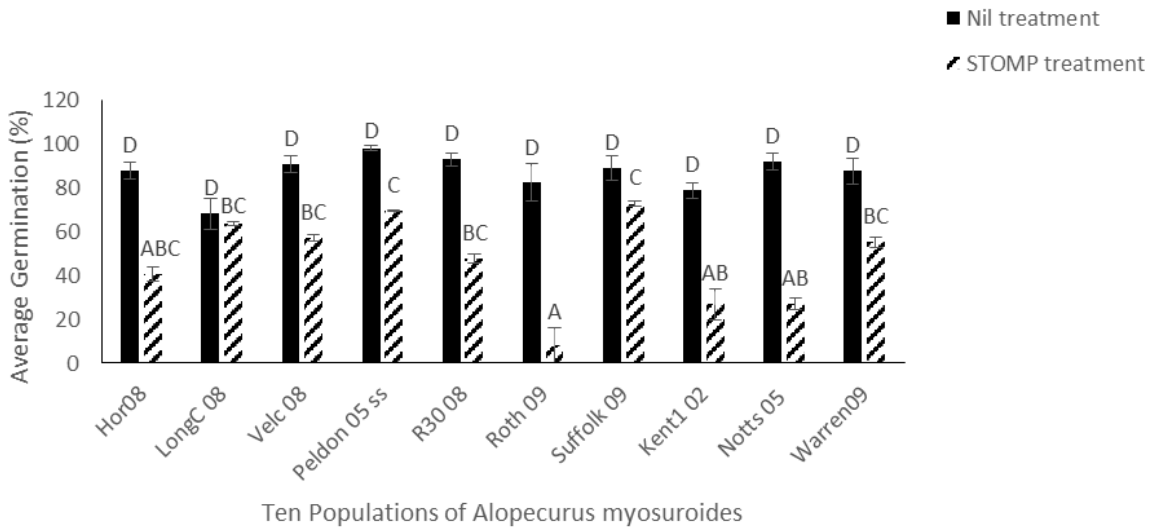


Figure 12. Bar chart of percentage germination in control vs. treatment with STOMP ® Aqua in ten populations of *A.myosuroides*.
Nil treatment ((potassium nitrate (2g/L)) vs. STOMP ® Aqua (5ppm) two weeks after treatment. (n = 3 replicates per population containing 20 plants). ANOVA analysis was performed, bars with different letters indicates significant difference at (P <0.05).

Whilst in contrast: R30, Warren, Velc and LongC are not significantly different to one another, and are not significantly different to Peldon or Suffolk (labelled with C). This result indicates that there is a clear difference between the TSR and WTS populations to those which could be termed temporarily as MHR, based on their similar response to treatment of pendimethalin as Peldon 05 ss (MHR). It was essential to compare these results, to those in response to treatment with cycloxydim (LASER®) to enable the populations to be termed: TSR, WTS or MHR.

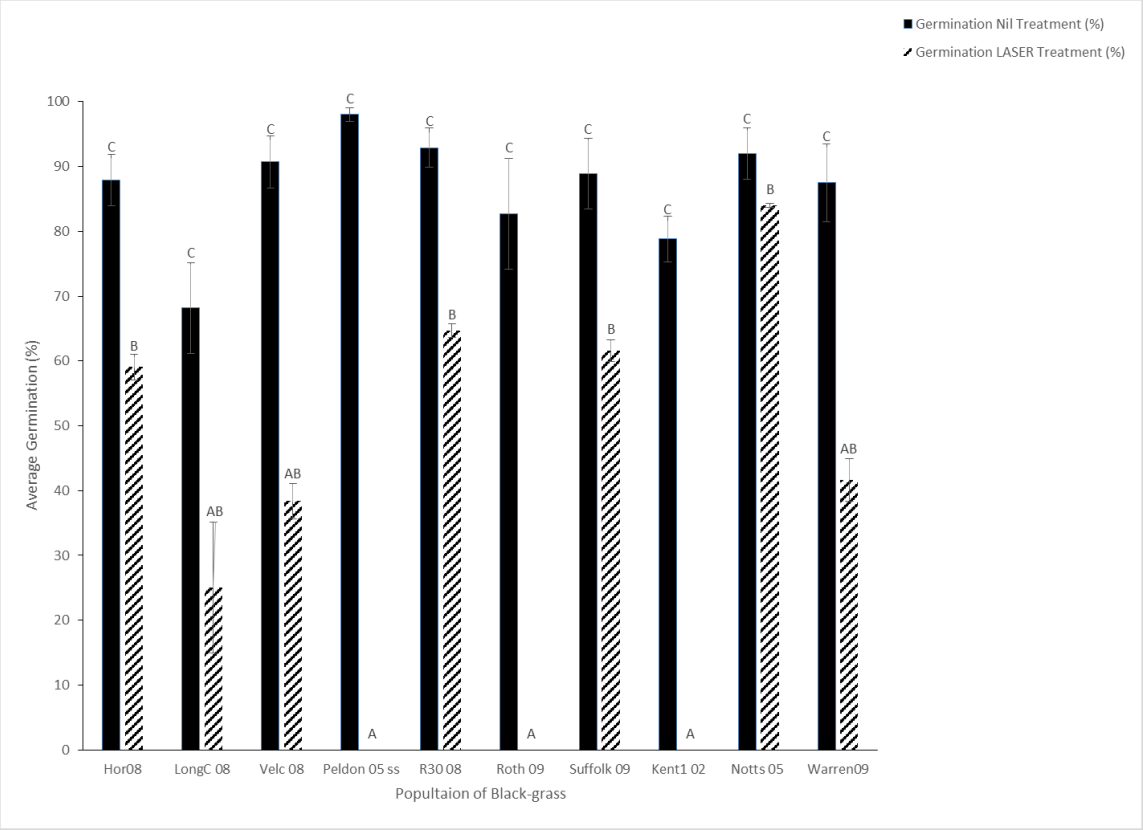


Figure 13 Bar chart to show the germination assay results with ten populations of *A.myosuroides* following treatment with LASER ®.
Nil treatment ((potassium nitrate (2g/L)) vs. LASER® (5ppm) two weeks after treatment. (n = 3 replicates per population containing 20 plants). ANOVA analysis was performed, bars with different letters indicates significant difference at (P <0.05).

Figure 13 highlighted that Roth 09, Kent1 02 and Peldon 05 ss had 100% loss in germination following treatment with LASER® and were significantly different to their controls (p value 0.000). The three populations were also significantly different to Hor 08, R30 08, Suffolk 09 and Notts 05. These four populations decreased in percentage germination but were the least affected, especially Notts 05, with 12% loss in germination. This result indicated that Notts 05 possessed a clear trait correlating to TSR in the presence of LASER®. Although, the populations LongC 08, Velc 08 and Warren 09 were not significantly different to those at either end of the resistance scale in this treatment. Interestingly, these populations were

potentially more herbicide resistant than the remaining seven populations in the presence of LASER®.

In conclusion, the ten populations and their treatment with LASER® and STOMP® Aqua in the Rothamsted rapid resistance test provided an indication of their resistance traits. Hor 08, LongC 08, R30 08, Velc 08 and Warren 09 appeared to be resistant to both MoAs and were temporarily termed MHR. Notts 05 still indicated the highest level of resistance to cycloxydim. Roth 09 was the most susceptible overall and Peldon 05 ss indicated resistance to pendimethalin but not cycloxydim. These results only provided a snapshot of the resistance traits that the populations possess. Therefore, a further series of analyses were required which would look at different MoAs through the optimisation of the Syngenta Quick Test (Boutsalis, 2001).

3.3 Syngenta Quick test

In order to gain a better understanding of the resistant traits that the wild grasses possessed it was also essential to carry out the Syngenta Quick-test (Boutsalis, 2001). This allowed the known and unknown populations used in the Rothamsted rapid resistance test to be assessed against group 2 MoAs, The culmination of the results from the two methods, enabled the preliminary classification of the populations as either TSR, MHR or WTS. The results however, were limited as they merely indicated the presence of resistance based on phenotypic changes which were qualitatively assessed rather than quantitative. The Syngenta quick test utilised the commercially available herbicides Atlantis® WG and Cheetah® Gold in a greenhouse experiment. They were applied to the known and unknown populations at doses above and below the recommended field rates.

The primary objective was to investigate the populations' response to herbicides which would act on different sites of action. The herbicide Atlantis® WG which acts on Acetyl-lactase synthase (ALS) enzyme, contained idosulfuron and mesosulfuron which belongs to the Group B chemical family of sulfonylureas. Cheetah® Gold contained: dicolfop, fenoxaprop and sethoxydim which belong to the chemical families Cyclohexanediones ('dims') and Aryloxyphenoxypropionates ('fops') constituents of Group A. All of which act on the ACCase enzyme. The data from the spray trial was coupled with the results from the petri dish assay, and the findings from literature to provide resistance traits for the populations.

3.3.1 Results

The ten unknown populations of black-grass were sown in triplicate and exposed to five concentrations of two herbicides including a nil control. The two herbicide treatment groups

were isolated in separate glasshouses and plants were assessed based on percentage damage: at 7, 14 and 21 days after treatment (DAT). Included in the spray trial were the three known populations Peldon (MHR), Notts (TSR) and Broadbalk (WTS) to enable a point of comparison. The concentrations used for Atlantis®WG were: 0,100, 400, 800 and 1600 g/Ha, which included the recommended field rate for control of 400 g/Ha. Cheetah® Gold was applied at 0, 0.625, 2.5, 10 and 20 l/Ha, which did not specifically include the field rate of 1.25 l/Ha. Instead a half field rate and doses of double and above were used for Cheetah® Gold.

The reason for these high application rates is linked to the increasing dependency on chemical control, and the limited chemistries available in the agrochemical industry. Atlantis® WG, is becoming increasingly relied upon as it has provided good control of black-grass and rye-grass which are an increasing problem within the UK. It was essential that the ten populations were exposed to herbicides which belonged to different chemical families than those used in the Rothamsted Resistance test. The reason for this was to ascertain a greater degree of certainty concerning their resistance traits.

When the percentage damage was assessed at 7, 14 and 21 DAT the plants phenotypic changes were compared to their respective nil treatments. Also, the damage as a result of herbicidal treatment was investigated in the known populations which enabled the unknown populations to be associated with MHR, TSR or WTS traits. In Figure 14 the three known population traits WTS, TSR and MHR are shown respectively in response to the field rate of Atlantis® WG and double the field rate for Cheetah® Gold. The images indicate that for the populations Broadbalk 07 also termed Roth 07, and Notts 05 are severely damaged 21 DAT with Atlantis at field rate. This visually indicated that these two populations were not resistant to ALS acting herbicides. In contrast, Peldon 07 appeared phenotypically unaffected 21DAT with Atlantis® WG. There has been confirmed resistance to ALS inhibitors iodosulfuron and mesosulfuron (‘Atlantis’) in 293 populations of *A.myosuroides* in the UK as of 2008 (Moss et al., 2009). Therefore, it was proposed that this Peldon 07 population must contain a codon mutation of Pro197Thr conferring ALS resistant trait (Moss et al., 2009, Hull et al., 2008).

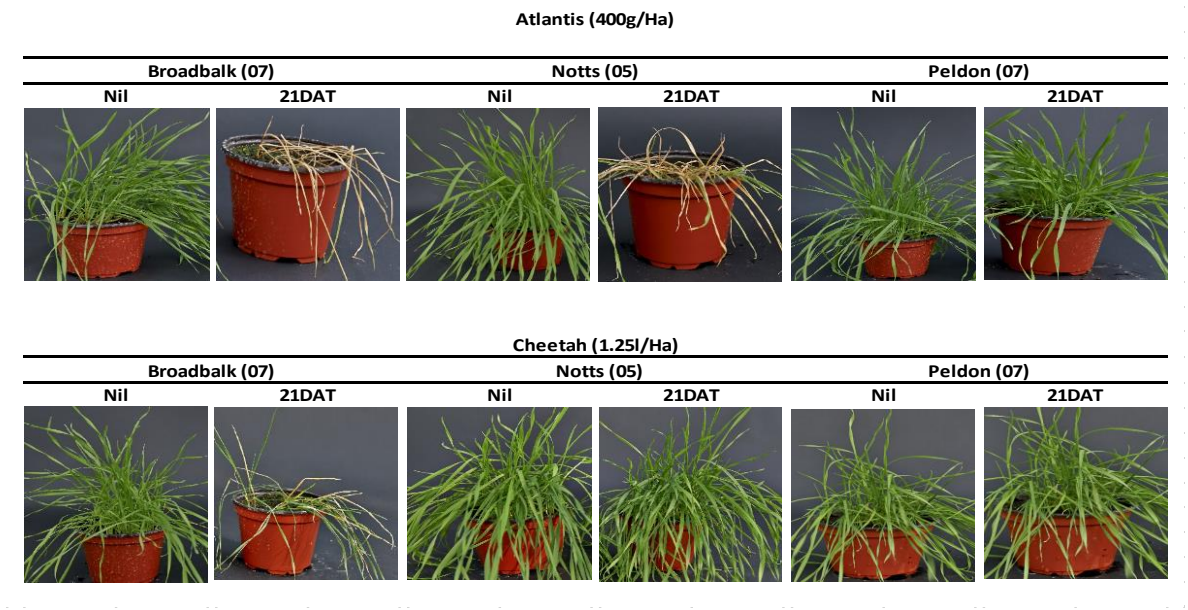


Figure 14. Spray trial images of *A.myosuroides* three known biotypes following exposure to herbicides.
Images demonstrate the visual damage encountered in Broadbalk (WTS), Notts (TSR) and Peldon (MHR) black-grass following exposure to Atlantis (400 g/Ha) Cheetah (1.25 l/Ha), standard field rates 21 days after treatment (DAT) in a glasshouse.

Figure 14 also illustrates that the only population affected by both commercial herbicides is Broadbalk (07), which meant that the resistant trait WTS was assigned to the population. Contrary to this, population Peldon (07) appeared unaffected 21 DAT after treatment with both commercial herbicides. This result indicated that the population had the capacity to detoxify herbicides from differing chemical classes confirming the trait MHR. Finally, Notts (05) showed resistance to the action of Cheetah® Gold on the enzyme ACCase, but remained susceptible to the action of Atlantis. This phenotypic response correlated with the TSR trait.

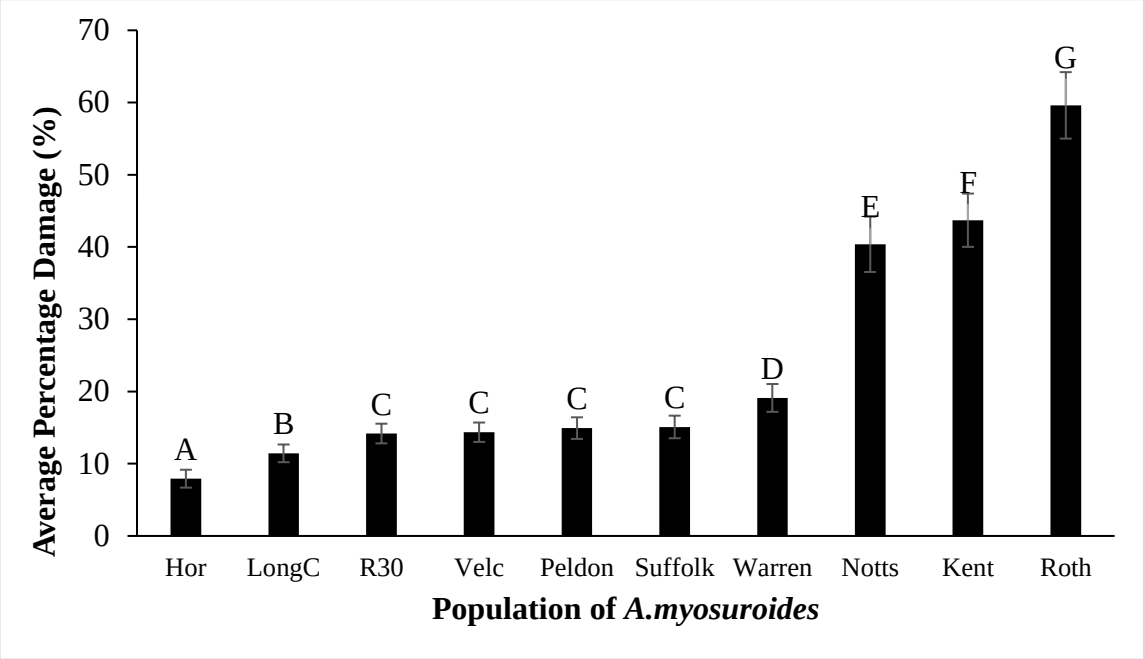


Figure 15 Graph to show the relationship between location and the average percentage damage to ten populations of *A.myosuroides* after treatment with Atlantis® WG.
The relationship between ten populations and the average percentage damage over time across all treatments with Atlantis. (n = 3 replicates per population containing 20 plants). ANOVA analysis was performed, bars with different letters indicates significant difference at ($P < 0.05$).

In Figure 15, it is clear to see that there is an increase in the overall level of damage displayed in the populations Notts, Kent and Roth irrelevant of concentration or days after treatment. All three were affected significantly differently to each other. There was an expectation that these three would be the most damaged, especially populations Kent and Notts. As they did not possess the target-site alteration capable of resisting ALS inhibiting herbicides (see Table 4). Interestingly, R30, Velc, Peldon and Suffolk were not significantly different to one another (Figure 15). In reference to Table 4, the characteristics displayed by Velc, Peldon and Suffolk were similar to each other as they contained the highest percentage of ALS mutations (32.5 - 50 %). The only odd population was Suffolk, which despite not containing any pre-identified ALS mutations (Table 4) displayed 67 % resistance 21 DAT with Atlantis® WG. This result indicated that the population must have had the enhanced capacity to detoxify ALS acting herbicide. The most resistant populations were Hor and LongC in Figure 15 which in reference to Table 4 contained 50 % and 42 % ALS mutations respectively. These results correlated to the expected behaviour. The two populations were not just significantly different to the remaining eight but also to each other. This result, highlighted the level of variation that exists between different populations especially those from different geographical locations. The starkest contrast is the difference between Hor and Roth, which had an average difference in damage of nearly 50 %. This result merely confirmed the assumption that they were at

opposite ends of the resistance trait spectrum. Therefore, Hor was labelled as MHR and Roth as WTS. Figure 15 also highlighted, that of the populations which were not labelled TSR (Notts) or WTS (Roth and Kent) less than 50 % damage was achieved even at four times the field rate application or 21DAT. This result could be associated with the "loss of chemical control" as a result of high dependency on limited chemistries. This has been noted to place high selective pressures on populations of *A.myosuroides*. Therefore, the herbicidal treatment history of these populations would provide a better idea of whether Atlantis® WG had been extensively used. Thereby, placing a selective evolutionary pressure on the resistant populations, or the likes of Suffolk which previously had no known ALS mutations but demonstrated over 50 % resistance.

Table 4 Ten populations of *A.myosuroides* descriptions and associated resistance characteristics.
Characterised according to:*(Marshall et al., 2013), **(Moss, 2007), ***(Moss, 2014), include mutations X^{197} and Y^{574} in amino acid sequence, and includes mutations A^{1781},B^{2027},C^{2078} and D^{2041}. The presence of mutations is given as a percentage of selected populations with the stated mutations. The shaded columns indicate previously published data and data from Rothamsted Research, Hertfordshire whilst the unshaded is data from this research.

Population	County	Location	ALS Mutations (%)$^{X \& Y}$	ACCase Mutation (%)^{A,B,C,D}	Enhanced Metabolism	Lethal Dose Atlantis (400g/Ha) % damage 21 DAT	Lethal Dose Cheetah (1.25l/Ha) % damage 21 DAT
Roth 09**	Hertfordshire	Broadbalk	0	0	N	85	100
LongC 08*	Oxfordshire	Chalgrove	42	8.5	Y	28	15
Notts 05*	Nottinghamshire	Notts	0	22.5	Y	81	6
Kent1 02***	Kent	Kent-Survey	0	0	Y	75	13
Hor 08*	Oxfordshire	Oxford	50	25	Y	16	28
Suffolk 09***	Suffolk	Suffolk survey	0	27.5	Y	33	17
Peldon 05**	Essex	Peld02 Sulfolk survey	50	0	Y	25	17
Velc 08*	Lincolnshire	Velcourt	32.5	31.5	Y	25	10
Warren 09	Bedfordshire	Conts Atl G/Ha60	31.5	18.75	Y	49	21
R30 08*	Cambridgeshire	Huntingdon	45	27.5	Y	25	15

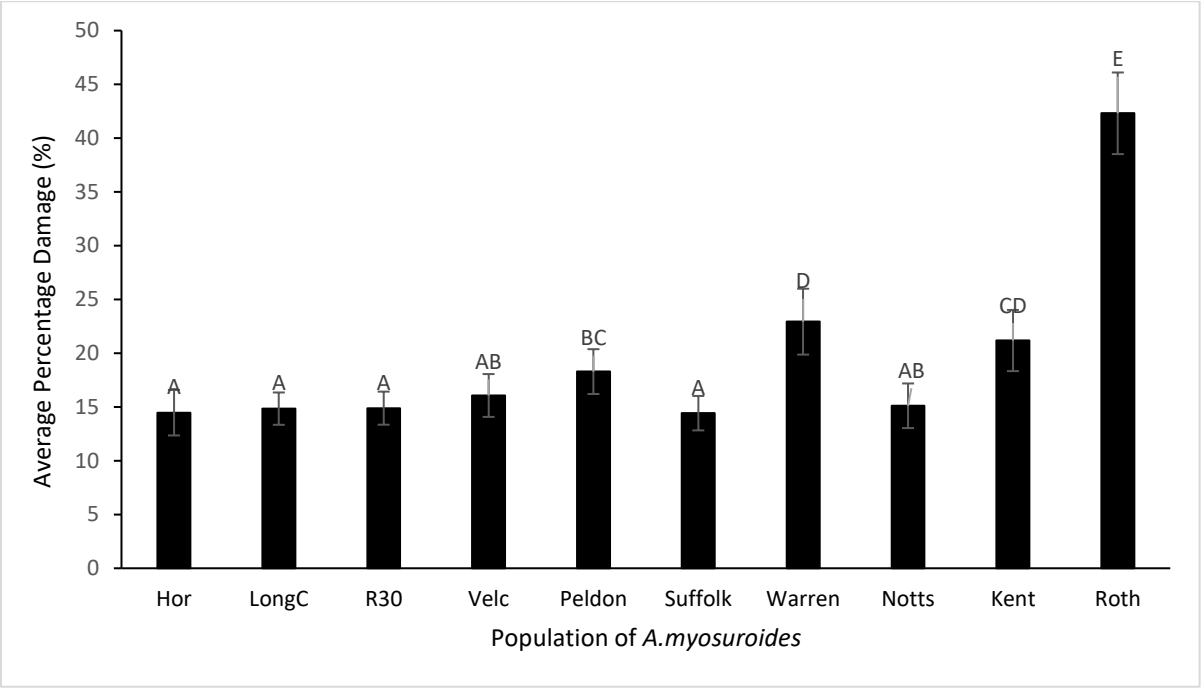


Figure 16. Bar chart to show the relationship between location and percentage damage to ten populations of *A.myosuroides* after treatment with Cheetah® Gold.
Relationship between ten populations and average percentage damage irrelevant of concentration or time. (n = 3 replicates per population containing 20 plants). ANOVA analysis was performed, bars with different letters indicates significant difference at ($P < 0.05$).

Table 4 indicated that Roth showed 100 % damage at field rate application from Cheetah® Gold 21 DAT. Whilst, Figure 16 confirmed that Roth remained the most susceptible population of black-grass regardless of the concentration or the time following treatment with Cheetah® Gold. This result combined with those from Atlantis® WG, STOMP® and LASER® indicated that this population was a viable marker of WTS and was subsequently used as a susceptible standard in the screen with ten unknown populations. Interestingly, when the data from the screens with Cheetah® Gold and Atlantis® WG were compared the populations: Hor, LongC, R30, Velc and Peldon appeared resistant to both MoAs. This result was later confirmed by the data in Table 4 with all but Peldon having ALS and ACCase mutations. These results meant that all the populations could be temporarily termed MHR as they had demonstrated a capacity to detoxify actives from different chemical classes (groups 1, 2 and 3). Although, of the five populations it could be hypothesised that Peldon may have acquired the capacity to metabolise Cheetah, as studies by Moss (2007) hadn't previously identified any mutations in the codon which conferred resistance to ACCase (Table 4) but the population had shown 83 % resistance to Cheetah in the spray trial.

From the data in Table 4 the percentage mutations identified in previous studies correlated to data in Figure 15 and Figure 16, as Hor 08, LongC 08 and R30 08 presented the highest percentage of mutations for ALS and ACCase resistance and showed the lowest level of damage at field rate application for both Cheetah® Gold and Atlantis® WG. Further to this, these populations were also some of the least affected populations following treatment with STOMP® and LASER®, which indicated that they potentially were more representative of MHR than Peldon.

3.2 Immunoblot detection of Resistance

The screened samples were used in a subsequent metabolic analysis which was carried out to aid a quantitative analysis at Agriculture, Food and Rural Development, Newcastle University. A final method of analysis utilised a protein approach which focused on the over-expression of GSTF1 by immuno-blotting by western blot in the various populations of black-grass and other wild grass species.

3.2.1 Assessing the over-expression of GSTF1

The populations of black-grass from this study and other wild grasses (Rye-grass and Wild-Oats) were tested by immune blot detection by western blot to see if they expressed the protein GSTF1. GSTF1 was selected as a marker for MHR as glutathione transferases have been identified as playing a role in oxidative stress tolerance, in addition to detoxifying xenobiotics through the catalysed conjugation with glutathione (Cummins et al., 1999).The standard samples of black-grass were exposed to *Zm*GSTF1-2 and used in comparison western blots to establish if the unknown populations of black-grass expressed the plant specific phi class glutathione, and could therefore further confirm previous predictions about the populations resistance characteristics.

3.2.1.2 *Results*

The antiserum raised to maize (*Zea mays* L.) type I GST heterodimer *Zm*GSTF1-2 was exposed to crude protein extracts from black-grass populations Peldon (MHR) and Rothamsted (WTS). Figure 17 indicates the characteristic expression polypeptides of 28kDa, 27kDa and 25kDa. The lower polypeptide, 25kDa was detected in both populations, whilst the antiserum selectively recognised the upper polypeptides, 27kDa and 28kD. These were expressed at high levels in the herbicide-resistant (MHR), but not the herbicide susceptible (WTS) or plants showing herbicide resistance due to a modified target site sensitivity. This result correlated with Cummins et al. (1999) findings of Peldon and Rothamsted. The anti-

*Zm*GSTF1-2 serum was used in western blots of extracts from the ten unknown populations of black-grass plants with differing herbicide resistance traits.

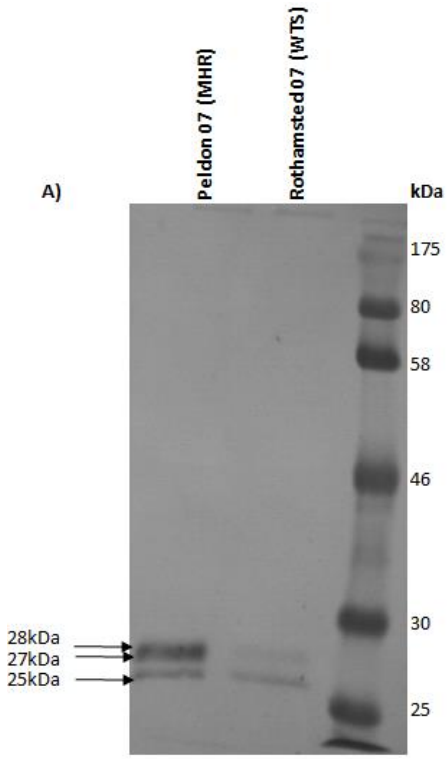


Figure 17. Western blot of crude protein extracts from Peldon (MHR) and Rothamsted (WTS) *A.myosuroides*.
A.myosuroides populations on a 12 % discontinuous gel were probed using antiserum *Zm*GSTF1-2 at a dilution of 1:1000. Samples loaded at 50 ng per 15 µL and ran alongside 10 µL pre-stained broad range protein marker 7-175 kDa (NEB, UK).

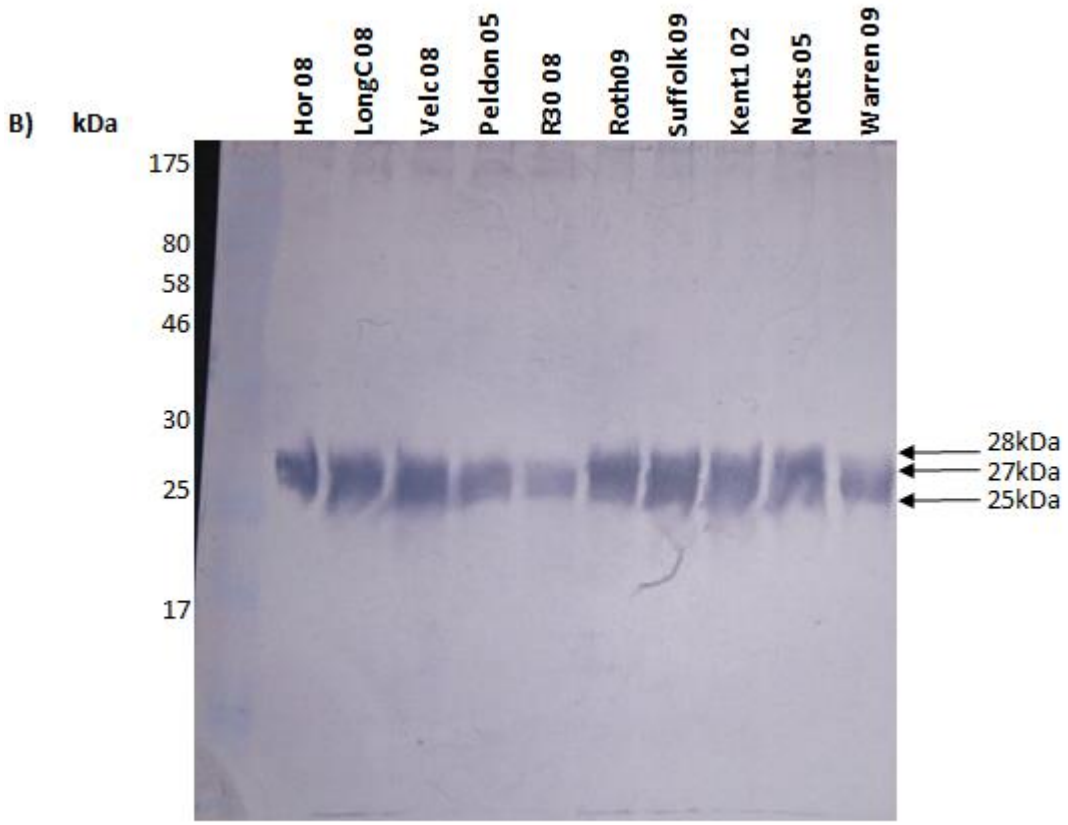


Figure 18 Western blot of crude extracts from ten black-grass populations of *A.myosuroides*. Ten populations of *A.myosuroides* with differing resistance characteristics were run on a 12 % discontinuous gel were probed using antiserum *Zm*GSTF1-2 at a dilution of 1:1000. Samples loaded at 50 ng per 15 µL and ran alongside 10 µL pre-stained broad range protein marker 7-175 kDa (NEB, UK).

In Figure 18 there wasn't a clear presence/absence relationship between the expression of the three polypeptides in the ten populations when probed with the *Zm*GSTF1-2 antiserum. This was not the predicted result. Populations Roth, Kent and Notts were expected to express only the polypeptide 25 kDa as were characterised as WTS and TSR respectively. Whilst the remaining populations had displayed characteristics which were associated with MHR trait so were anticipated to display the three polypeptides. However, these populations did not clearly over-express the upper polypeptides more than the TSR or WTS counterparts. This image indicated that the plant phi class glutathione transferase was expressed in all ten populations. This result meant that all the plants had the potential capacity to detoxify herbicides, due to the link between the associated up-regulation of GSTF1 and herbicide resistance (Cummins, 1999). Therefore, the results from this screen did not provide the expected insight into the resistance traits of the ten populations. As the result is qualitative, like those of the Syngenta spray trial it proved difficult to acquire a definitive answer for which populations were MHR, TSR or WTS. Interestingly, the populations that were predicted to show expression of only the lower 25kDa polypeptide: Notts 05, Kent1 02 and Roth 09 appeared to give a stronger

expression of this polypeptide than Peldon 05. The epitope recognised by the antibody is fixed; the populations may have proteins with different sequences but this seems unlikely due to the results in Figure 7. However, this result did indicate that the populations may simply have bound to this particular epitope more efficiently therefore, the ten populations needed to be subjected to a more specific antiserum which could specifically identify the protein *Am*GSTF1 to a larger extent than that of the antiserum raised from maize.

3.3 Discussion

This study on characterising herbicide resistance indicated the importance of a combined approach. A combined approach meant that the previous results regarding the ten populations' ALS and ACCase mutations could be phenotypically tested and compared (Marshall et al., 2013, Moss, 2007, Moss, 2014). Whilst the inclusion of a test based on immunoblotting was insightful, it was not as sensitive for *Am*GSTF1 as originally hoped. All these methods provided a qualitative picture concerning their resistance traits without the implementation of a quantitative genomic analysis.

The Rothamsted rapid resistance test (RRR) provided the quickest method of phenotypic analysis, as results were achieved after 2 weeks. However, the preliminary work to ensure uniformity in germination means that the test results realistically took 5- 6 weeks to be achieved. Therefore, as a research tool, the test was time and space efficient in comparison to the normal pot testing. The assay provided a good measure of the resistance the populations had. The assay had previously been optimised to detect resistance to 'Fops' and 'Dims' with the use of cycloxydim, an indicator of TSR (Moss, 1999b). Cycloxydim is not affected by enhanced metabolism which gives virtually complete resistance to all 'fops' and 'dims'. The overall advantage of using the petri-dish assay as a preliminary screen for resistance meant that results were generated in a relatively short time whilst providing an indication of the mechanisms present, although, one of the key issues with the existing method was the lack of including ALS acting herbicides. As a result, the populations had to be tested for ALS resistance using the traditional pot testing; Syngenta Quick test as currently the RRR test hasn't been optimised for the inclusion of ALS herbicides.

The Syngenta Quick test provided a better analysis of herbicide resistance to the two commonly used classes of herbicides, ALS and ACCase inhibitors. The preparation of this particular screen was essentially impractical as a research tool. It was more time and space consuming, especially for the number of populations and treatment conditions. The screen relied on the visual interpretation which was also a qualitative assessment. This method of

analysis is subjective since plants are naturally subjected to biotic stresses in the FERA glasshouses despite trying to control as many variables as possible. It is also worth noting that for some populations subjected to a single herbicidal treatment of varying concentrations, the plants initially appeared damaged however they later showed signs of recovery. This indicated that in some populations their rate of metabolising the xenobiotic was simply slower, but they possessed the natural capacity to detoxify the compounds. Despite screening various application concentrations, the approach commonly used in the field is a succession approach where pre and post-emergence treatments are used to control wild grasses in arable crops, so to reflect the conditions in the field it might have been better to use this approach. Therefore the results in this assessment are potentially biased to those with resistance to post-emergence herbicides. Whilst this technique was very useful for verifying the expected results of those seen in Table 4, the interpretation remained qualitative. A future analysis using pot testing as a method of characterisation might be altered to include, chlorophyll assessments, radio-labelled actives and GPOX activity assay, all of which could provide quantitative data thereby, enabling the resistance traits to be categorised depending on their behaviour towards differing MoAs, the rate at which they are metabolised and the pathways used to detoxify. Overall the phenotypic assessments of the populations identified that due to the high level of variation within and between populations some populations did not behave as predicted. For instance Warren 09, possessed 31.5 % ALS mutations yet was damaged by more than 50 % at field rate application of Atlantis® WG. The presence of variation was hypothesised to be linked to the fact that black-grass is a cross-pollinating species.

The final method of analysis therefore, relied on the implementation of an immunoblot based test. The intention was to screen the populations with the *Zm*GSTF1-2 antiserum and establish whether they were MHR, WTS or TSR depending on their expression of GSTF1. The western blot analysis using the antiserum raised in rabbits, to a maize antigen *Zm*GSTF1-2 on the known populations, proved somewhat diagnostic for MHR and WTS populations. The results indicated that there was a characteristic expression of 28 kDa, 27 kDa and 25 kDa polypeptides with the lowest polypeptide detected in both populations. Whilst the MHR population, when screened selectively recognised the upper polypeptides. The *Zm*GSTF1-2 antiserum appeared to be a useful antibody to detect GSTF1, a known gene which exerted a regulatory control on the metabolism of herbicides, conferring resistance (Cummins et al., 2013). When the antibody was used to screen the ten populations it was apparent that the antibody was not as diagnostic as originally anticipated. Therefore, there was strong indication that an antibody raised from MHR Peldon, would perhaps be more diagnostic and

prove a more viable biomarker for resistance in black-grass populations. Therefore, the next stage of the analysis was to focus on identifying a more specific antibody which was raised to *Alopecurus myosuroides*, rather than maize which is a homologous species.

To conclude, all the methods of characterising the resistance traits were independently useful but proved the most informative when the data was combined. The issues arising from the techniques highlighted the need for a genomic assessment of black-grass and a better, more selective antibody marker to detect multiple herbicide resistance. The techniques had identified that the petri-dish analysis was useful as a preliminary screen and would subsequently be used in combination with an immunoblot test in subsequent populations of wild grasses.

Chapter 4. Anti-*Am*GSTF1-serum

4.1 GSTF1 as a protein biomarker for herbicide resistance

4.1.1 Introduction

The aim of this chapter is to identify a protein marker for herbicide resistance which is specific to black-grass and could be implemented as an in-field test diagnostic. Current tests for resistance either involve transplanting plants to a glasshouse for spray-trial analysis or petri-dish growth analysis using seed collected prior to crop harvesting (Reade, 2000). The issue with these tests are that they are space and time consuming, expensive and the results were often delivered too late to be effective in the current cropping season. Therefore, a more effective test would need to be quick, cheap and easy to use with the capacity to provide results before the application of post-emergent herbicides. This would enable the alternative strategies to be adopted where necessary.

In the 1960s, glutathione S-transferases (GSTs), now more commonly termed glutathione transferases, were identified in animals for their involvement in both metabolism and detoxification of drugs (Wilce and Parker, 1994). GSTs in plants were initially identified in 1970, as soluble proteins located in the cytosol and commonly had molecular weights of around 50 kDa, composed of two polypeptide subunits (Dixon et al., 2002). Subsequently, the capacity for plants to detoxify xenobiotics (foreign compounds) using GSTs was explored, for example, maize survived herbicidal injury due to its capacity to conjugate the chloro-s-triazine with GSH, a reaction catalysed by GST (Edwards and Dixon, 2000a). We now know that soluble glutathione transferases are encoded by large and diverse gene families. To date 14 distinct classes have been identified in plants (Munyampundu et al., 2016). The classes of particular interest in detoxification are Tau (GSTU) and Phi (GSTF) classes (Sheehan et al., 2001, Frova, 2003). Of these, phi GSTs were first purified and cloned from maize in the 1980s due to their active involvement in herbicide detoxification (Timmerman, 1989).

Subsequently a large number of phi GSTs have been cloned from a variety of plants, including *Am*GSTF1 from black-grass (Cummins et al., 1999). Previous studies have identified the significance of *Am*GSTF1 as a protein which is highly expressed in MHR black-grass populations, such as ‘Peldon’, but not in target-site resistant (TSR) or WTS biotypes (Cummins et al., 2009, Cummins et al., 1999). Assays for GST expression have been required to determine the abundance of *Am*GSTF1 in black-grass. While strongly expressed in MHR black-grass, *Am*GSTF1 has low activity toward herbicides such as fenoxaprop suggesting detoxification is not its primary role.

GSTs are incredibly versatile enzymes which catalyse a wide range of reactions. This involves the conjugation of glutathione to electrophilic compounds to form more soluble peptide derivatives (Labrou et al., 2015). The GS-conjugate is then usually transported to the vacuole and further catabolized (Brazier-Hicks et al., 2008b, Walczak and Dean, 2000). There is an increasing awareness of the structure-function relationships in compounds which are detoxified by GSTs.

Despite the large number of GSTs present in plant genomes most of the characterised GST homologues share a relatively conserved gene structure, especially within the major tau and phi classes (Figure 19). The highly conserved region indicates that natural selection has continually eliminated forms with mutations and as such, the conservation of protein-coding sequences leads to the presence of identical amino acid residues as analogous regions of the protein structure. This provided an opportunity to develop a biomarker for herbicide resistance for use in an in-field diagnostic which would be highly sensitive to black-grass.

Previous studies utilised an antiserum raised against maize *Zm*GSTF1-2 (heterodimer) in screens of soluble protein extracts from wild-type sensitive (WTS) and multiple herbicide resistance (MHR) black-grass. The antiserum identified the immunoreactive GSTF band in MHR plants. Cummins et al. (1999) demonstrated, that MHR black-grass expressed four closely related GSTF isoforms (approximately 95% amino acid sequence identity). Following the expression of the respective recombinant proteins in *E.coli*, it was found that the yielded polypeptides co-migrated in gel electrophoresis with the GSTF band in MHR plant extracts.

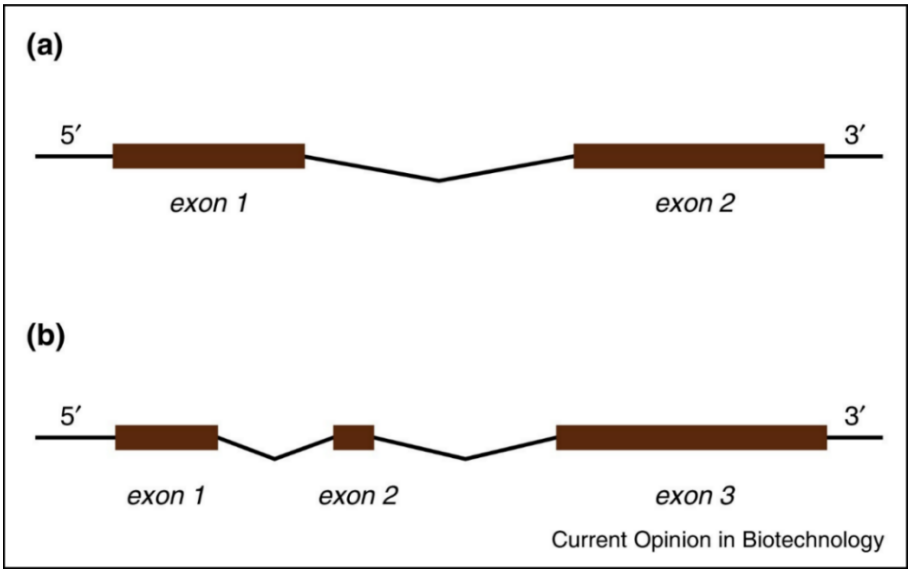


Figure 19. Common gene structures of the dominant plant GST classes.
(a) The one-intron- two exon structure normally found in vascular plants specific to tau GSTs; (b) The two intron-three exon structure characterises phi class GSTs in plants from Labrou et al., 2015.

Therefore, for the purpose of this study and due to the sequence similarity between all four isoforms of *Am*GSTF1 (Figure 20) the isoform *Am*GSTF1c was selected for expression and recombinant protein production. This sequence was also selected as it had previously been used in studies by the Edwards group so it was a point of reference (Cummins, 2013).

Figure 20. Predicted Amino Acid Sequence of *Am*GST2 clones showing alignment with the most similar GST sequences determined in crop plants.
*Am*GST2 sequences aligned with type I maize GST *Zm*GST I (accession X06754). Residues present in all sequences are shown with an asterisk while residues differing within the respective *Am*GST sequences are underlined. (source:(Cummins et al., 1999))

This study aimed to identify how *Am*GSTF1 could be used as an in-field marker of resistance. Current markers have relied on orthologues of GSTF1 in closely related species rye-grass, maize, wheat and rice (Cummins et al., 1999, Cummins et al., 2013, Cho et al., 2007, Cho and Kong, 2005, Cummins et al., 2003b). Therefore, there was a need for a more specific antiserum raised against the Peldon polypeptide (*Am*GSTF1-2) for use in western blots and an ELISA (enzyme-linked immunosorbent assay). This would then potentially be used for the quick detection of GSTF1 abundance in black-grass plants.

When establishing whether to develop a polyclonal or monoclonal antiserum the advantages and disadvantages were reviewed see Table 5. Ideally the antiserum aimed to have greater sensitivity and specificity it was hypothesised that this would be achieved as a result of using the recombinant protein *Am*GSTF1 from Peldon black-grass as the immunogen. For the purpose of this study it was decided that a polyclonal antiserum was raised due to the fact that the developmental protocols were shorter, it was suited for use in a sandwich ELISA and produces an antiserum consisting of a mixed

pool of immunoglobulin molecules that bind to several epitopes making it useful in detection and capture. These factors were coupled with the fact that there were time and financial constraints which made the production of a monoclonal antibody less appealing despite having numerous benefits including reduced risks of unexpected cross-reactivity and the fact that a monoclonal is almost indefinitely be renewable (Table 5).

Table 5 The advantages and disadvantages of raising a monoclonal or polyclonal antibody for the purpose of this study.

	Advantages	**Disadvantages**
Monoclonal	• If cell culture conditions are kept stable the production of monoclonal antibodies can be almost indefinitely renewable. • Stable production provides a unique homogeneity feature to these antibodies meaning more reproducible experiments. • Very low background compared to polyclonal. • Reduced risks of unexpected cross-reactivity	• Takes time and requires high technical skills • Developmental protocols take more than 3 times as long as polyclonal for the generation of a stable hybridomas and isolation of clones requires several cycles of cell expansion in vitro. • Epitope is unique among the populations of monoclonal antibodies meaning slight change in conformation can dramatically reduce binding efficiency.
Polyclonal	• Moderate technical skills • Multi-epitope specificity makes them useful in detection and capture. • Development protocols relatively short (down to 2 months) • Upon denaturation, although some epitopes may be lost due to conformational changes, the probability that one or more epitopes remain intact and bind to antibodies is still high.	• Multi-epitope recognition leads to high cross-reactivity • The presence of high quantity of non-specific antibodies often leads to high background levels. • Production is animal dependent and may dramatically differ from one individual to another.

4.1.2 Protein Analysis for the detection of F1

4.1.2.1 *Zm*GSTF*1-2 Detection*

To identify herbicide resistance in various biotypes of *Alopecurus myosuroides* and biotypes of other wild grasses Cummins *et al.* produced the anti-*Zm*GSTF1-2 heterodimer serum which was used to identify phi class GSTs in a range of crops and model species (Cummins et al., 2003b). Cummins *et al*. work forms the basis for this study, as the anti-*Zm*GSTF1-2 antiserum was used in an initial protein assessment. Crude extracts from all 10 populations were probed with antiserum *Zm*GSTF1-2, which detected a band of approx. 25 kDa presumed to be *Am*GSTF1. This result did not correlate to the relative resistance of the plants found in the spray and petri-dish analysis.

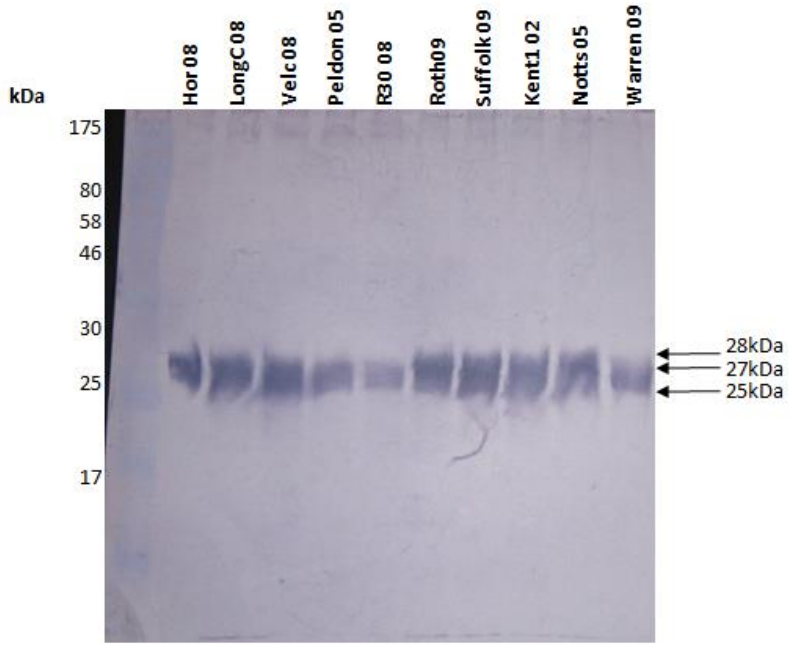


Figure 18 Western blot of crude extracts from ten black-grass populations of *A.myosuroides*.
Ten populations of *A.myosuroides* with differing resistance characteristics were run on a 12 % discontinuous gel were probed using antiserum *Zm*GSTF1-2 at a dilution of 1:1000. Samples loaded at 50 ng per 15 µL and ran alongside 10 µL pre-stained broad range protein marker 7-175 kDa (NEB, UK).

The antiserum *Zm*GSTF1-2 was known to detect the over-expression of GSTF1 in MHR populations but the band was of low abundance in TSR and WTS populations. In Figure 18 the Notts and Roth populations were predicted to detect a faint 25 kDa polypeptide, as they were termed TSR and WTS respectively, whilst Suffolk, Hor, Long and Peldon were expected to over-express the aforementioned polypeptide, since the relative resistance of the plants in the spray and petri-dish analysis indicated that they were most representative of MHR. However, it was apparent that the immune detection in Figure 18 was not as discriminating as originally hoped. The populations Roth and Notts appeared in this qualitative analysis to express the 25 kDa polypeptide

more highly than Peldon (MHR). The results of this immunoblot indicated that there was a need for a more specific antiserum with the potential to be used in quantitative analysis ELISA. There was an expectation that the new antibody could provide novel proteins which may be explored concerning herbicide resistance. There were other existing markers for immuno detection of GSTs including classes tau and lambda although these markers did not show differentiation between those populations that were MHR and WTS.

4.1.2.2 *Detection of GSTL and GSTU*

Protein markers raised for the immuno-detection of lambda and tau class GSTs had previously been used to examine populations of black-grass. However, there was poor distinction between populations of varying levels of resistance to herbicides. In Figure 21 crude protein extractions from the populations Peldon 05 (MHR) and Roth 07 (WTS) were separated by SDS-PAGE, western blotted and probed using antisera raised to lambda (L), tau (U) and phi (F) classes of GSTs, all of which were known to be associated with the detoxification of xenobiotics in cereals (Edwards et al., 2005). The antisera had previously been raised against wheat tau TaGSTU1-1 (Cummins et al., 1999), wheat cla30 lambda (Theodoulou, et al. 1999) and maize phi class *Zm*GSTF1-2 (Cummins et al., 1999). Previous studies had identified that MHR black-grass population contained similar levels of GSTUs as WTS populations (Cummins, et.al. 2009). MHR populations constitutively expressed GSTFs, whilst they were only detectable in WTS populations that had been safened (Cummins et al., 2009). The results in Figure 21 confirmed previous literature findings, as the expected polypeptide 27 kDa was present in both Peldon 05 and Roth 07 populations at equal levels for both GSTL and GSTU enzymes. Although the results indicated that Peldon 05 possibly expressed the 27 kDa polypeptide more strongly, it was a qualitative analysis which made it difficult to confirm. The expression of GSTF enzyme was constitutively over-expressed in Peldon 05 (MHR) but not in susceptible black-grass Roth 07.

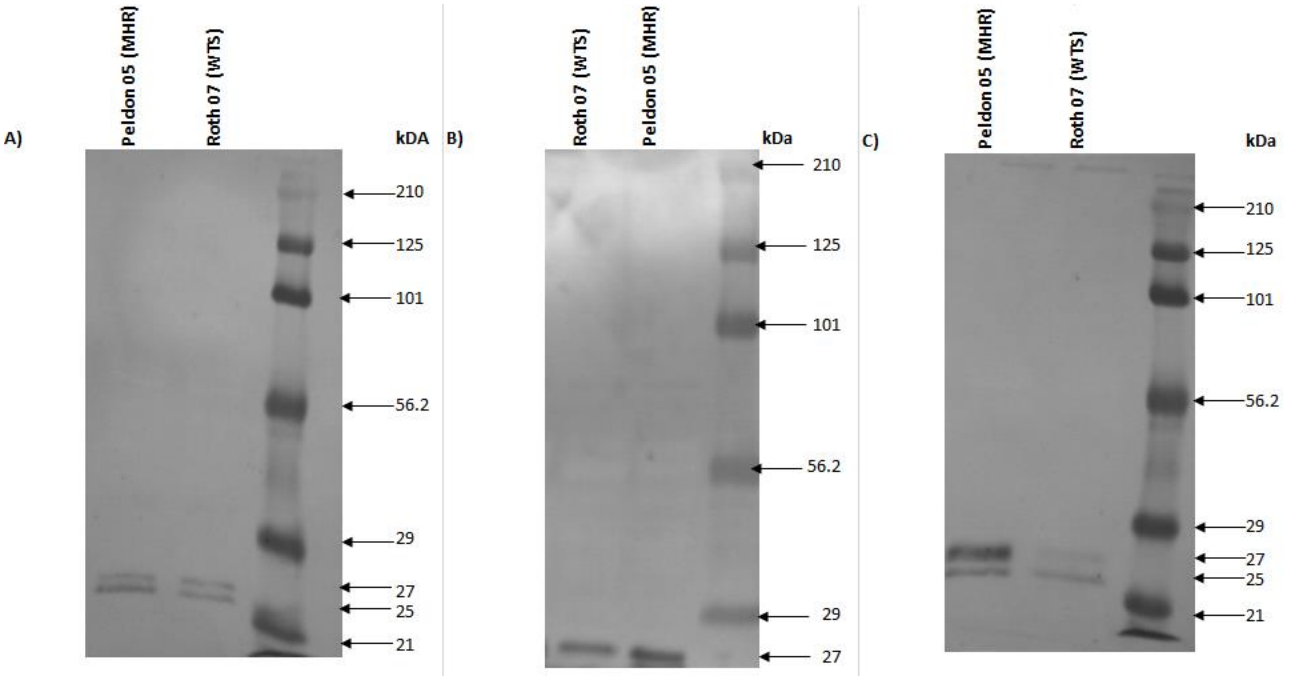


Figure 21. Western blot analysis of two known *A.myosuroides* populations (Peldon 05 (MHR) and Roth 07 (WTS)) probed with Lambda, Tau antisera raised from wheat, and Phi raised from maize.
Peldon 05 (MHR) and Roth 07 (WTS) crude protein probed using: A) Anti-*Ta*GSTL B) Anti-*Ta*GSTU1-1 and C) Anti-*Zm*GSTF1-2 at a dilution of 1:1000 the populations were run on a 12 % discontinuous gel samples loaded at 50 ng per 15 µL and ran alongside 10 µL pre-stained broad range protein marker 7-175 kDa (NEB, UK).

From Figure 21 the expression of GSTL and GSTU is visible but are apparently unrelated to herbicide resistance when MHR and WTS populations were compared whereas, when the two biotypes were probed using *Zm*GSTF1-2 there was a noticeable difference in the expressed patterns of polypeptides. This result indicated the significance of GSTF class and why it has remained the protein of choice when detecting resistance by immunoblot. However, due to the fact that WTS was known to express GSTF after exposure to safener treatment, *Zm*GSTF1-2 antiserum was perhaps not the best suited for continued analysis of resistance traits in black-grass populations (Cummins et al., 2009). Therefore, there remained a clear need for a more sensitive and specific antiserum for the detection of resistance traits across various populations of black-grass. This antiserum also needed to be compatible for use in a quantitative analysis as well as the standard qualitative methods.

4.1.2.3 *CDNB assay in Populations of black-grass*

The focus of this chapter has been on the diagnostic nature of immuno-detection by western blot. This method of analysis not only assessed the detection capacity of the over-expressed F1 protein in black-grass, but also highlighted the existing limitations of the current antibodies. It was therefore essential to establish a potential quantitative analysis of resistance in the populations of black-grass. The method of choice was the GST catalysed substitution of glutathione for the chloro group of the

xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974). The CDNB assay measured the rate of GST activity in samples based on a colorimetric assessment.

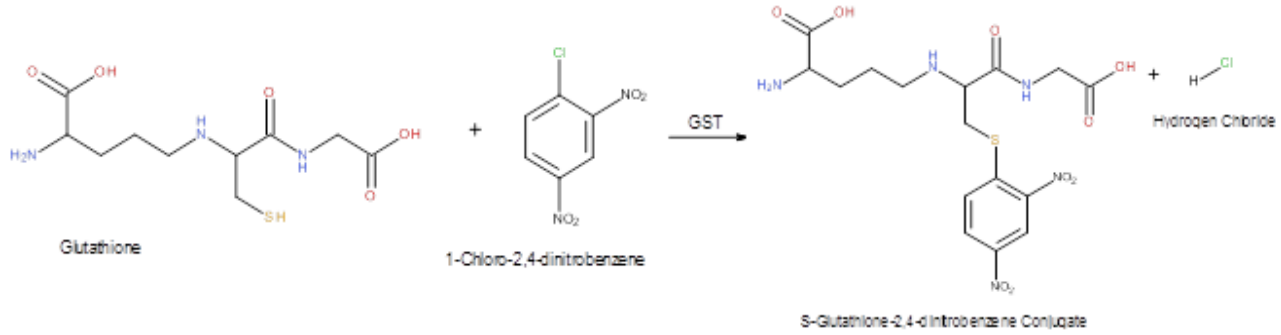


Figure 22. Reaction Scheme for CDNB and GSH (Sigma-Aldrich, USA)
Glutathione catalyses the conjugation of L-glutathione to CDNB through the thiol group of the glutathione, the reaction product, GS-DNB conjugate absorbs at 340nm. Rate of absorption is directly proportional to GST activity.

This assay was ideal for assessing the xenobiotic-detoxifying activity of the unknown and known populations GSTs. The schema for the conjugation of the thiol group of glutathione to the CDNB substrate was illustrated in Figure 22. The rate of increased absorption at 340 nm was directly proportional to the GST activity in the sample (Habig et al., 1974,Wilce and Parker, 1994, Mannervik and Danielson, 1988).

The assay was used to measure the activity of the ten unknown populations of black-grass (Table 6). Table 6 indicated that the differences in activity towards CDNB directly correlated to the level of resistance in the known populations of black-grass. For example, Hor 08 which had the highest level of TSR mutations to both ALS and ACCase and indicated the greatest MHR trait also had the highest level of activity towards the substrate CDNB. The populations associated with the phenotypes, Kent1 02, Roth 09 and WTS had the lowest activity towards CDNB. The results from this study proved to correlate with the relative resistance of the plants in the spray and petri-dish analysis.

Table 6. Substrate specificity of *Am*GSTF1 in populations of *Alopecurus myosuroides*
Measurements taken 6 times. Mean specific activities shown.

Population	Specific Activity (nKat/mg)
Recombinant *Am*GSTF1-Strep	586.92
Hor 08	619.21
LongC 08	543.98
Velc 08	486.11
Peldon 05	399.31
R30 08	422.45
Roth 09	40.51
Suffolk 09	370.37
Kent1 02	46.30
Notts 05	162.04
Warren 09	393.52

4.2 *Am*GSTF1 novel biomarker

4.2.1 Introduction

The intention of this investigation was to generate an antibody which had greater specificity to the detection and over-expression of the plant specific phi class protein in black-grass, *Am*GSTF1. The antibody was also intended to be used in an ELISA (enzyme-linked immunosorbent assay) to enable quantitative analysis. Also, the development of an ELISA enabled the future use of this biomarker on a lateral flow device (LFD) which would be practical for in-field testing.

Prior work identified the significance of *Am*GSTF1 in relation to the role it plays in conferring MHR in black-grass and in transgenic host plants (Cummins et al., 2013). The role of *Am*GSTF1 in MHR was explored through the constitutive expression in transgenic *Arabidopsis thaliana*, a plant that is otherwise herbicide sensitive. The transgenic plants exhibited resistance to multiple herbicides through similarities in their secondary, xenobiotic, and antioxidant metabolism mechanisms to those termed MHR (Cummins et al., 2013). Following a transcriptomic analysis of the *Am*GSTF1-expressors, the changes in biochemistry were not the result of any changes in gene expression (Menne, 2007). Cummins. et al (2013) noted that *Am*GSTF1 exerted a direct regulatory control on metabolism, through accumulating protective secondary metabolites and the co-ordinated up-regulation of a subset of endogenous detoxification enzymes in the transgenic *Arabidopsis thaliana*. Of strong significance, these results mirrored the biochemical changes that had already been observed in MHR black-grass plants relative to wild-type sensitive plants (Cummins et al.,

2009). Therefore these results strongly suggested that *Am*GSTF1 played a key role in eliciting MHR in both a transgenic host plants and in black-grass.

4.2.2 Expression and purification of *Am*GSTF1

4.2.2.1 Expression of AmGSTF1

*Am*GSTF1 was cloned into pET-24(+) vector and expressed in *E.coli*, followed by purification using an S-hexyl GSH column as described (see section 2.5.2-2.5.4). In Figure 23 there is an example chromatogram which was produced when the *Am*GSTF1 protein was partially purified on an S-hexyl GSH column. The eluted protein was analysed using western blot. In each of the four eluted fractions a single polypeptide band of ~25 kDa was detected using anti-*Zm*GSTF1-2-antiserum (Figure 24).

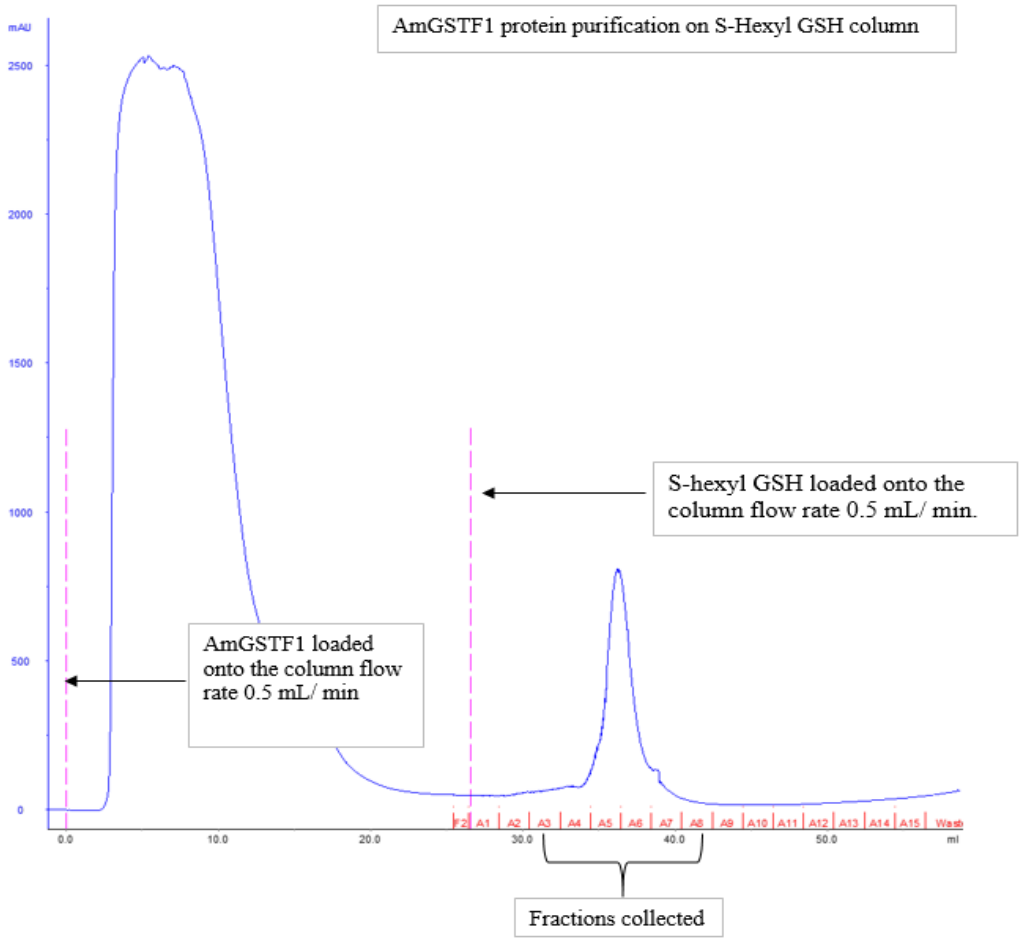


Figure 23 Chromatography of *Am*GSTF1 purification on S-hexyl glutathione column.
Crude E.coli lysate containing 5 mL of *Am*GSTF1 was loaded onto the column at a flow rate of 0.5 mL/min, and then eluted with S-hexyl GSH. The fractions were collected for later analysis to determine r*Am*GSTF1 concentration.

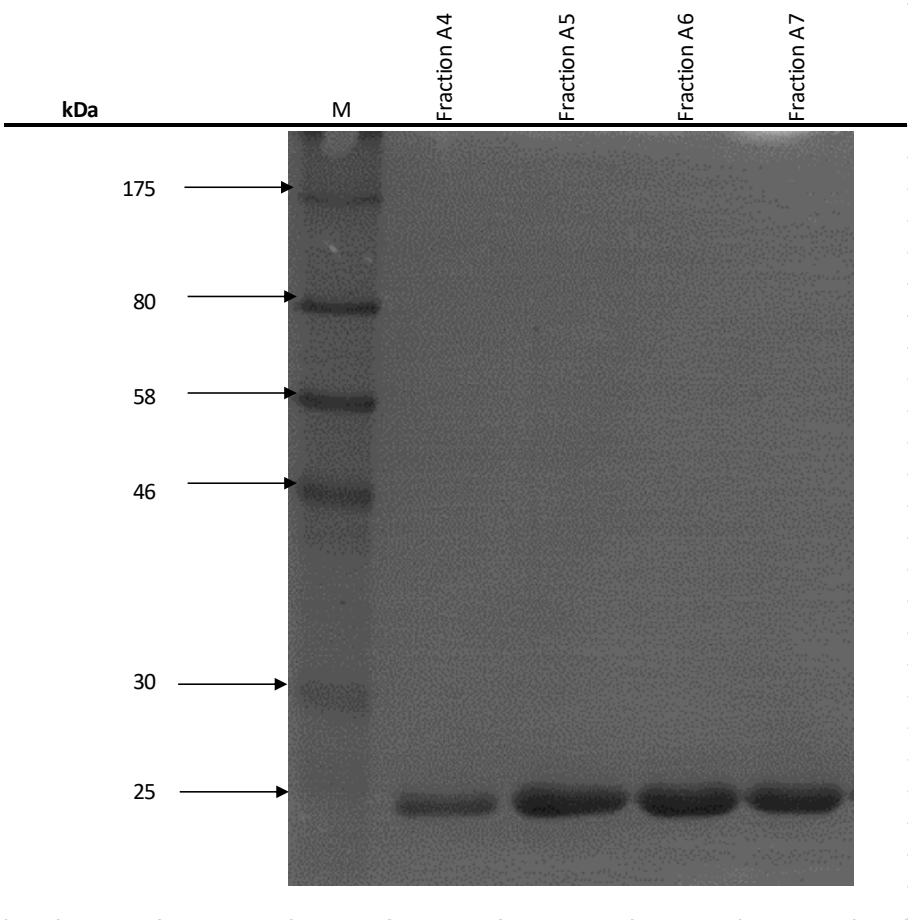


Figure 24 SDS-PAGE and western blot detection of *Am*GSTF1 purified protein following S-Hexyl-GSH column purification.
Eluted fractions were loaded at a concentration of 50 ng/ µL and were probed with *Zm*GSTF1-2 antiserum at a dilution of 1:1000.

4.2.2.2 Hydrophobic Interaction column purification of AmGSTF1

A hydrophobic interaction column was used to remove S-hexyl GSH through the addition of ammonium sulphate [$(NH_4)_2 SO_4$], from a concentrated salt solution to an elution without salt. The protein fractions containing the recombinant protein were pooled, dialysed for 16 h at 4 ^{0}C in 0.05M potassium phosphate buffer, 1.5 M [$(NH_4)_2SO_4$, pH 7] in a volume of 5 L. The dialysed samples were concentrated into a volume of 1 mL prior to use on the hydrophobic interaction column (1 mL GE Healthcare Resource PHE R10 column). The eluted protein was collected and measured at 280 nm to assess the protein concentration. An example chromatogram indicated the point at which the eluted proteins were collected (Figure 25). The eluted protein was analysed again by immunoblot where a single band was detected, in each of the fractions much like those seen in Figure 24. Fractions at each stage of the purification were also collected and their activity assayed using CDNB (Table 7). The results indicated that the specific activity improved following each step of the purification.

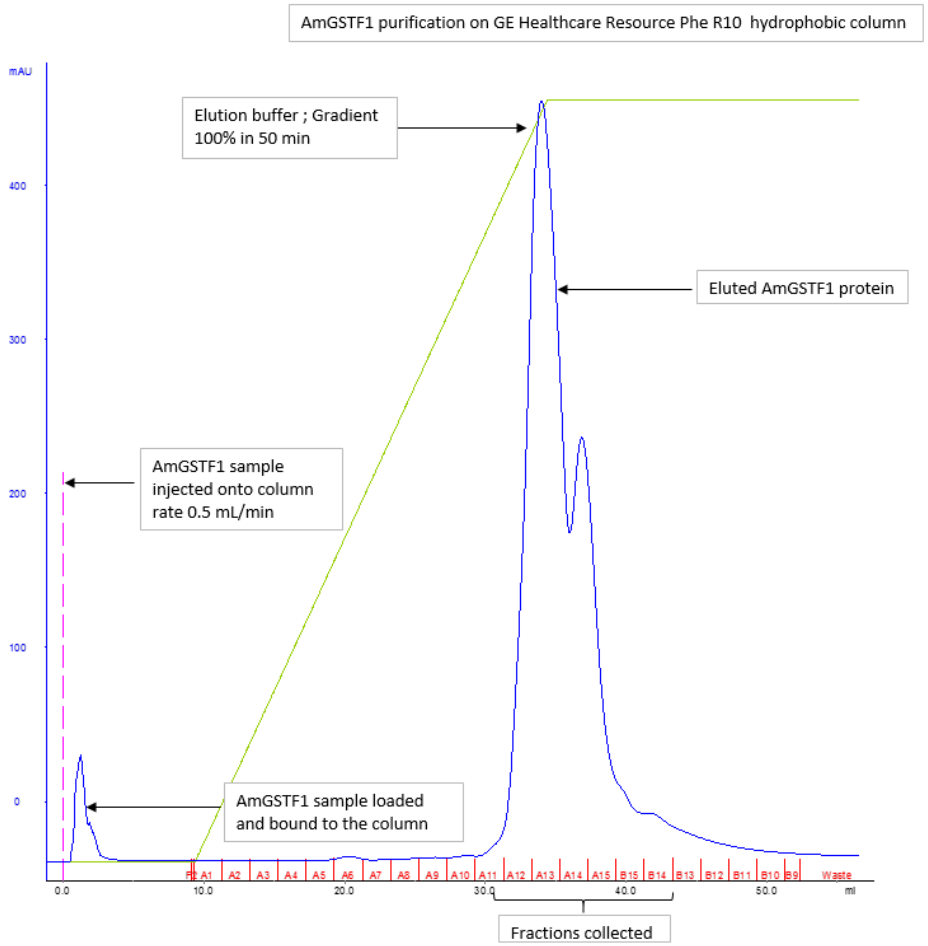


Figure 25. Chromatograph of *Am*GSTF1 purification using a hydrophobic interaction column.
GE Healthcare Resource Phe R10 hydrophobic interaction column (HIC) used in the purification of 5 mL of partially purified r*Am*GSTF1 injected onto the column at a rate of 0.5 mL/min, whilst the elution buffer (containing no salt) continued to flow over the column at a rate of 0.5 mL/min until a 100 % gradient was reached the eluted rAmGSTF1 fractions were collected for later analysis to determine the protein concentration.

Table 7. Substrate specificities of recombinant *Am*GSTF1 during purification.
CDNB: 1-chloro-2,4-dinitrobenzene used to measure *Am*GSTF1 protein. Measurements taken 6 times. Mean specific activities shown. Purification is calculated by dividing by specific activities. Recovery is calculated using total activity.

Stage of *Am*GSTF1 Purification	Total Protein (mg)	Total activity (nKat)	Specific Activity (nKat/mg)	Purification (n-fold)	Recovery (%)
Crude Protein *Am*GSTF1	14.28	682.87	47.82	1.00	100.00
S-Hexyl GSH (Affinity purification)	13.08	1166.67	89.19	1.87	170.85
Hydrophobic interaction column	10.06	891.20	88.59	1.85	130.51

The next stage involved generating a column for the affinity purification of Anti-*Am*GSTF1-antiserum. Therefore, the newly purified *Am*GSTF1 protein was bound to a column matrix.

4.2.3 Production and preliminary assessment of Anti-*Am*GSTF1 Antiserum

Polyclonal antibodies were raised to the recombinant protein *Am*GSTF1 as described in 2.7. In Figure 26, the western blots are shown to illustrate the key stages of antibody development, A illustrates the response of MHR and WTS to the pre-immune serum which indicated no existing cross reactivity. Western blots B and D in Figure 26 indicated cross reactivity to a polypeptide of approximately 60 kDa. It was apparent that the cross reactivity was likely to be associated with the immunisation rather than dietary, as it would have shown up in the pre-bleed. Another noticeable difference was the response to the GSTF1 protein. The anti-*Zm*GSTI-II recognises a smeary 25 kDa GST subunit polypeptide in the MHR protein extracts, however the anti-*Am*GSTF1-serum reacted with three polypeptides of Mr 25kDa, 24 kDa and 22 kDa. The *Am*GSTF1 polypeptides were weakly detected in WTS black-grass particularly polypeptides at Mr 25 kDa and 22 kDa.

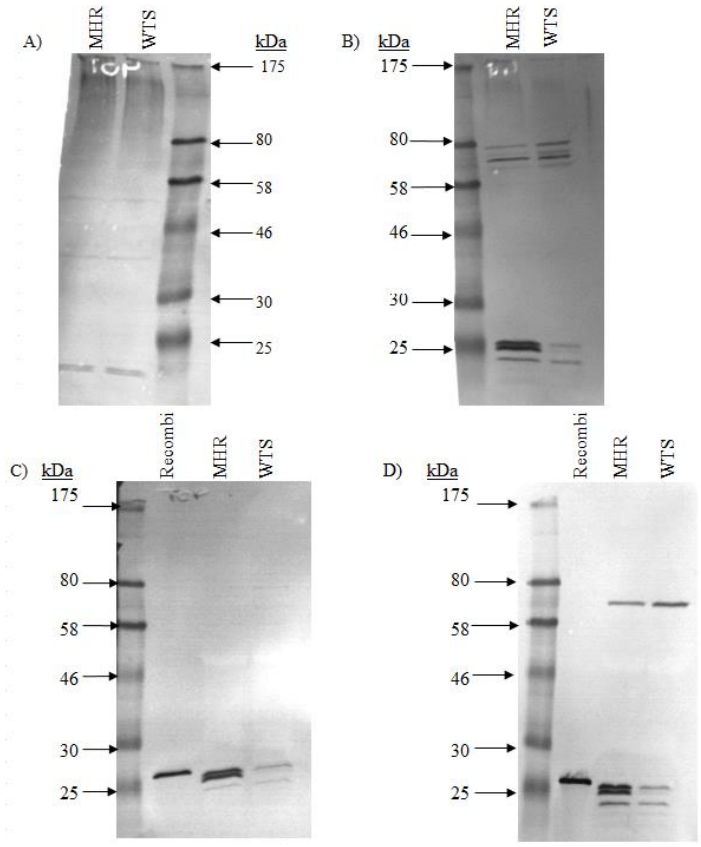


Figure 26. Western blot images of the Anti-*Am*GSTF1 serum response to crude plant protein extracts from *Alopecurus myosuroides*.
Crude protein samples from MHR and WTS were loaded at a concentration of 50 ng / 15 µL and blots were probed with the anti-*Am*GSTF1 serum at a dilution of 1:1000. A) Pre bleed response, B) First bleed day 39, C) Second bleed day 53 and D) final bleed day 67. The purified recombinant strep tagged-*Am*GSTF1 protein was loaded at a concentration 50 ng / 15 µL and included in C and D as an internal standard.

The inclusion of the recombinant *Am*GSTF1-strep tagged protein produced a positive control on the immunoblot. The sample only produced one single polypeptide of 27 kDa which is likely to be linked to the fact that it was a purified protein rather than a crude protein extract. To use the antibody in a quantitative analysis via an ELISA format, the cross-reactivity to the 60 kDa polypeptide needed to be removed. The next stage of development focused on the purification of anti-*Am*GSTF1-antiserum and then the reassessment of its detection limit. It was also key to establish how robust the antiserum was for detecting *Am*GSTF1 in a blind screen of ten populations of black-grass, rye grass and wild oats with varying degrees of resistance.

4.2.4 Purification of Anti-*Am*GSTF1 Sera

4.2.4.1 *Generation of affinity column for purification of anti-AmGSTF1-antiserum*

The recombinant protein *Am*GSTF1 was coupled to the resin of a column provided in the AminoLink® Plus immobilisation kit (Thermo Scientific; Illinois, USA). This column was used to purify the anti-*Am*GSTF1-antiserum, as the proteins were covalently immobilised on the beaded agarose making it a valuable tool for affinity purification. This technique enabled the clean capture of the antibody without cross contaminants present and had a coupling efficiency which was generally greater than 85 % with antibodies, this was crucial with limited supply of the antibody. The coupling reaction evolved around the principle of reductive amination see the schema in Figure 27. The AminoLink® Plus resin was used with a coupling buffer of pH10 as described (see section 2.7.3.1) and left overnight prior to the purification of the Anti-*Am*GSTF1-serum.

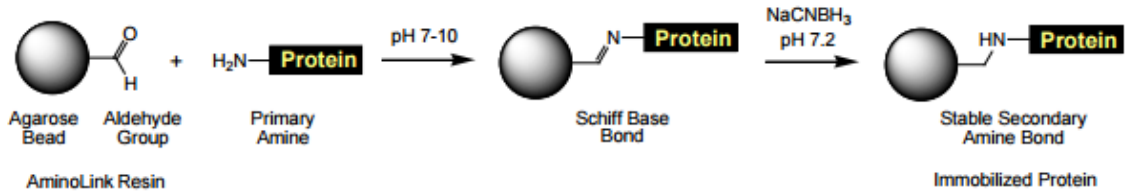
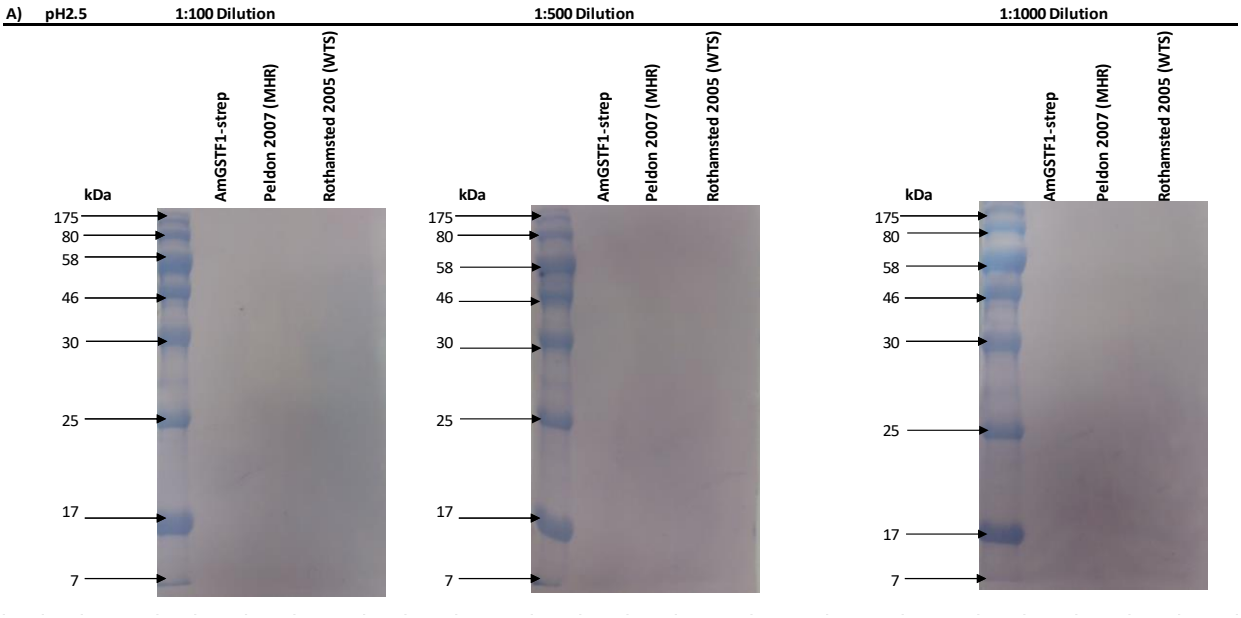
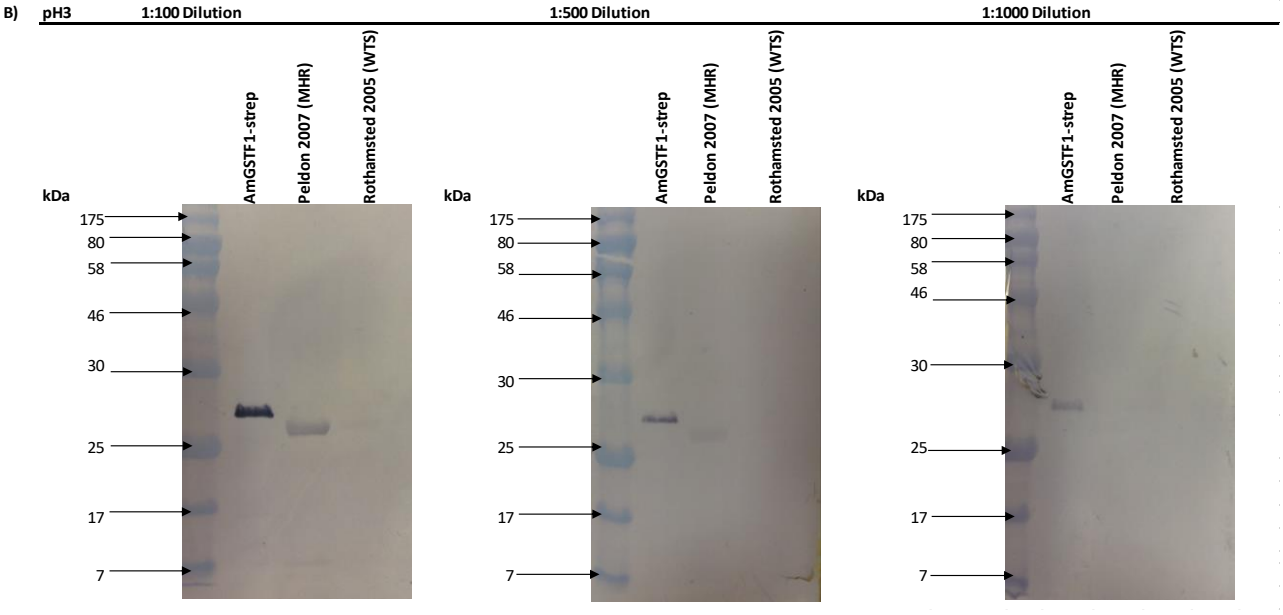


Figure 27. General Structure and reaction scheme of AminoLink Plus Resin.
(Thermo Scientific, Illinois, USA)

4.2.4.2 *Purification of anti-AmGSTF1-antiserum*

Anti-*Am*GSTF1 serum needed to be purified to isolate the group of polyclonal antibodies which bind specifically to *Am*GSTF1. Ammonium sulphate precipitation of the *Am*GSTF1-antibody was used as it stabilises proteins, reduces lipid content, concentrates immunoglobulins from a crude source, and retained the samples in their native form. The Anti-*Am*GSTF1 serum was precipitated over a 2 h period as described (see section 2.5.7). The ammonium sulphate was then removed during clarification through the use of HiTrap desalting column (Sephadex G-25 media). This method was selected over the standard method of dialysis as it was a faster exchange rate. It was more efficient to use a column which was packed with Sephadex-G25 as this had the capacity to separate based on size. This meant that the antibodies were separated from the ammonium sulphate and other small molecules. The concentrated Anti-*Am*GSTF1-sera was eluted and subsequently applied to the *Am*GSTF1 AminoLink Column. The antibody fraction eluted at pH 4, 3 and 2.5 was neutralised and then analysed. Crude protein extracts from MHR and WTS black-grass populations were assessed by SDS-PAGE to identify the immunoselectivity of the newly purified antiserum.


A) pH2.5
1:100 Dilution
1:500 Dilution
1:1000 Dilution
AmGSTF1-strep
Peldon 2007 (MHR)
Rothamsted 2005 (WTS)
kDa
175
80
58
46
30
25
17
7


B) pH3
1:100 Dilution
1:500 Dilution
1:1000 Dilution
AmGSTF1-strep
Peldon 2007 (MHR)
Rothamsted 2005 (WTS)
kDa
175
80
58
46
30
25
17
7

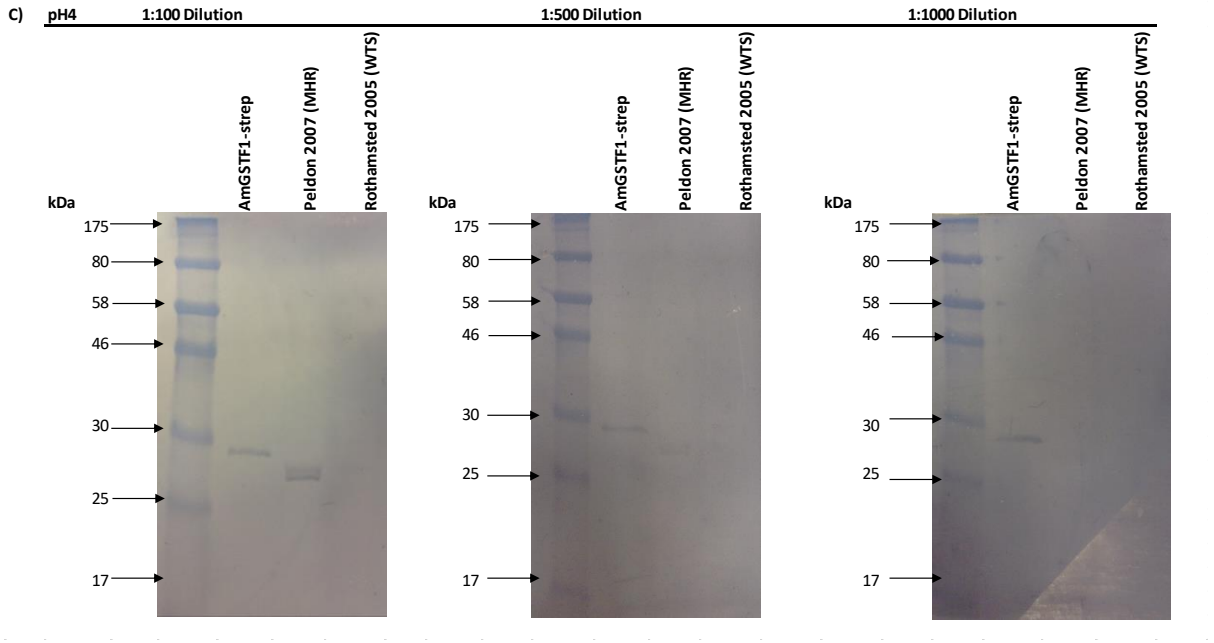


Figure 28 Western blots following elution of purified Anti-*Am*GSTF1 antibody at different pH conditions.
Western blots: A) pH 2.5, B) pH 3 and C) pH 4. Each purified antibody was diluted at three different dilutions: 1:100, 1:500 and 1:1000 and were used to detect both crude protein extracts from MHR and WTS populations of black-grass and purified recombinant *Am*GSTF1-step tagged protein loaded at 50 ng/ μL to assess Anti-AmGSTF1 antibody sensitivity.

4.2.4.3 Assessment of affinity purified antibodies

The newly purified Anti-*Am*GSTF1 antibody was assessed to establish its selectivity of *Am*GSTF1 in wild grasses, particularly black-grass. Although, latterly it was also decided that it would be interesting to determine the antibody's detection in *Lolium rigidium* (ryegrass) and *Avena fatua,* (Wild oats) as well. The ten populations of black-grass as described in chapter 3 were used to establish the selectivity of the newly purified Anti-*Am*GSTF1 antibody. In Figure 29 there was still a distinctive three band polypeptide pattern which was detected in immunoblots probed in Figure 26. The recombinant *Am*GSTF1-strep tagged protein was included, as a positive control. Populations Roth 09 and Notts 05 showed faint banding patterns however this was predicted as they are WTS and TSR populations respectively. The expected expression of *Am*GSTF1 in WTS and TSR was less than the constitutive expression in MHR populations of black-grass. Intriguingly, the relative abundance of the *Am*GSTF1 polypeptides of differing relative molecular mass varied between MHR populations, suggesting the presence of multiple component isoenzymes. From the results potentially novel patterns of polypeptides had been identified although the results remain qualitative.

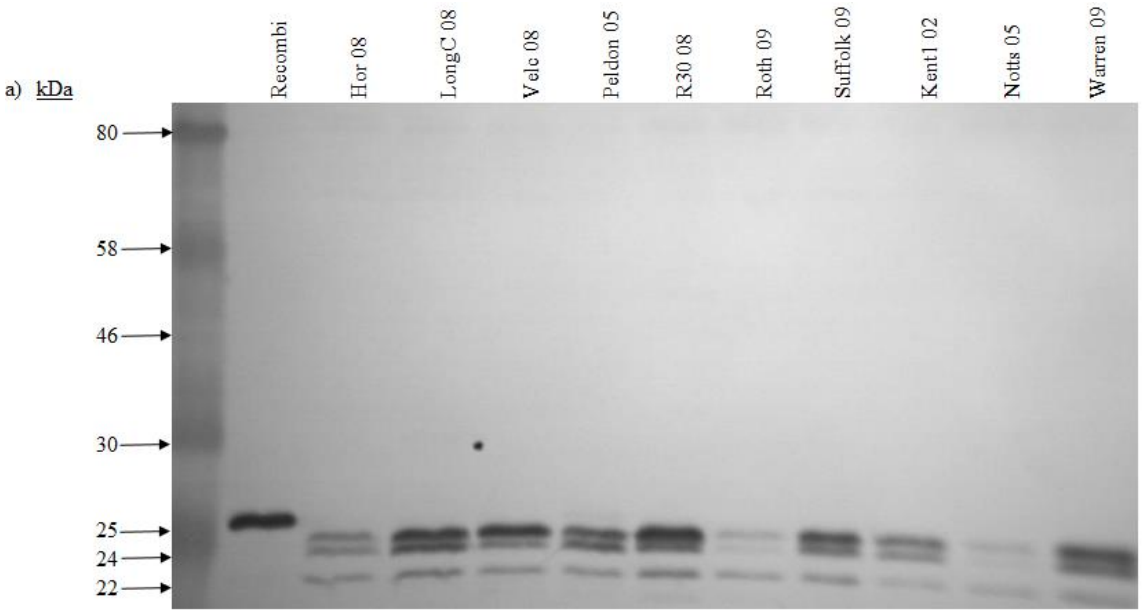


Figure 29. Immuno blot of crude protein extracts from ten populations of *A.myosuroides* when probed with anti-*Am*GSTF1 antibody.
Crude protein samples from ten black-grass populations with differing herbicide resistance characteristics. The recombinant purified strep-tagged *Am*GSTF1 and crude samples were loaded at 50 ng/ 15µL and probed with the Anti-*Am*GSTF1 antibody at a dilution of 1:500.

It was hypothesised that the detection of potential *Am*GSTF1 orthologues could be explored further in rye grass and wild oat populations with varying degrees of herbicide resistance. These populations were probed using the Anti-*Am*GSTF1 antibody and the resulting western blots did not demonstrate the same three polypeptide pattern (See Appendix-Figure S 1 and Figure S 2), instead there were faint bands present in some cases with Mr of approximately 27 kDa and 25 kDa. There was an expectation that the homologues of the *Am*GSTF1 protein would be present, however due to the specificity of the new antibody it is possible that the detection in these species wasn't as clearly defined due to the lack of epitopes in common. Although the analysis of the anti-*Am*GSTF1 serum showed the sensitivity towards *Am*GSTF1, the results were qualitative, therefore the next stage focused on the introduction of an ELISA a quantitative analysis. Before utilising an ELISA consideration was placed on the use of colorimetric detection, chemiluminescent detection and fluorescent detection as a means of quantitatively analysing an immune blot. Unfortunately, all of these techniques relied on access to either a spectrometer to detect protein levels (colorimetric detection) or Charge-coupled device (CCD) cameras with appropriate filters (Chemiluminescent or fluorescent detection) all equipment that was not easily available. Therefore, proceeding with optimising an ELISA seemed the most appropriate option especially as the chemiluminescent technique is less commonly used due to issues with linearity of results.

4.3 ELISA *Am*GSTF1

4.3.1 Introduction

Studies using enzyme-linked immunosorbent assays (ELISAs) indicated the numerous advantages of moving the detection of GSTF1 to a quantitative system. The positives included: the number of screens that could be carried out, the opportunity to reliably reproduce results and the speed in which results are obtained. The implementation of an ELISA for *Am*GSTF1 detection was more advantageous than the existing western blot system used to study the proteomic expression of resistance. Reade (2000), developed an ELISA to detect the GST abundance in black-grass plants after a monoclonal was raised against the Peldon (*Am*GSTF1) polypeptide. This test assessed plants which survived herbicidal treatment, and the results provided an aid for the prediction of field performance at sites where black-grass control was poor. The test was carried out on plants at 2-3 leaf stage which meant results were generated in three days. Therefore results could be obtained before post-emergence herbicide application. Using Reade's findings it was possible to see that the polyclonal Anti-*Am*GSTF1-antibody could be used in an ELISA. Furthermore, the system could readily be transformed for use in a lateral flow device (LFD). The production of an LFD offered the opportunity for a novel in-field testing of herbicide resistance in black-grass populations with the potential for further use in other wild grasses. Therefore, the study focused on the detection of *Am*GSTF1 using the polyclonal Anti-*Am*GSTF1 antibody and assessed its capacity in populations of wild grasses which included black-grass, wild oats and rye grass.

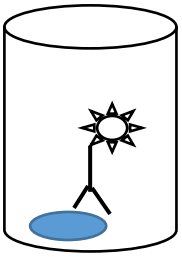
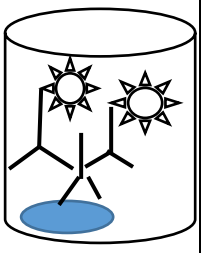
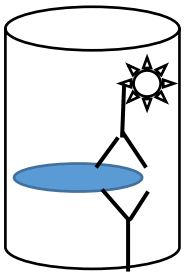
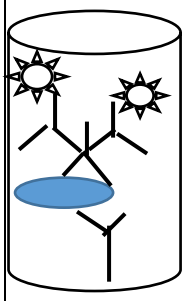
Over recent years there has been an increased reliance within the agricultural sector on immunodiagnostic tests, especially enzyme-linked immunosorbent assays (ELISAs) normally associated with the healthcare sector (Engvall and Perlmann, 1971, Engvall and Perlmann, 1972, Chirkov et al., 1984). The potential of such methods for the direct detection of infectious agents such as viruses or pathogens in plants has been proven successful and was a point of interest for this study (Kingsnorth et al., 2003, Bandte et al., 2016, Lacroix et al., 2016, Voller et al., 1976). To establish the most appropriate type of ELISA for the detection of glutathione transferases it was helpful to determine whether any such assay currently existed. Cummins et al (2013) identified parallels between the overexpression of the human pi class of glutathione transferase (GSTP1) and the plant specific phi class glutathione (GSTF1). GSTP1 over expression was associated with multiple-drug resistance (MDR) in tumours, whilst GSTF1 over expression was associated with MHR plants. The over expression of GSTP1 protein was used in an ELISA as a healthcare diagnostic, to detect MDR tumours (Ma et al., 2015, Kolwijck et al., 2009, Pandey et al., 2010). Given the similarities in function between the mammalian and plant glutathione transferases, the

project focused on exploring the detection of *Am*GSTF1 protein in crude plant protein extracts, using an ELISA format. The issue remained as to whether the ELISA needed to be a direct assay, indirect assay or a capture assay (‘sandwich’).

4.3.2 *ELISA Development*

Reade et al. (2000) investigated the GST abundance in black-grass plants following herbicide treatment using a monoclonal antibody raised against the Peldon *Am*GSTF1 polypeptide. As a result of this investigation and a comparison between the various ELISA formats, the strategy implemented was an indirect sandwich ELISA (See Table 8.) The indirect sandwich ELISA relied on the sample antigen being sandwiched between a primary and secondary antibody and in this case it was the polyclonal unlabelled primary antibody (Anti-*Am*GSTF1-serum) and a secondary detection antibody (anti-rabbit IgG (whole molecule)- Alkaline Phosphatase antibody, raised in goat (Sigma-Aldrich,USA)) see the diagrammatic representation in Table 8. On the addition of the substrate the enzymes linked to the antibody produced a fluorescent signal. The change in fluorescence occurred as the enzyme substrate-chromogen developed and this change was directly proportional to the amount of bound sample antibody. The more antibody present in the sample, the stronger the fluorescence in the test wells.

Table 8 Table outlining the advantages and disadvantages of different enzyme-linked immunosorbent assay (ELISA) techniques

Type of ELISA	Protocol Description	Advantages	Disadvantages	Schematic
Direct	An antigen is coated to a multiwall plate and is detected by an antibody that has been directly conjugated to an enzyme. This can be reversed so an antibody can coat the plate and an antigen be used for detection.	It is faster as fewer steps are required. Cross reactivity of secondary antibody is eliminated	Immunoreactivity of the primary antibody may be adversely affected by labelling with enzymes. No flexibility in choice of primary antibody label from one experiment to another.	
Indirect	Antigen coated to a multiwall plate is detected in two stages. First an unlabelled primary antibody (specific for antigen) is applied. Next, an enzyme-labelled secondary antibody is bound to the first antibody.	Increased sensitivity, since more than one labelled antibody is bound per primary antibody. Flexibility as different primary detection antibodies can be used with a single labelled secondary antibody. Cost saving as fewer labelled antibodies are needed.	Cross reactivity might occur with the secondary antibody, resulting in nonspecific signal. An extra incubation step is required in the procedure.	
Sandwich	This typically requires the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping part of the antigen molecule. The capture antibody is coated onto the plate then the sample solution is added. A second detection antibody is added and helps measure the concentration of the analyte. **Direct Sandwich**- if the detection antibody is conjugated to an enzyme. **Indirect Sandwich**- if the detection antibody is unlabelled and a second detection antibody is needed.	High specificity, as two antibodies are used the antigen/ analyte is specifically captured and detected. Suitable for complex samples as the antigen doesn't require purification prior to measurement. Flexibility and sensitivity, as both indirect and direct can be used. Suitable for use with polyclonal for both capture and/ detection	Issues with using polyclonal antibodies need to ensure that variability is present in the polyclonal allowing for both capture and detection of the analyte.	 

The initial stages of development involved applying a known concentration of the antigen (200 µg/ mL of recombinant *Am*GSTF1 protein) to the well of microtiter plate, from which a 2-fold dilution series of antigen was carried out across the plate (200 µg/ mL to 0.78 µg/ml). The antigen was fixed to the surface through simple adsorption of the protein to the plastic surface. These samples of known antigen concentrations constituted a standard curve used to calculate antigen concentrations of unknown samples. The standard took into consideration the optimum fold dilution series of the known antigen, the length of time for exposure and the dilution series in which the primary and secondary antibody was applied. This was then used as a standard protocol which enabled the detection and quantification of *Am*GSTF1 as a proportion of the total protein in unknown samples. A concentrated solution of non-interacting protein (BSA) was added to all the plate wells which ensured that all sites in which nonspecific adsorption could occur on the plate were blocked after the serum samples were added. The results for the detection of *Am*GSTF1 were quantified at 405 nm on a spectrophotometer and the standard curve was used to calculate the amount of *Am*GSTF1 in crude protein extracts.

4.3.3 ELISA Screening

The results from the ELISA screening of the ten black-grass populations indicated that Hor, Roth and Notts were significantly (p value 0.000) different to the remaining 7 populations (Figure 31). When the results of the detection for *Am*GSTF1 in the ELISA were compared to the results of the immunoblot (Figure 29) the same three populations did not display the three polypeptide bands as intensely as the other black-grass populations. Figure 31 also indicated that the WTS population Roth had the lowest expression of *Am*GSTF1, whilst Notts and Hor characterised as TSR and MHR respectively were the next lowest, although Notts and Hor were significantly (p value 0.001) different to one another. Whilst the remaining seven populations, expressed *Am*GSTF1 to the highest level and were denoted ‘D’ as they were not significantly different from one another. This mirrored the results of the western blot in Figure 29 as these seven populations were the ones that expressed the three banding polypeptide pattern with greater intensity, as anticipated for populations termed “MHR”. The results also indicated that there was an average 5.5 fold-increase in the expression of GST in the seven populations in comparison to the WTS (Roth 09) population.

Therefore, it can be hypothesised that the ten populations had varying degrees of resistance which can be associated to their varied expression of *Am*GSTF1. The differences between the immunoblot and ELISA could be linked to differences in sensitivity, as it is possible that the ELISA became

saturated during the screenings, especially when comparing the results to the established a standard curve (Figure 30) .

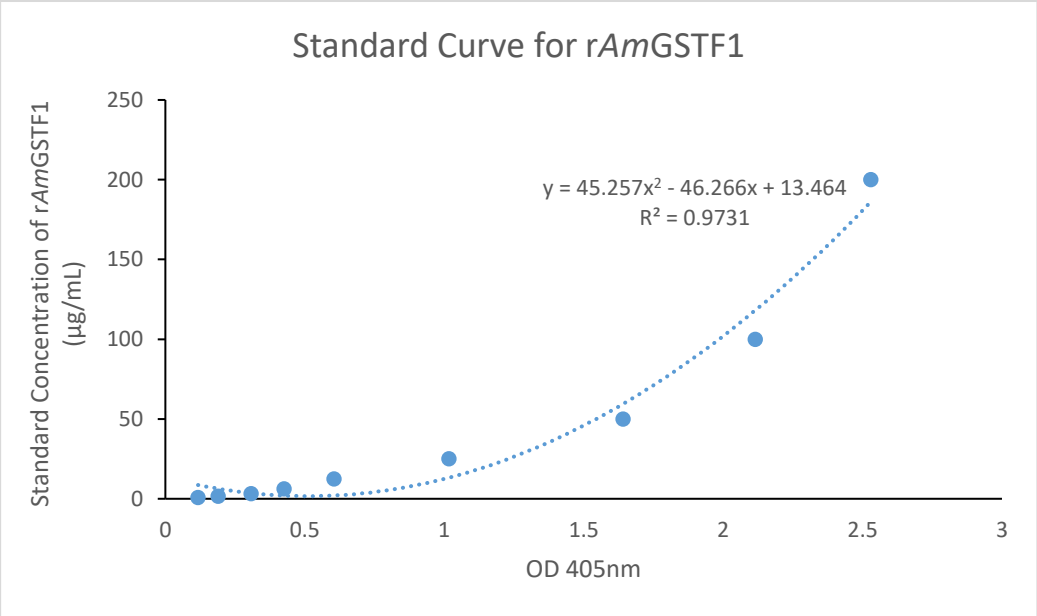


Figure 30 Standard curve for r*Am*GSTF1
Concentrations of r*Am*GSTF1 ranging from 0.781 µg/mL to 200 µg/mL were used to establish a standard curve based on the optical density (OD) 405nm of the samples when screened using an ELISA. (n = 3 replicates per concentration).

The ELISA screen did provide an opportunity to determine whether qualitative results from the immunoblot could be translated into a quantitative analysis, making the two techniques somewhat comparable in the absence of techniques or equipment which would normally quantify an immunoblot. However, it is possible to see that the “indirect” ELISA used in screens of black-grass, rye grass and wild oats needs further refinement.

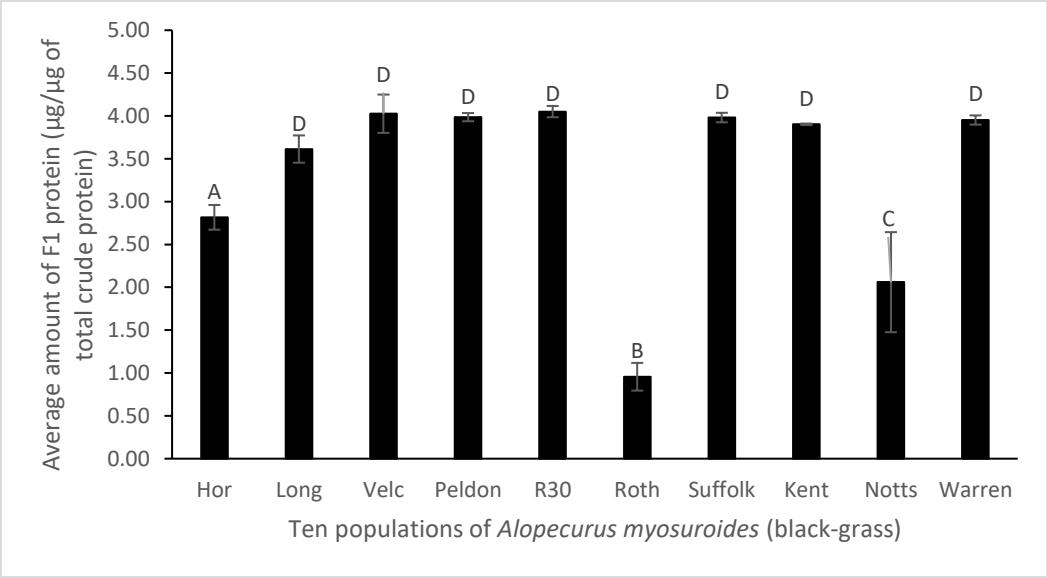


Figure 31 Bar chart showing the amount of *Am*GSTF1 orthologue expression in ten populations of *Alopecurus myosuroides*.
The ten populations were assayed by ELISA with each population loaded at 200 µg/ mL of crude protein extract in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to a final volume of 100 µL and probed with the primary antibody (Anti-*Am*GSTF1 serum diluted 1:500) and secondary antibody Anti-rabbit IgG (whole molecule) Alkaline phosphatase. (n= 3 replicates per population). ANOVA analysis was performed, bars with different letters indicates significant difference at ($P < 0.05$).

The ELISA was subsequently used on sixteen populations of rye grass (*Lolium rigidium*) and three wild-oat populations (*Avena fatua*) to determine whether the expression of *Am*GSTF1 homologues was similar to that seen with *Am*GSTF1 in crude protein extracts from black-grass. In Figure 32, the expression of *Am*GSTF1 orthologues was not too dissimilar to that seen in the crude protein extracts from black-grass. In contrast, the detection of *Am*GSTF1 orthologues in the western blot analysis with these populations was not successful (See Appendix- Figure S 1 and Figure S 2). The likely reasons for the results in Figure 32 was either the binding efficiency of the ELISA was much more sensitive, and/ or the amount of crude protein added was greater than in a western blot so the ELISA was saturated. Whilst the reason for the reduced expression of *Am*GSTF1 orthologues or lack of expression in the western blot maybe associated with the fact the antiserum was raised to the black-grass GST, or the way in which the antigen was presented in the ELISA. The rye grass and wild oat populations utilised in the screens had previously been assessed for resistance in studies by Cocker (2001, 2000); those which hadn't were screened by Rothamsted (Table 9).

In ryegrass, Trajan is a susceptible population (Table 6) however despite the results of the ELISA identifying this population as expressing the *Am*GSTF1 orthologue significantly lower (p value 0.000) than the remaining fifteen populations, it still contained a substantial amount of protein and

its expression was also greater than Roth09 in black-grass. Therefore, from the ELISA results it proved difficult to confirm that Trajan is a susceptible population. At the opposite end of the scale, Isk French expressed the *Am*GSTF1 orthologue at higher levels than Trajan and Adams, and has been designated as ALS TSR population (Table 9). Populations Clev (ACCase NTSR population) and Oak (MHR), were the next populations to over-express *Am*GSTF1 orthologue significantly above the standard susceptible Trajan. Surprisingly, Raven did not behave as anticipated as the data suggested that the population was comparable to the susceptible population.

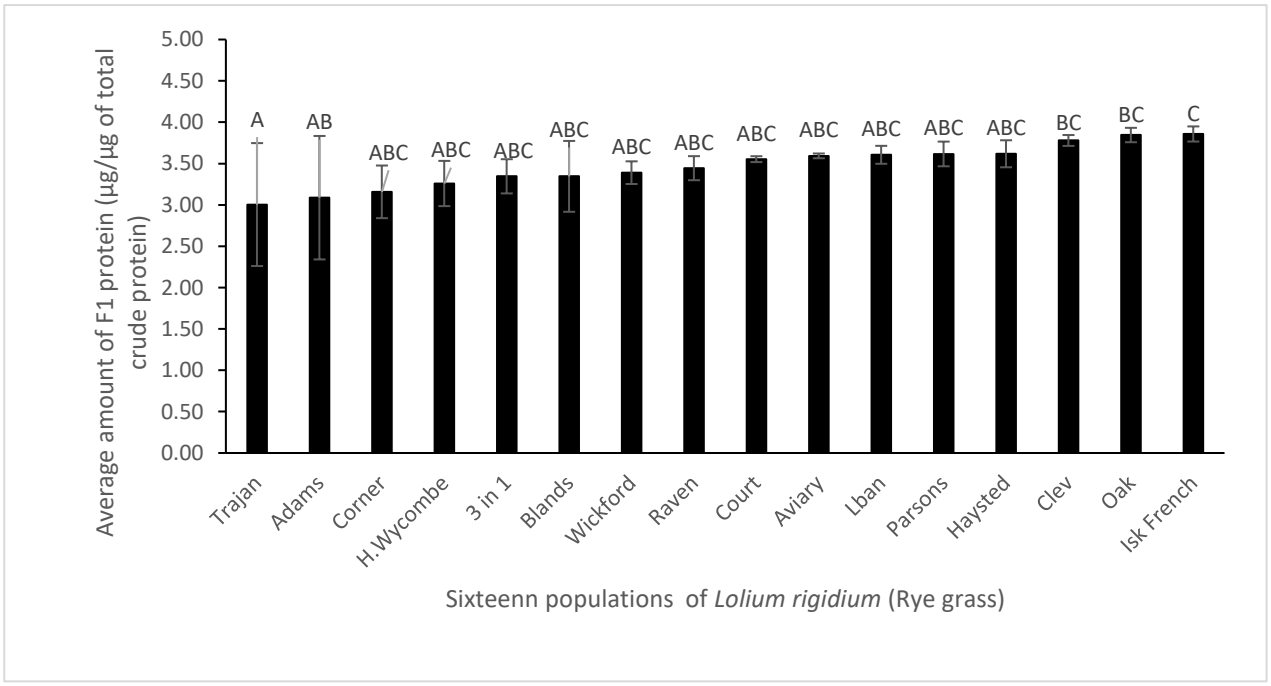


Figure 32 Bar chart showing the amount of AmGSTF1 orthologue expression in sixteen populations of *Lolium rigidium*.
The sixteen populations were assayed by ELISA with each population loaded at 200 µg/ mL of crude protein extract in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to a final volume of 100 µL and probed with the primary antibody (Anti-*Am*GSTF1 serum diluted 1:500) and secondary antibody Anti-rabbit IgG (whole molecule) Alkaline phosphatase. (n = 3 replicates per population). ANOVA analysis was performed, bars with different letters indicates significant difference at ($P < 0.05$).

Table 9 Resistance characteristics of sixteen populations of *Lolium rigidium* (Rye-grass) and three populations *Avena fatua* (Wild-oat).
A) Population denoted * data unknown, ** referenced in (Cocker et al., 2001). All other populations characterised by Rothamsted Research, Hertfordshire UK. "R" rating system according to Moss, S., (Moss et al., 2007). B) Population denoted * indicate referenced in (Cocker et al., 2000)

A) Rye-Grass (*Lolium rigidium*)

		ACCase		ALS		
Population	**County**	R rating to diclofop in pot test	TSR	R rating to Atlantis in pot test	R rating to sulfometuron in pot test	Syngenta ALS TSR assay
Trajan (SUSCE standard)**	*	S	X	S	S	*
LBAN (ACCase TSR standard)	Yorkshire	RRR	✓	S	S	*
CLEV (ACCase NTSR standard)	Wiltshire	RRR	X	S	S	*
Raven	Yorkshire	S	X	S	S	*
Aviary	Warwickshire	RR	X	S	S	*
Hayshed	Durham	RRR	X	S	S	*
Adams	Durham	RRR	✓	R?	S	*
Corner	Oxon	RRR	X	RR	R?	X
B-Lands 2	Essex	RRR	✓	RR	R?	X
Wickford	Essex	RRR	✓	RR	R?	X
High Wycombe	Bucks	RRR	X	RR	S	X
Oak	Kent	RRR	✓	RR	R?	X
Court	Kent	RRR	✓	RR	S	X
Parson	Oxon	RRR	X	RR	S	X
3 in 1	Shropshire	S	X	RR	S	X
ISK (French ALS TSR)	France	*	*	RRR	RRR	✓

B) Wild Oats (*Avena fatua*)

Population	**Resistance Traits**
T/11*	NTSR standard
T/41*	Fop specific ACCase TSR
New Hall	Most resistant population ever found to ACCase and ALS

The expression of *Am*GSTF1 homologues was also explored in wild-oat samples (Figure 33). The only sample that was significantly different was identified in population T11. This population was previously characterised as NTSR standard population (Cocker et al., 2000). The remaining two wild-oats populations are both resistant to ACCase inhibiting herbicides, however New Hall has been described in previous data as the most resistant population to ACCase and ALS inhibiting herbicides yet its expression of *Am*GSTF1 orthologue was not as high as T11 (Table 9).

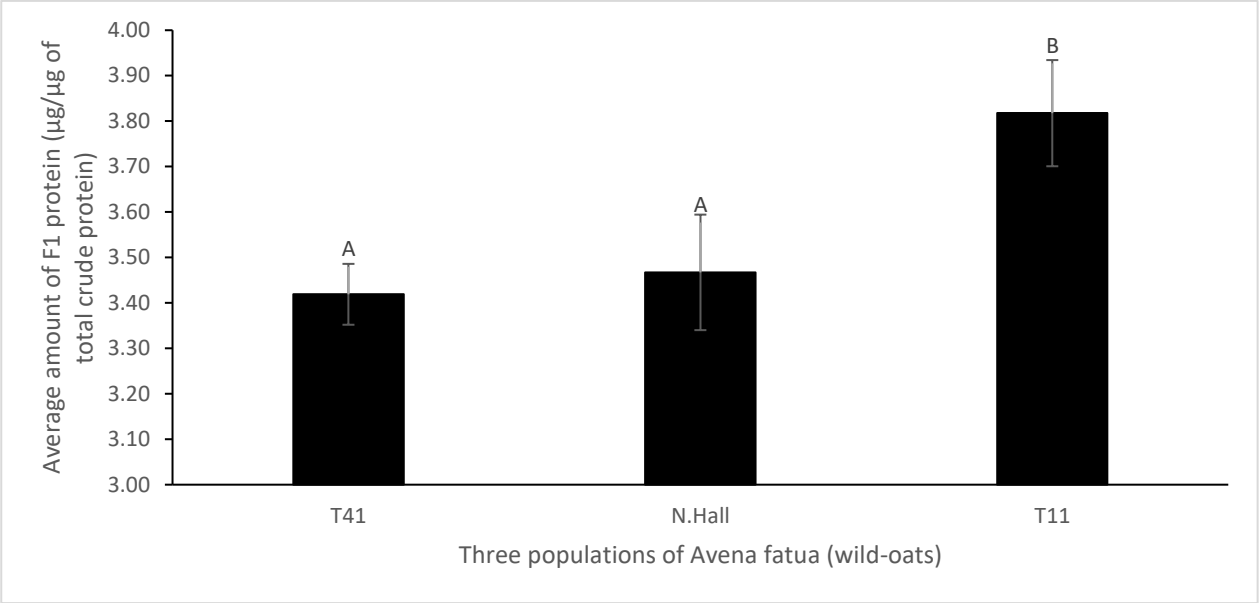


Figure 33 Bar chart showing the amount of *Am*GSTF1 orthologue expression in three populations of *Avena fatua*.
The three populations were assayed by ELISA with each population loaded at 200 µg/ mL of crude protein extract in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to a final volume of 100 µL and probed with the primary antibody (Anti-*Am*GSTF1 serum diluted 1:500) and secondary antibody Anti-rabbit IgG (whole molecule) Alkaline phosphatase. (n = 3 replicates per population). ANOVA analysis was performed, bars with different letters indicates significant difference at ($P < 0.05$).

The results from these screens indicated that the expression of *Am*GSTF1 orthologues were detected in both rye-grass and wild-oat populations. This meant that this method of assessment was versatile enough to be used in wild grasses other than black-grass. However, the studies on the different wild-type populations of the black-grass, rye-grass and wild oats indicated that there were potential limitations. Although as this assay was implemented in the latter stages of the project there was limited time to adapt the assay to account for some of the issues.

4.4 Novel Virus Biomarker

4.4.1 Introduction

In parallel to purifying the newly raised Anti-*Am*GSTF1 an investigation of a novel virus biomarker for MHR in black-grass was carried out with Federico Sabbadin (Edwards group member). The Edwards group had previously sequenced the transcriptome of both annual rye grass and black-grass populations using a non-targeted next generation sequencing (NGS) approach (Roche 454 and Illumina). The analysis was originally carried out to provide a more detailed understanding of the complexity leading to MHR resistance. It was known that selective pressures of abiotic and biotic stresses influenced the rate in which resistance traits were inherited (Powles and Yu, 2010, Cummins et al., 2013). Therefore, the Edwards group focused on looking for endophytes in black-grass and rye-grass, with specific interest in the potential role that microorganisms had as an environmental driver of MHR. Therefore the aim of the subsequent study I was involved with, was to identify whether there was a potentially more discriminatory marker for MHR in black-grass than *Am*GSTF1.

Federico Sabbadin identified the complete RNA genome of three viruses in the NGS datasets (Figure 34). These sequences were subsequently subjected to BlastX annotation and phylogenetic analysis based on the RNA1 sequences (Appendix 1- Figure S 3). This analysis indicated that two sequences were homologous to the dsRNA family of *Paritiviridae* and were subsequently termed *Alopecurus myosuroides partitiviridae* 1 (AMPV1) and *Alopecurus myosuroides partitiviridae* 2 (AMPV2) (Sabbadin, 2017). The third virus sequence found in black-grass showed some protein sequence similarity to the *Rhabdoviridae* family, but was most comparable to the *Varicosavirus* Lettuce big-vein (LBVaV) (Appendix 1-Figure S 3) (Sasaya et al., 2002, Walsh, 2012). The virus was termed *Alopecurus myosuroides varicosavirus* 1 (AMRV1). When the screen included samples from annual rye-grass two putative viral sequences were identified. These sequences indicated shared homology with the RNA2 of AMPV2.

The results indicated that there was similarities between the viral sequences identified in both black-grass and rye-grass. Subsequently, the occurrence an abundance of these new viral markers was assessed in both black-grass and rye-grass. The results indicated that the viral sequences were higher in the MHR plants than WTS in black-grass only.

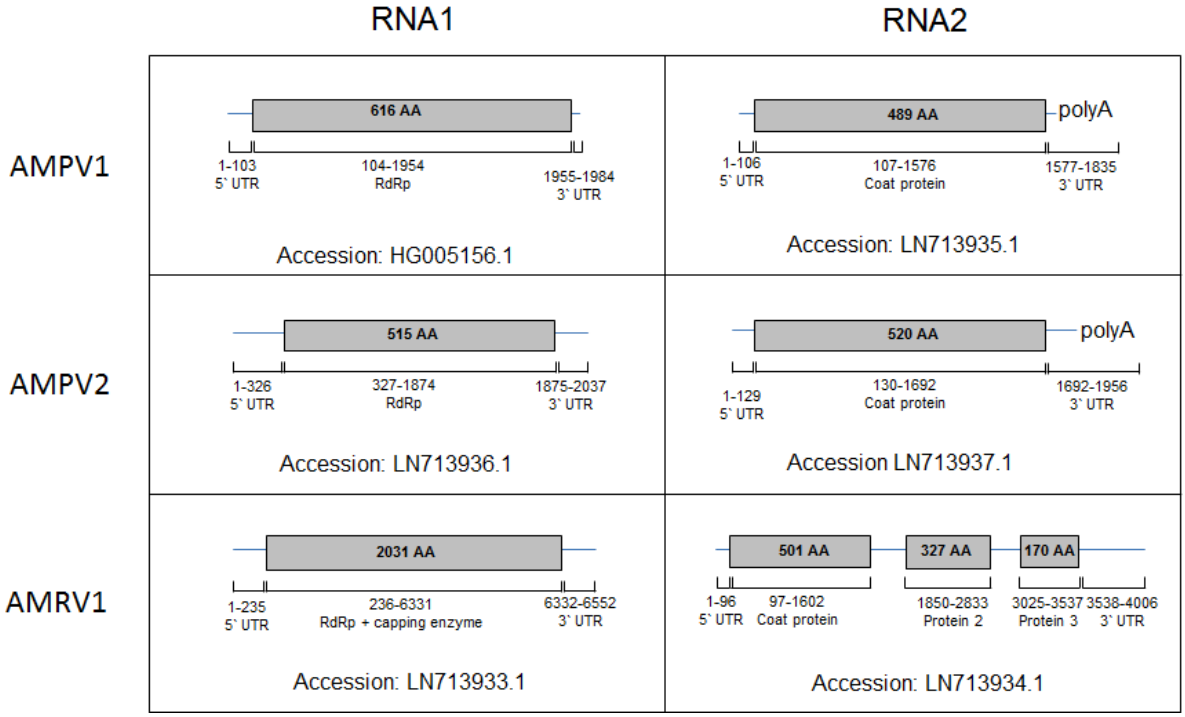


Figure 34 Schematic representation of the fully assembled genome sequences of the three viruses found in *A.myosuroides*.
Total nucleotide and protein length of the coded ORFs are indicated and the accession number for AMPV (*Alopecurus myosuroides partitiviridae*) and AMRV (*Alopecurus myosuroides varicosavirus*) (Sabbadin, F. 2017).

As a result of these novel findings it was of interest to carry out an extensive screen of these viral sequences in black-grass populations. The screen used RT-PCR to amplify the viral sequences in ten black-grass populations from different locations in the UK which included three populations of known resistance Peldon (MHR), Notts (TSR) and Roth (WTS).

4.4.2 Results

The results of the RT-PCR used viral sequences of interest identified in Figure 34 and Federico Sabbadin designed oligonucleotide primers for these sequences. The amplified sequences related to the RdRP gene of RNA1 and the coat proteins of RNA2 for AMPV1, AMPV2 and AMRV1 respectively. These primers were used to screen the ten previously characterised black-grass populations mentioned earlier in this chapter (Marshall et al., 2013, Moss, 2014). Figure 35 presents the results which indicated that the viruses being screened for were widespread, with at least one of the six RNA sequences expressed in all the populations that were screened. Interestingly, the relative abundance of the amplified products was very variable across all 11 populations. However, the populations which exhibited metabolism-based MHR had the greatest abundance of viral sequences. The majority of the populations indicated in the RT-PCR that they were able to amplify

both RNA1 and RNA2 components of each virus. Although Notts 2005 (TSR) for example only amplified one of the components, the RNA encoding RdRp of AMPV1 was expressed but there was no expression of the coat protein. A similar situation was seen in population Roth 2009 (WTS).

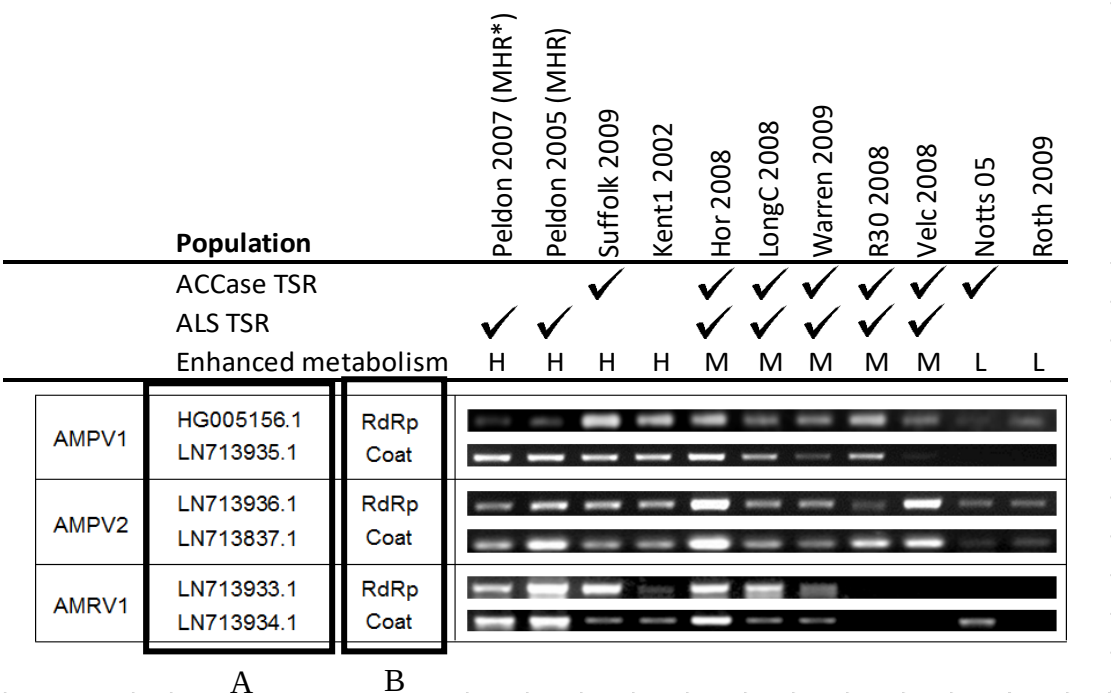


Figure 35 RT-PCR of the viral sequences for RNA1 and RNA2 of the three viruses identified in *A.myosuroides* populations.
The populations all had differing levels of resistance to herbicides. AMPV1,AMPV2 and AMRV1 denote the three viruses accession numbers found in box [A] which correlate to either the sequence for the RNA2 coat proteins or the RNA-dependent RNA polymerase (RdRp) gene of RNA1 found in box [B]. For reference the population *Peldon 2007 was used in the initial transcriptomic analysis and was compared to the ten populations.

Interestingly, the relative abundance of the both RNA1 and RNA2 was highly variable. For example, both Peldon 2007 and 2005, the RNA encoding the coat protein of AMPV1 was more abundant than the RdRp sequence (Figure 35). The results of this analysis also highlighted the issue of exploring an out-crossing species weed, like black-grass. As within the populations tested it was already known that individuals varied in their susceptibility to herbicides based on the occurrence of TSR mutations (Marshall et al., 2013, Marshall and Moss, 2008). The results of both Peldon 2007 and 2005 varied in the abundance of the six RNA sequences, which was testament to this. As despite the different years of collection they were harvested and sourced from the same location, thereby indicating a level of pre-existing variation, which may have been exasperated by the fact that for each population the samples were pooled.

Although the initial screens by Sabbadin, F. (2017) indicated the potential for a novel virus biomarker to detect MHR in wild grasses, these results indicated that the previously undescribed viruses were widespread in black-grass populations in the UK. However, due to the varied abundance within and between populations with the same resistance traits the viral sequences were not ideal for further protein studies. The most abundant viral sequences AMPV1 and AMPV2, did not invoke any visible physiological effects. They were members of the *Alphapartitiviridae* group and are known to be stably inherited in many crops and wild species (Roossinck, 2015). However, as the findings of this study were delivered relatively late, there was no time to assess the TSR populations for their incidence of viral infections and the means of infection. It would have been interesting to know the extent of variation that existed within a population through isolating individuals within a population and testing for the viral sequences. It would also have been useful to carry out the Koch's postulates experiment to see the inheritability of the viral traits, and whether this primed the plant against further abiotic and biotic stresses (Dixon et al., 2011).

4.5 Discussion and conclusion

GSTs have been associated with involvement in detoxifying herbicides, through the conjugation of xenobiotics (Cummins et al., 2013). As described in the introduction, the plant xenome is known for its associated upregulation in response to xenobiotics and involves a four phase pathway of detoxification (Cummins et al., 2011, Edwards et al., 2011). The general aim therefore was to develop a more specific and sensitive diagnostic for the detection of *Am*GSTF1, which was involved in phase two of xenobiotic detoxification. This meant that subsequently the analysis could move towards a quantitative analysis. The developed and purified antibody proved to be more sensitive to *Am*GSTF1 protein than originally anticipated, although further refinement is still required especially for its use in an ELISA. Interestingly, the anti-*Am*GSTF1 serum detected a novel pattern of three polypeptides of 25 kDa, 24 kDa and 22 kDa when used in screenings of black-grass. Intriguingly, the relative abundance of the *Am*GSTF1 polypeptides of differing relative molecular mass varied between MHR populations, suggesting the presence of multiple component isoenzymes. The three bands were detected throughout the screens of black-grass material although to varying degrees, it could be hypothesised therefore that this was due to the increased sensitivity of the newly synthesised polyclonal antibody, and /or a result of improved epitopes.

For future development of this diagnostic approach, the identified polypeptides cDNA needs to be sequenced so that the specific isoforms of *Am*GSTF1 a,b,c or d can be identified (Cummins et al., 1999). As the polypeptides were expressed differentially, this means that there is potential to

identify different types of MHR and the pathways conferring their resistance. The integrity of the proteins that were identified by the newly raised antiserum were assessed and were found not to be the result of protein degradation. There is also potential to explore the crystal structure of the antigen-antibody substrate through crystallography, thereby helping establish the exact tertiary binding structure of the antigen in different populations of black-grass. The results of which, may offer potential new avenues of control or a greater understanding of the variation of resistance between and within populations of the same biotype. Although the antiserum identified three polypeptides, there remained a few issues with the protein assessment as it was qualitative and remained optimised for detecting GSTF1 in black-grass. Therefore, an ELISA was used to screen populations of different wild grasses and generate a quantitative analysis. Initially the assay was a promising method to quantify and differentiate between MHR, TSR and WTS populations of black-grass. The assay also offered an opportunity to quantify the differences both within and between populations of wild grasses. As black-grass is known to be a cross-pollinating species there are high levels of variation within populations (Marshall et al., 2013).

Despite the ELISA being optimised with the recombinant *Am*GSTF1 protein there were limitations with the detection. These limitations were seen in the results (Figure 31, Figure 32 and Figure 33) as the assay appeared to be saturated, which potentially masked the extent of GSTF1 expression (Figure 30). On reflection it was likely that as the assay was developed using recombinant *Am*GSTF1 there were issues when samples were extracted from crude protein, or a cross-reactive residue from the extraction which limited the detection. Therefore, further development was required as it had potential to be used in a wide variety of resistant wild grasses. The investigation of a potential viral biomarker indicated a growing awareness of the presence of persistent viruses in crops and weeds. This investigation offered an insight into the role of the microbiome on the plants' health through the expression of AMPV1, AMPV2 and AMRV1 viruses, by focusing on the potential for the infections to affect the plants health and general defence to abiotic and biotic stress. Marquez et al. (2007) indicated an interesting mutualistic association between a fungal endophyte and a tropical panic grass which enabled the two to grow at high soil temperatures. This was an example of a three-way symbiosis enabling tolerance to an abiotic stress. Therefore, further work will be needed to establish whether the viral infections are beneficial to MHR wild grasses as they may have been priming the populations for further stresses eg. Herbicidal treatments. This required the use of a Koch's postulates experiment to establish both the means of transmission and the subsequent generations resistance traits.

In conclusion, the new Anti-*Am*GSTF1 serum was found to be sensitive in an immunoblot and indicated that there was further potential for its use in an ELISA. This assay method requires further development but appeared to be a promising assay. The assay had increased sensitivity to different biotypes of wild grasses, it was inexpensive, quick and provided a quantitative analysis of resistance. Although, a novel virus was identified this did not prove diagnostic for resistance; despite the virus's presence in MHR 'Peldon' in the initial screen, other MHR black-grass populations did not constitutively express the virus. Whilst some described as WTS did, meaning that the virus could not be used as a reliable marker for herbicide resistance. The presence of virus sequences remained a point of interest but currently a causative link between infection of black-grass with either AMPV or AMRV and the occurrence of herbicide resistance remains unclear. However the literature indicates that such infection should enhance the plants' tolerance to abiotic stress and general fitness (Dixon et al., 2008, Marquez et al., 2007) thereby providing an improved genetic pool for MHR to evolve. Further study regarding the links between herbicide resistance and the infection of persistent viruses will be required.

Chapter 5. Novel Molecular Diagnostics

5.1 Genomic Analysis of Xenome Genes

5.1.1 Introduction

In recent years, the use of DNA sequencing data has changed due to the development and emergence of next generation sequencing (NGS), which has inevitably increased our knowledge of genomics exponentially (Egan et al., 2012). NGS has been used in plants since its early development where its main purpose was for plant breeding. NGS has been used in plants as it provided a genetic analysis of: sequence conservation, abiotic and biotic stress markers and most interestingly, the identification of metabolism-based resistant biomarkers. It is for this reason that NGS was employed in this study. NGS technologies provided many advantages for characterising transcriptome-wide gene expression, namely the digital quantification of known reference sequences (Morozova and Marra, 2008). It was also possible to use the system on non-model species such as weeds, as a reference transcriptome could be used even when no previous transcriptome data was available, enabling the quantification of relative expression levels.

As this project previously outlined, current assays for the detection of MHR in wild grasses are both time consuming and expensive. The current assays relied on were principally, spray trials or petri-dish growth analysis, all of which were dependent on seed collected prior to crop harvesting (Reade, 2000). Despite the sensitive Anti-*Am*GSTF1 antibody development and use as a protein markers, there was still a necessity to develop a quantitative diagnostic for herbicide resistance in black-grass. This chapter focuses on the implementation of NGS analysis of *Alopecurus myosuroides* most and least resistant populations, Peldon (MHR) and Roth (WTS) respectively by IonTorrent. The aim was to identify potential functional biomarkers of metabolic based resistance by exploring the regulation of xenome genes. Previous studies had identified that MHR black-grass was associated with the up-regulation of xenome genes. Subsequently it was identified that the rates of detoxification were dependent on the classes of graminicides the plants were exposed to.

This study aimed to develop a diagnostic ‘toolkit’ which provides: an opportunity (a) to establish whether MHR is linked to all potential routes of herbicide detoxification, (b) to quantitatively analyse resistance and (c) to predict the economic future and the environmental implication of chemical mechanisms of control. The results also offered an opportunity to further decipher the complexity of xenome regulation in response to abiotic and biotic stresses. Furthermore the information would help decision making when graminicides chemistries were selected, ensuring

less susceptible chemistries were applied. This genomic analysis of the xenome has the capacity to be coupled with data from the proteomic screens which provided a two-tiered approach to detecting and quantifying herbicide resistance in a wide range of black-grass populations. It also provided a real-time data approach. RNA-seq transcriptome analysis was used on black-grass to identify and validate the differential expression of specific genes associated with MHR, TSR and WTS individuals. It was hypothesised that the expression of the xenome markers would differ depending on the associated metabolic resistance. These markers could then be used as a decision tool to aid the implementation of the appropriate alternative strategies when facing herbicide resistance.

Previous studies have investigated the transcript expression in grass weeds including: *Lolium rigidium* (Gaines et al., 2014, Duhoux et al., 2015, Duhoux and Delye, 2013), *Alopecurus myosuroides* (Gardin et al., 2015), *Festuca arundinacea* (tall fescue) (Hu et al., 2014) and a wild relative of wheat (Bouyioukos et al., 2013). However, with respect to data produced from the screens none of the biomarkers identified have been taken any further forward. No biomarkers currently exist in a format like that of micro-array which are used as a research diagnostic tool with the potential for in-field analysis.

In relation to these studies, a transcriptomic analysis of *Lolium rigidium* identified genes involved in metabolism-based resistance to diclofop Gaines, et al. (2014). The study identified two P450s, one nitronate monooxygenase (NMO) and one glucosyltransferase (GT) as constitutively highly expressed in nine field-evolved metabolically resistant *L.rigidium* populations. This study confirmed prior knowledge concerning the resistant populations' capacity to rapidly metabolise diclofop in comparison to the susceptible counterpart (Yu et al., 2013). It was apparent that the four contigs were not just constitutively over expressed in the Australian resistant population but also in a genetically unrelated French metabolic diclofop-resistant *L.rigidium* population. The evidence from this study highlighted three things: (1) increased expression of these four genes contributed to diclofop resistance, (2) diclofop resistance was under quantitative genetic control with the high expression of multiple genes conferring resistance and (3) linked to inheritance studies (Busi et al., 2013, Busi et al., 2011).

These results combined with Gardin et al. (2015) analysis of black-grass indicated that gene regulation was at the root of herbicide response and of MHR. These transcriptomic analyses offered an invaluable insight into the genetic basis of herbicide stress responses, and the differences both between resistant and susceptible plants before and after herbicide application. It represented a

major step forward in understanding the evolutionary and ecological functions of genetic traits, which ultimately had an impact on plant fitness (Gaines et al., 2014).

It was predicted that in this investigation based on Gardin et al (2015) analysis of black-grass three cytochrome P450s were identified which shared homology to those found in *Lolium rigidium* (Gaines et al., 2014) and *Lolium sp* (Duhoux et al., 2015) respectively. Therefore, there was potential for homologs of cytochrome P450s to be found in the MHR population of black-grass. It highlighted that there was potential for biomarkers which could be associated with MHR in black-grass and other resistant wild grasses.

5.2 Next Generation Sequence analysis of *Alopecurus myosuroides*

Understanding the success behind herbicide resistant black-grass required unravelling the genetic basis of MHR. This was achieved through Next Generation Sequencing (NGS) which generated a comprehensive transcriptome sequence (RNA-Seq) which produced both qualitative (transcript sequences) and quantitative data (transcript expression level) with a high level of sensitivity and accuracy (Lister et al., 2009, Martin and Wang, 2011). The NGS analysis was performed by colleagues in the group and the full results are in preparation for publication. The data abstracted from the study presented here is restricted to the identification of the DNA biomarkers used in this PhD project by the author.

NGS was used on the Rothamsted and Peldon populations which had the lowest and highest capacities to detoxify herbicides respectively. The populations were grown for 14 days and used to prepare cDNA libraries in biological triplicate. Subsequently, these were used for RNA-Seq analysis by *De novo* IonTorrent sequencing. The analysis was sequencing through synthesis where base composition was determined through the detection of chemiluminescence. This reaction was formed by nucleotide incorporation during the synthesis of a complementary DNA strand by DNA polymerase (Egan et al., 2012). As there was no existing reference sequence, overlapping RNA sequences (contigs) were assembled to construct unigenes corresponding to the expressed genome of WTS and MHR plants. This analysis was termed *de novo*.

5.2.1 Next Generation Sequence Results of *Alopecurus myosuroides*

The Edwards Group preliminary *de novo* NGS results generated a total of 38,3149 contigs which were assembled and blasted against the uniprot database, of which 2908 were matched to genes identified in the uniprot database (Magrane and UniProt, 2011). The unigenes were assessed based on the differential transcript abundance which was calculated as the fold-difference in the number

of successful alignments between reads and contigs in MHR compared to WTS lines. After normalisation, 4,724 unigenes based on relative abundance were identified as significant, as they varied more than 2-fold difference between MHR and WTS populations. The original analysis identified that MHR black-grass had 1537 up-regulated and 1371 down regulated transcribed genes in comparison to WTS. However, further analyses performed by the Edwards group included the use of Mercator annotation tool which assigned the genes with specific functions (Lohse et al., 2014); subsequently the range of gene classes identified decreased.

The analysis performed by the Edwards group identified numerous pathways of interest. Primary metabolism, in MHR plants had a lower proportion of transcripts associated with glycolysis, carbohydrate metabolism and fermentation, all of which are photosynthetic components of plants. In comparison, the WTS genes involved in lipid, cell wall, nucleic acid and amino acid biosynthesis were lower in abundance than in MHR. As predicted genes associated with xenobiotic metabolism were highly upregulated in MHR plants. This was the most interesting link between genotype and phenotype, as MHR in black-grass had previously been associated with an increase in thiol glutathione, a marker of detoxifying enzyme activities (Cummins et al., 2013) from these preliminary findings it was necessary to investigate the upregulation of the xenome genes in MHR black-grass in greater detail.

5.2.1.2 *Regulation of xenome genes in MHR, TSR and WTS black-grass*

In the preliminary step of validation, qPCR primers were prepared for each of the twenty-four genes in order to quantify the relative level of transcription in WTS and MHR plants relative to those of the transcriptome sequencing. The 24 genes were selected based on their initial transcriptional expression levels in MHR vs WTS, as they were found to be in the top 20 upregulated for each of the xenome phases. In selecting the potential biomarkers it was predicted that the inclusion of markers from all four phases of the xenobiotic detoxification pathway would give a more detailed explanation behind the mechanisms of MHR. A few pathogenesis related proteins were also included as there was potential for these putative resistance biomarkers to be linked to abiotic and biotic stresses. Therefore, due to the number of biomarkers selected to represent each phase of the xenome there was an increased chance that a clearer genetic understanding of the pathways could be gained, so much so that a collection of markers could potentially help distinguish between TSR, MHR and WTS more quantitatively. The known TSR population Notts 05 was also included in the initial qPCR screen to aid the identification of any markers which may prove diagnostic for TSR over MHR. Interestingly, the overall picture was the same when comparing the qPCR validation

and that of the IonTorrent analysis for MHR and WTS. Although, the level of up-regulation in qPCR data was generally lower than that of the IonTorrent analysis. *Am*OPR1 was 70 fold more abundant in the MHR plants as compared to WTS, in comparison of a 101 fold change from the IonTorrent sequencing. The qPCR result did not alter the significance of the differential gene expression. It was clear to see similar patterns of differential induction (MHR vs WTS) with the other biomarkers as well, see Table 10.

Table 10 qPCR biomarkers used to screen Rothamsted (WTS), Peldon (MHR) and Notts (TSR) *A.myosuroides* and the relative fold-change in expression level.

Represents all 24 genes used in the screen of well characterised *A. myosuroides* populations and are categorised according to the phase of detoxification in the plant xenome they are associated with. Data normalised using the expression of Rothamsted (WTS) with the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) vs. the expression of the genes of interest*.

				Rothamsted (WTS)		Peldon (MHR)		Notts (TSR)	
Phase of Detoxification	Contig name	Description	Reference Name	Average fold-change	stdev	Average fold-change	stdev	Average fold-change	stdev
Phase 1**									
	R00041432	P450 secologanin synthase	c1432	1.14	0.12	4.02	0.26	4.8	0.2
	R00030509	P450 secologanin synthase	c0509	1.09	0.08	3.01	0.19	6.9	2.14
	R00027925	P450 secologanin synthase	c7925	1.04	0.08	3.43	0.08	3.21	2.27
	R00027289	Isoflavone hydroxylase	c7289	1.06	0.05	2.29	0.12	4.13	0.52
	R00232027	OPR1	c2027	0.97	0.04	51.49	2.01	1.5	0.24
	R00052459	OPR1	c2459	1.30	0.35	71.09	7.36	2.13	0.58
	Rm00002116	OPR1	c2116	1.15	0.14	50.90	2.02	1.54	0.6
	R00029421	Carboxylesterase	c9421	1.00	0.18	4.41	0.48	1.62	0.2
Phase 2									
	R00029215	Zeatin UGT	c9215	1.21	0.19	14.38	1.51	1.1	0.9
	R00007921	GSTU6-like	c7921	1.04	0.04	5.31	0.49	0.98	0.57
	R00030700	GSTU6-like	c0700	1.01	0.09	18.40	0.11	NA	NA
	R00005793	GSTU6-like	c5793	1.15	0.15	24.30	0.90	5.1	1.25
	R00096975	GSTU6-like	c6975	1.00	0.08	27.77	1.14	2.72	0.52
		GSTF1	GSTF1	1.10	0.10	8.30	0.39	0.93	0.04
	R00010869	Aminotransferase	c0869	1.03	0.03	27.94	5.93	3.13	1.41
	R00029959	Cellulose synthase	c9959	1.39	0.34	6.03	0.95	1.67	0.34
Phase 3									
	Rm00043661	ABC transporter	c3661	1.08	0.15	1.71	0.10	0.85	0.07
	R00030815	MATE transporter	c0815	1.13	0.13	4.16	0.25	9.21	3.02
Phase 4									
	R00000345	Thiol methyl transferase	c0345	0.93	0.08	18.06	0.28	3.36	0.6
	Rm00016513	Thiol methyl transferase	c6513	0.84	0.14	18.30	0.67	7.02	1.85
	Rm00004119	Thiol methyl transferase	c4119	1.06	0.09	14.91	0.56	5.14	1.2
Other									
	R00029303	Pathogenesis related protein	c9303	0.98	0.04	1.22	0.15	2.63	0.44
	R00003857	Pathogenesis related protein	c3857	0.97	0.05	16.58	0.31	2.47	0.28
	R00004163	Gag-pol retrotransposon	c4163	0.85	0.17	4.32	0.27	42.47	4.45

*Samples were run in technical triplicates which were generated from three biological replicates

**Colour of phases linked to diagram in Figure 3

The initial validation screen of 24 gene biomarkers indicated the potential for developing biomarkers representing all phases of the xenome. There were several genes that exhibited differential expression across all three resistance characteristics, MHR, TSR and WTS.

In Phase I the CYPs were up-regulated in MHR (2-fold) more than WTS, although due to the nature of the partial sequences obtained when carrying out the NGS analysis coupled with the complexity of the gene family, it proved impossible for the Edwards group to assemble any of the contigs as full length sequences (Nelson, 2008). However, the Edwards group initial NGS analysis indicated that half of the up-regulated contigs were separated into Clan 71 and Clan 72, of which CYP72, CYP76 and CYP81 families belonged to Clan 71 whilst CYP709 families were most similar to Clan 72.Orthologues of the black-grass CYPs have been found to be up-regulated in late watergrass (*Echlinoahloa pyllopogon*) biotypes. These biotypes have subsequently been identified to have developed resistance to multiple herbicides, including fenoxaprop-ethyl (Yun et al., 2005). Similarly in annual ryegrass (*Lolium rigidium*), the majority of CYPs that have been found to be associated with up-regulation in biotypes with resistance to iodosulfuron and mesosulfuron, pyroxsulum or diclofop were members of either the CYP81 or CYP72 subfamilies (Duhoux and Delye, 2013, Gaines et al., 2014). With regards to TSR the CYPs were also up-regulated however there was a noticeable difference between the expression of the contig R00030509 which was over expressed 6.9 fold in comparison to MHR's 3.01 fold-change. This indicated the potential for CYPs as a marker to differentiate between MHR and TSR populations.

Of all the genes used in the preliminary screen for Phase I, the most significantly expressed were OPRs. Table 10 indicated that the three genes ranged from 50 to 70 fold increased expression relative to WTS Rothamsted. OPR was also a potential biomarker to differentiate between MHR and TSR, as TSR still over-expressed the gene in comparison to WTS but was significantly down-regulated in comparison to MHR. Following a closer examination by the Edwards group of the OPR contigs it was identified that eight of the nine sequences from the original NGS analysis mapped to OPRs, were highly similar with overlapping coding regions. This enabled a theoretical full length OPR sequence to be constructed and was termed *Am*OPR1 (Figure S 4).

A further analysis of the relationship between the putative *Am*OPR1 and other plant OPRs indicated that *Am*OPR1 belonged to the larger phylogenetic group I (Abu-Romman, 2012) as opposed to the well characterised group II (Figure S 5). Group II members are known to

catalyse the conversion of 12-oxo-cis-10, 15-phytodienoate to 3-oxo(-cis-2-pentenyl) cyclopentane-1–octanoate, a key step in jasmonic acid (JA) biosynthesis (Schaller, 2001, Schaller and Weiler, 1997a, Vick and Zimmermann, 1979). Group I OPRs remain less characterised however they have been identified in *Arabidopsis*: *At*OPR1 and *At*OPR2, along with group II *At*OPR3 which are known for their capacity to transform the explosive 2,4,6-trinitrotoluene (TNT) to yield nitro-reduced derivatives (Beynon et al., 2009).

Several Phase II namely glutathione transferase genes were screened as they were known for their association with xenobiotic detoxification. From the NGS data MHR black-grass had the largest number of contigs which encoded phase II enzymes from both GST and UGT classes as upregulated in comparison to WTS plants (Table 10). Previous examination by the Edwards group identified that of the contigs belonging to the Phi class 20 out of the 24 shared high similarities to *Am*GSTF1a, *Am*GSTF1c and *Am*GSTF1d isoforms, of the protein previously identified as being functionally linked to MHR in black-grass (Cummins et al 2013).

From the preliminary screen contig GSTU6-like (R00096975) was over 25 times over-expressed in MHR compared to WTS and TSRs 2.7 fold-expression. This biomarker indicated the potential use as a marker for Phase II of the xenome detoxification pathway. The GSTs were also useful markers as they were suppressed in TSR populations which mirrored the behaviour seen when screened in an immunoblot test (Cummins et al., 1999). It was also discovered that the GST contigs from the NGS analysis accounted for 40% of the total xenome contigs identified in the MHR line, therefore the preliminary screen had identified the potential value of having a GST in any genomic screen for resistant biotypes in black-grass or wild grasses. In addition, a large number of tau class (GSTU) sequences were identified which included two contigs with high similarity to *Am*GSTU1. GSTUs are known to be the most abundant class of GSTs in plants, with clearly defined roles in herbicide detoxification in rice, wheat and maize (Cummins et al., 2011, Sharma et al., 2014, Hu et al., 2009, Milligan et al., 2001, Thom et al., 2002), although previous attempts to identify members of the GSTU class of protein as up-regulated in resistant black-grass had failed when raising an antiserum to *Am*GSTU (Cummins et al 1999).

Therefore, these results indicated the potential benefits of gene biomarkers for GSTs, especially for the tau class as it offered a mechanism of quantitative assessment in resistant black-grass as opposed to qualitative analysis by immunoblot (Cummins et al. 2009).

In Phase III the plant ABC transporters (ABCs) were found to be up-regulated in MHR 1.71 fold but only 0.85 fold in TSR black-grass. ABCs were found to play an important role in the transport of xenobiotics in to the plant vacuole (Martinoia et al 1993). The data from the NGS analysis indicated that a significant number of ABCs were down-regulated in MHR black-grass which was unusual in comparison to the other xenome genes identified in the study. The ABC utilised in this screen belonged to subfamily C which is commonly found to be localised in the vascular membrane (Rea et al., 2007). Further to this, the multidrug transporter proteins found to be up-regulated in MHR were Multidrug and Toxic Compound Extrusion efflux family proteins (MATEs). The MATE used in the preliminary screen was assigned to the phylogenetic cluster I (Chen et al, 2015) which has been recently characterised in *Arabidopsis thaliana* (AtATT12), *Oryza sativa* (OsMATE1) and *Medicago truncatula* (MtMATE1) (Takanashi et al., 2014). The MATE1 proteins have been identified in plants for their involvement in the transport of secondary metabolites such as alkaloids (Shoji et al., 2009), flavonoids (Zhao and Dixon, 2009, Debeaujon et al., 2001), regulation of disease resistance (Sun et al., 2011, Nawrath et al., 2002, Ishihara et al., 2008) and aluminium detoxification (Zhou et al., 2013, Wu et al., 2014). All of these indicated how MATE transporters played an important role in a wide range of biological processes in plants (Liu et al., 2016).

Phase IV markers consisted of the thiol methyltransferases (TMTs), enzymes involved in the downstream processing of thiol-containing catabolites of S-glutathionylated agrochemicals (Brazier-Hicks et al., 2008a). Further to this the Edwards group identified that the TMTs in this study were distinctive from the N-,O- and S-methyltransferases (Attieh et al., 2002), which made them increasingly interesting as biomarkers for herbicide resistance in black-grass.

Therefore, the results from the preliminary study indicated that the NGS results were comparable to the results from the real-time qPCR analysis of the candidate genes/ biomarkers, although the absolute fold-change between the samples tended to be lower in the qPCR validation in comparison to the IonTorrent analysis. The biomarker genes were in general upregulated in the MHR population relative to the WTS plants. This reflected the different reaction types associated with the four phases of xenome detoxification. To assess the robustness of the biomarkers the number of genes involved in the screen was significantly reduced. The genes selected were *Am*OPR1, CYP709, *Am*GSTF1, UGT, MATE, ABCI and TMT for real-time qPCR analysis. The rationale for the selection of these putative marker

genes was a combination of their expression in the preliminary screen involving 24 potential biomarkers, their relative sequence length and their similarity to known genes.

5.2.2 Xenome RNA biomarkers screening of Unknown Populations

To continue the investigation the previously characterised ten independent populations of black-grass showing a combination of WTS, TSR and MHR traits were screened. The genes used were grouped into the functional classes which underpinned the four phases of the xenome: three phases of detoxification, and the fourth phase associated with processing the conjugated and sequestered products of detoxification. The xenome genes selected to screen and validate ten different populations of black-grass were: *Am*OPR1, CYP709, *Am*GSTF1, UGT, MATE, ABCI and TMT, further to this a pathogenesis related protein (PRP) was also included. The specific focus in this secondary screen was on the genes involved in the four phases of xenobiotic detoxification (Table 11), with the phases colour coded to the xenome detoxification pathway in Figure 3.

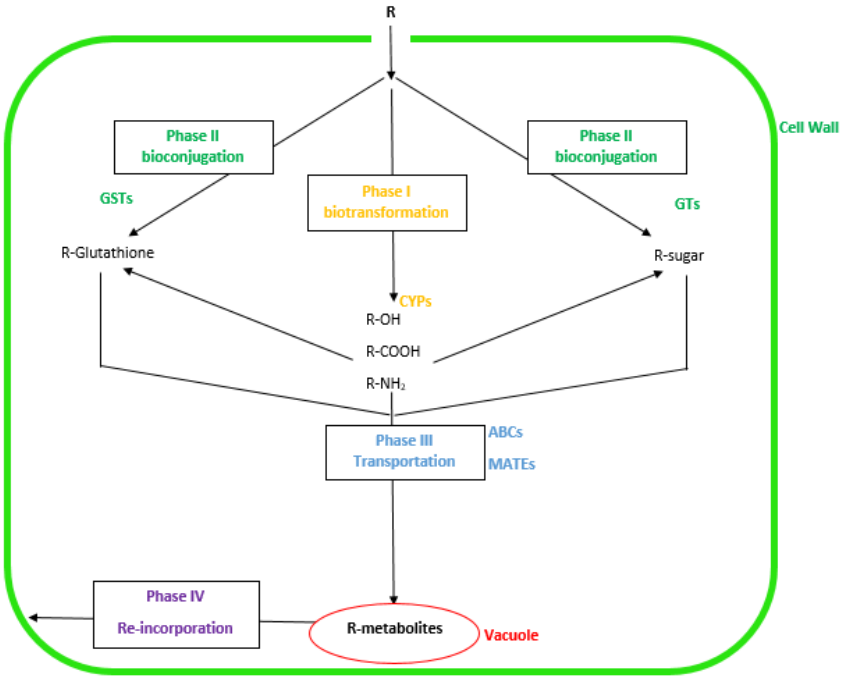


Figure 3 Generalised schema of the plant 'Xenome' indicating the four phases of xenobiotic detoxification.
R; xenobiotic, CYPs; cytochrome P450, GSTs; glutathione transferases, GT; glycosyltransferases, ABC; adenosine triphosphate binding cassette transporter and MATE; Multidrug and Toxic compound Extrusion protein.

The putative biomarkers: *Am*OPR1, CYP709, *Am*GSTF1, ZUGT, UGT, MATE, ABCI, TMT and PRP were tested in biological triplicate for their quantitative expression in the ten black-grass populations characterised earlier.

Table 11 Xenome genes utilised in the real-time qPCR analysis of ten populations of *A.myosuroides*.
The xenome genes are separated into the relevant phases of the detoxification pathways. The primers for *Am*OPR1 and UGT were different from the original screen (Table 10) the same house keeping gene was used to ensure the results would be comparable from the initial 24 gene screen.

Phase of Detoxification	Description	Reference Name	F primer	R primer
Phase 1 *				
CYP709	P450 secologanin synthase	c7925	TGATTCTTCAGAGGTTCTCCTTCTC	GGGCCGCAGCGTGAT
AmOPR1	OPR1	c2459	GGGCCGCAGATTAGTTTTGA	GTCATCGACTATCCCGGGAAT
Phase 2				
ZUGT	Zeatin UGT	c9215	GCAGCAAGCAGAGGTTCATCT	TCGCCGGACTCTGCAAAT
AmGSTF1	GSTF1	GSTF1	AGCATAAGAGCCCCGAGCAC	CCGTCCTGGAAAGCAGGGATTTG
UGT	GSTU6-like	c6975	TCCCTGGTCATCGTGCAGTA	GGGTCGGAGGAAAGCAATG
Phase 3				
ABCI	ABC transporter	c3661	TGTGGTGCAGGAAATGGTATTTT	TGGTCTGCTGCCCTGCAT
MATE	MATE transporter	c0815	CCTTCACCATCCTCCTCAACA	GCCATCCTGACCCAATCG
Phase 4				
TMT	Thiol methyl transferase	c0345	ACCCTCATGTACCTGCCTCAA	TCGAGCACCGTGGTGTTGT
Unknown				
PRP	Pathogenesis related protein	c3857	GCTTCGCCATCGAGGTGAT	GTACCCCCAGTGACGGAACTT
Reference Gene				
	Glyceraldehyde 3 P dehydrogenase		GAGGCTGGTGCTGACTACGTT	TGAGCTGCGGCCTTGTC

*Colour of phases linked to diagram in Figure 3.

5.2.2.1 *Results of real-time qPCR screening of Unknown Populations*

The results were presented in a heat map format which indicated the levels of relative expression in the different lines relative to the WTS Rothamsted population (Figure 36, Figure 37 and Figure S 6). The highly up-regulated genes are shown in red, moderately induced indicated in orange and down-regulated in green. From the data presented four of the xenome genes proved to be highly diagnostic for MHR the most quantitative were CYP709, GSTF1 and GSTU genes. In contrast, the UGT, MATE, ABCI and TMT genes proved poor indicators of MHR. Although OPR1 was a relatively sensitive marker of MHR, its relative expression was not symptomatic of the level of metabolism based resistance indicated by the biotypes of black-grass.

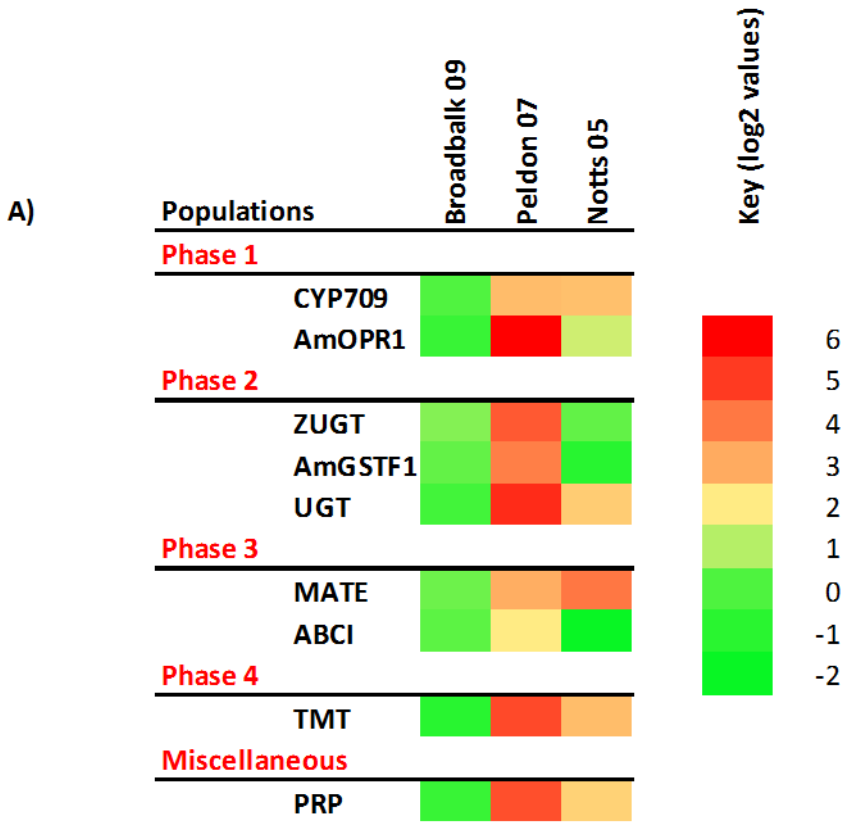


Figure 36."Heat map" of relative expression levels in three known biotypes of *A.myosuroides*. A) Three populations of Alopecurus myosuroides (black-grass) with characterised resistance: Broadbalk 09 (WTS), Peldon 07 (MHR) and Notts 05 (TSR), the relative expression levels were normalised in accordance to the expression of Broadbalk 09 (WTS) and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) and then the expression is represented as log2.

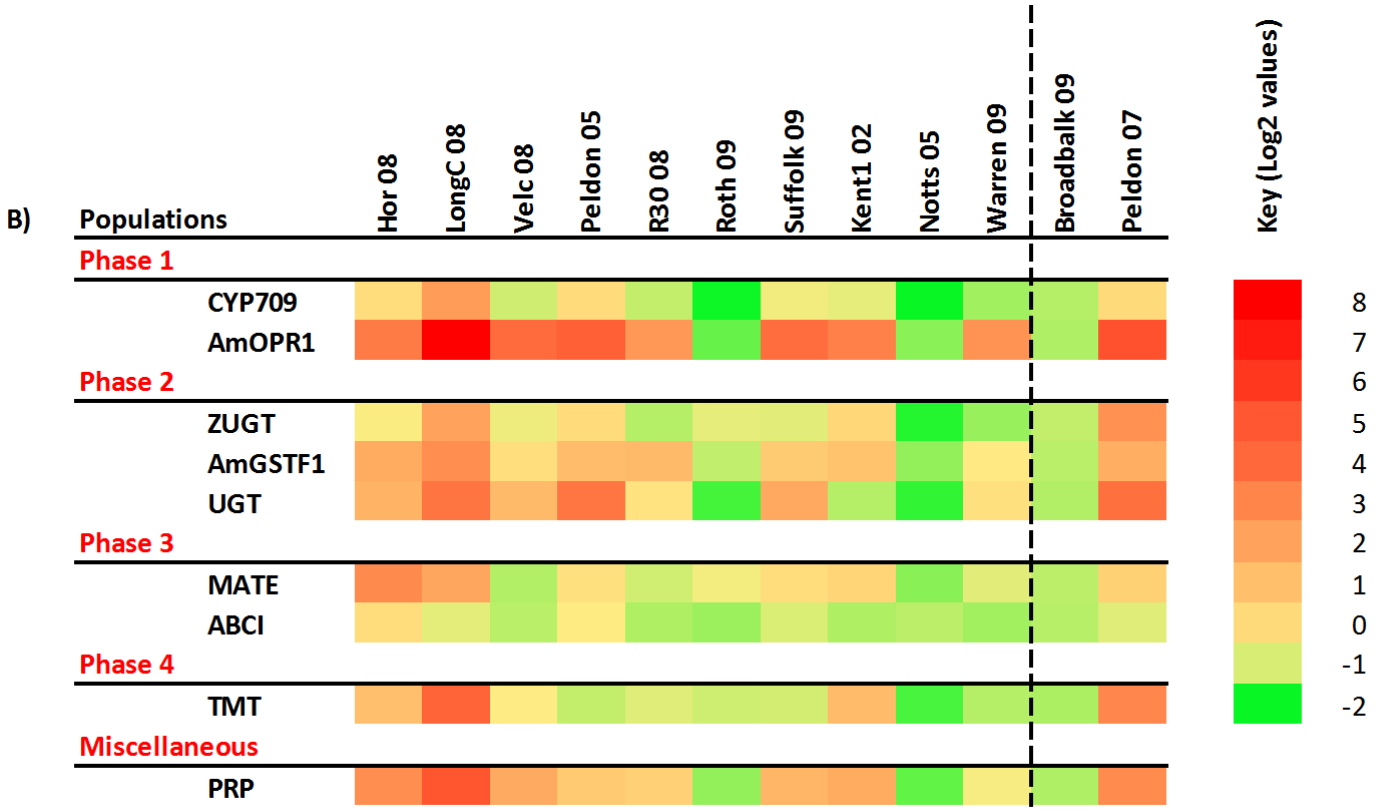


Figure 37 "Heat map" of relative expression levels in ten different biotypes of *A.myosuroides.*
B) Ten populations of black-grass which include known populations Broadbalk 09 (WTS) and Peldon 07 (MHR) the relative expression levels were normalised in accordance to the expression of Broadbalk 09 (WTS) and housekeeping gene and then the expression is represented as log2 glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*).

Three additional genes in our panel a UGT, a TMT (thiol methyl transferase) and a PR (pathogen related protein) have mixed behaviour but are generally up-regulated in MHR populations. However the most significant result is represented by *Am*OPR1 which is massively up-regulated in all lines except the WTS and TSR ones, reaching a 254 fold change in population LongC (Figure S 6). This result is of particular interest considering that it is the result of constitutive expression. i.e. no external treatment (such as chemical or physical stress) was applied to these plants. In contrast: UGT, MATE, ABC and TMT genes proved to be less use as indicators for MHR.

Whilst OPR1, commonly associated with its link to detoxification of TNT (Beynon et al., 2009) proved to be a sensitive marker for MHR, its level of expression was not associated with the level of metabolism based resistance. The remaining markers UGT, MATE, ABC and TMT genes did not prove to be indicative markers for MHR.

As already discussed, the role of OPR1s in plants remained unclear apart from those identified in Arabidopsis which had the capacity to transform and detoxify the explosive TNT (Beynon et al., 2009). Plant OPRs are separated into 3 classes: OPR1, OPR2 and OPR3, of which their classification is dependent on the enzymes substrate preferences (Strassner et al., 2002, Strassner et al., 1999, Schaller and Weiler, 1997a, Schaller and Weiler, 1997b). This biomarker was the most abundantly expressed across all the ten populations except those termed TSR and WTS biotypes.

*Am*OPR1 did not fall directly into the OPR1 class, but it appeared to share similar phenotype traits to those identified in rice, *Arabidopsis thaliana* (Agrawal et al., 2003, Agrawal et al., 2004, Sobajima et al., 2003) and maize (Zhang et al., 2005) genomes. Studies have indicated in monocots that OPR genes were rapidly and transiently up-regulated in response to a variety of biotic and abiotic stresses including wounding, signalling molecules and the presence of pathogens (Zhang et al., 2005), although despite the ongoing studies the function of OPRs in plants remained relatively unknown. *Am*OPR1 was still a potential novel biomarker which held great potential for future determination of xenobiotic detoxification in black-grass.

With regards to the other phase I enzymes which were upregulated in MHR plants but down regulated in WTS and TSR this included CYP709, derived from the CYP72 clan. The real-time expression of CYP709 was surprising as the population LongC had the greatest up-regulation of the gene, however it did not metabolise the herbicide chlorotoluron as

successfully as Velc (Table S 1). This population possessed approximately 30 % ALS and ACCase mutations, whilst in contrast LongC had 40 % and 8.5 % mutations respectively but did not metabolise the CYP mediated biotransformation of chlorotoluron as efficiently. Therefore, this biomarker relative expression was not indicative of the level of metabolism based resistance indicated by the biotypes of black-grass. Despite this, the marker was still discriminatory for WTS, TSR in comparison to MHR.

The xenome biomarker genes which were used in the study highlighted that MHR is associated with a multitude of regulated pathways rather than just a single pathway, as four genes proved to be diagnostic for MHR. Of particular interest were CYP, GSTF1 and GSTU genes which were the most upregulated and diagnostic and as expected, several transferases seem to be associated with MHR. GSTF1 is up-regulated in all MHR populations, with relative fold-change varying between 2.5 and 15.5. Another GST putatively annotated as GSTU6-like is also up-regulated in all MHR lines except for Suffolk, Notts (TSR) and Roth (WTS).

Phase II enzymes included *Am*GSTF1, ZUGT and UGT classes, which were shown to be upregulated in MHR relative to WTS. Interestingly, *Am*GSTF1 was not as strongly up-regulated in comparison to the UGTs despite its clear upregulation at protein level in MHR black-grass (Cummins et al., 2013). Whilst the GSTU-like protein was included in these screens because an ortholog was identified in Gaines (2014) analysis of *Lolium rigidium*. Therefore, from this study the results correlated to those found by Gaines as its expression was up-regulated in populations with MHR traits and those that possessed ALS or ACCase mutations. However, due to the difficulty of achieving a full sequence of this GSTU protein from the d*e novo* NGS analysis, the percentage similarity with those identified in Gaines (2014) proved difficult to analyse.

5.2.3 Xenome RNA biomarkers screening WTS black-grass following abiotic and biotic stress

The biomarkers *Am*OPR1, CYP709, *Am*GSTF1, *Am*GSTU, UGT, MATE, ABC, TMT and PRP were subsequently tested for their potential viability against a further set of WTS black-grass samples which were subjected to abiotic and biotic stresses. The inclusion of abiotic and biotic stresses was to establish whether there were any potential parallels between MHR and either abiotic or biotic stress responses in plants, and also to see whether any of these stresses may potentially hinder the diagnosis of MHR in the field. These stresses included:

drought, wounding, Rh (Rhizobacteria), aphid, heat, polyethylene glycol (PEG), salt, 10% nitrogen and safener treatments. The novel biomarkers were screened in biological triplicates for their relative quantitative expression in the WTS populations and two MHR populations. The results of this investigation are represented in the 'heat map' which highlighted the different levels of relative expression in the different lines when compared to the reference population WTS Rothamsted untreated, at Log2 (Figure 38). Genes which were upregulated are represented in red, moderately induced orange and down regulated in green.

It was hypothesised that in order to survive the particular stress abiotic or biotic, that an induced and associated fitness cost to the plant would occur and that this may be a similar response pathway used to enable herbicide resistance in plants. This study would then help to see whether some plants have evolved resistance to herbicides due to fitness costs, and are therefore primed for herbicide resistance prior to agrochemical pressures.

5.2.3.1 *Results of real-time qPCR screening of biotic and abiotic stressed populations*

The results of the real-time qPCR analysis indicated that the stress treatment of 10 % nitrogen was most representative of herbicide resistance as the up-regulation of the most commonly associated genes CYP709 and *Am*OPR1 were seen under this condition. In contrast most of the other treatments resulted in the down-regulation of the xenome markers, especially ABCI and TMT markers.

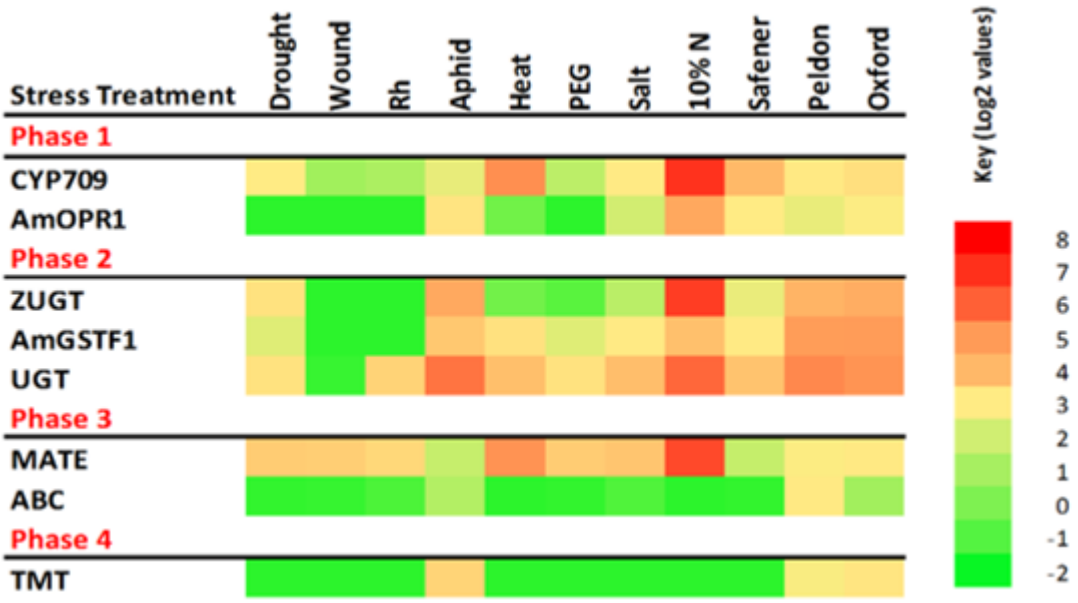


Figure 38 "Heat-map" of xenome biomarkers relative expression in abiotic and biotic Rothamsted (WTS) *A.myosuroides*
Population of Rothamsted (WTS) screened against 8 genes which covered the four phases of the detoxification pathway. Peldon and Oxford populations of *A.myosuroides* were included for comparative reasons. The relative expression levels were normalised in accordance to the expression of Broadbalk 09 (WTS) and housekeeping gene (*G3PDH*) and then the expression is represented as log2.

The data from this diagnostic screen mirrors that known about a plant's capacity to respond to stresses. Maize (*Zea mays*) had previously been studied at a transcriptional level to identify the impacts of nitrogen presence on the crop (Yang et al., 2011). In recent years there has been a dramatic increase in the use of nitrogen-containing fertilisers to aid crop yield productions. Therefore, using model species *Arabidopsis thaliana* the effects of nitrogen regimes of a plants growth development have previously been studied using microarray analysis. It was identified that a plant's response to limited nitrogen resulted in extensive changes in the primary and secondary metabolism, along with numerous changes to the regulatory genes and pathways and protein synthesis (Wang et al., 2003, Scheible et al., 2004, Bi et al., 2007). However, less is known about nitrogen response in cereals like *Oryza sativa* or *Zea mays* (Yang et al., 2011, Beatty et al., 2009). From this recent analysis it is apparent that there is still a lack of understanding a plant's response to differing nitrogen conditions in the field. However, if a genetic understanding was achieved then it would be possible to improve the yield potential through nitrogen utilisation. It was interesting to see from the real-time qPCR data that under the drought, wounding and Rh treatments all the biomarker expression was lower, since there was an expectation that the WTS plants would express these genes as a defence mechanism. CYP72A39 was found to be over-expressed in barley

(*Hordeum vulgare*) cultivars but not in seedling development under stresses and particularly pathogen challenges (Nguyen, 2006). This CYP belonged to CYP72A family like that used in our study, therefore it was predicted that it would have been over-expressed. The biomarker *Am*OPR1 was still a potential biomarker for MHR in the stress study, since 5 of the 9 treatments suppressed the expression. However, it was anticipated that *Am*OPR1 would rapidly and transiently be up-regulated in response to wounding and the presence of aphids (Zhang et al., 2005). Although the results in Figure 38 indicated that wounding suppressed its expression whilst in contrast the presence of aphids resulted in an over-expression. Therefore, *Am*OPR1 still needed further investigation to determine its exact role within black-grass and establish its potential use as a biomarker not only for herbicide resistance but for both abiotic and biotic stresses.

From this independent study it was apparent that having a clearer understanding of the pathways that a plant relied on to survive both abiotic and biotic stresses was crucial. As this would enable farmers to exploit the relevant mechanisms to improve crop yields. In the instance of black-grass, having the capacity to suppress its expression of these vital survival pathways could result in death or a reduction in the seed bank of resistant biotypes.

5.3 Discussion

In this work, *de novo* transcriptome assembly, coupled with differential expression analysis revealed a complex set of enzymes and pathways linked to multiple herbicide resistance (MHR) in the crop weed black-grass. Up-regulation of distinct detoxification enzymes clearly indicated a key role of transferases, especially Phi and Tau class GSTs and some UGTs, while the contribution of P450s and ABC transporters was only moderate. Furthermore, as proof of concept for the development of a diagnostic tool, the expression of 8 target genes was analysed in 10 black-grass populations. This identified *Am*OPR1 as a potentially new powerful biomarker of MHR, which was expressed less in the herbicide sensitive and TSR population.

From this analysis, several classes of xenome genes were found to be induced in the NTSR populations, with specific cytochrome P450 and GST gene families found to be semi-quantitative biomarkers of MHR when tested in population sample sets using qPCR. These biomarkers have the potential to be both diagnostic for resistance and potentially unique tools to study the molecular evolution of NTSR in the field by analysing the pathways up-/ down-

regulated. The biomarkers were relatively successful in the biotic and abiotic screen as there were still differences in the relative expression of all 8 genotypes across the different stresses.

In both real-time qPCR analysis studies the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) was used, although in the stress study this house keeping gene was not ideal. As a result, some of the expression levels may have been falsely represented therefore if the study was repeated the use of β-tublin or actin might have been better suited.

As NGS technologies continue to improve, their scope and application will correspondingly expand within and across scientific disciplines. Within plant biology there is much still to be gained with regards to increasing plant breeding and evolutionary studies. It was also increasingly apparent that the number of fully sequenced plant genomes would enable a greater understanding of genetic, genomic, developmental and evolutionary processes which are responsible for the complexity surrounding plant life on earth (Egan et al., 2012). In the instance of black-grass the sooner a comprehensive analysis of the plant xenome is achieved the easier it will be to identify specific pathways and mechanisms that could offer back control. The ALOMYbase (Gardin et al., 2015) was currently the largest comprehensive study of black-grass transcriptome and proved a useful comparative tool in this study.

Chapter 6. Discussion

6.1 General Discussion

Herbicide resistance in plants was originally documented 40 years ago, and was associated with GSTs which had the ability to detoxify herbicides through the conjugation with GSH (Ryan, 1970, Frear and Swanson, 1970). Subsequently, herbicide resistant weeds were viewed as the greatest biotic threat to global agricultural practices, with an annual loss of £0.5 billion in the UK (Basu et al., 2004). Consequently, there has been large focus on understanding the mechanisms causing weeds of cereal crops to adapt to herbicides (mainly the wild grasses annual ryegrass (*Lolium rigidium*) and black-grass (*Alopecurus myosuroides*)). As a result, a multitude of GSTs belonging to the classes' phi (F) and tau (U) were identified, characterised and proven to detoxify a subset of xenobiotics via the conjugation with GSH in crop plants: *Zea mays, Triticum aestivum* and *Glycine max* (Dixon et al., 1997, Andrews et al., 2005, Cummins et al., 2003a). Research suggested that these GSTs in crops could either directly detoxify herbicides and/ or operate as GPOXs, by utilising GSH as a cofactor to detoxify hydroperoxide species which usually accumulated in crops following herbicidal treatments to generate hydroxylated derivatives (Sommer and Böger, 1999, Cummins et al., 2003a). Essentially, detoxification is aided by components of the xenome namely GSTs and are capable of detoxifying the reactive oxygenated downstream products.

When the research extended to populations of weed species, the invasive weed species of these crops displayed evolved herbicide resistance, which was understandable given the close proximity to the resistant crops. The mechanisms utilised by the weed species originate from an enhanced expression and/ or activity of the herbicide-detoxifying GSTs. Anderson and Gronwald (1991) discovered atrazine resistance in the weed, velvetleaf which displayed an enhanced capacity to metabolise herbicides through a GSH-conjugate catalysed by GST. Since the initial report, a further four MHR weed species including black-grass have now been identified (Cummins et al., 1997, Hall et al., 1997, Bakkali et al., 2007).

Cummins *et al.,* confirmed that specific GSTFs play a pivotal role in MHR black-grass (Cummins et al., 2013). This research identified similarities between the over-expression of the plant phi class GST and the mammalian over-expression of phi 1, which has a known role in multiple drug resistance (MDR) in human tumour cells (Cummins et al., 2013). The study

identified that MDR-inhibiting pharmacophore 4-chloro-7-nitro-benzoxadiazole also inhibits *Am*GSTF1 activity leading to restoration of herbicidal control of MHR black-grass. Importantly, these phi class GSTs are constitutively expressed in protein extracts of MHR biotypes but absent in herbicide-sensitive and ACCase target-site resistant biotypes (Cummins et al., 1999). Therefore, *Am*GSTF1 appears to be a key component of MHR, although most interestingly the research identified that there was still a potential for future mechanisms of control to be explored for MHR in invasive weed species.

The studies presented in this thesis aimed to address the question; 'Is GSTF1 the most appropriate biomarker for metabolic based herbicide resistance?'

The initial stage involved identifying the suitability of biomarkers for MHR in the invasive weed black-grass, by first investigating the role of the xenome in MHR and the proactive methods used to detect associated resistance in the field. This work built on the discovery by Cummins *et al.*, of *Am*GSTF1 in the MHR weed black-grass and utilised a NGS analysis as an 'omic' approach to identifying potential novel molecular biomarkers (Cummins et al., 2013, Cummins et al., 1999). This detailed analysis offered an opportunity to generate a diagnostic which would allow an informed weed management strategy to potentially counteract the phenomena.

To examine the diagnostic protein biomarker *Am*GSTF1, several populations of black-grass were initially characterised using a combined approach of phenotype and immunoblot analysis. The results indicated that there were populations which had various levels of resistance to contrasting herbicide chemistries compared to a susceptible standard (Figure 14 and Table 4) in the phenotype analysis. Subsequently, a protein analysis was carried out on the aforementioned populations (Figure 18) with the *Zm*GSTFI-II antiserum. Given the initial sensitivity in Figure 17 where there was a clear visible distinction between MHR and WTS biotypes, it was anticipated that the immunodetection would be successful in the larger screen of ten populations with varying resistance traits. However, the immunodetection (Figure 18) using *Zm*GSTFI-II was not as discriminating as originally hoped as the populations Roth 09 and Notts 05 (TSR) appeared in this qualitative analysis to express the 25 kDa polypeptide more highly than Peldon (MHR). Therefore, one of the biggest issues surrounding this biomarker stemmed from its use as a qualitative marker rather than quantitative marker. Therefore, to interrogate *Am*GSTF1 role further, a more sensitive and specific antiserum for the detection of resistance traits across various populations of black-grass was required.

A further concern with the existing protein marker related to the use on WTS biotypes following exposure to safeners, where GSTFs expression was not too dissimilar to MHR (Cummins et al., 2009). In this preliminary assessment the inclusion of a CDNB assay indicated that differences in activity correlated to the level of resistance in the known populations of black-grass (Table 4 and Table 6). For example, Hor 08 which had the highest level of TSR mutations to both ALS and ACCase and indicated the greatest MHR trait also had the highest level of activity towards the substrate CDNB. The populations associated with the phenotypes, Kent1 02, Roth 09 and WTS had the lowest activity towards CDNB. Despite the correlation in activity to resistance traits in the phenotype, this did not correspond to the protein assessment. Therefore, it was essential to develop a novel diagnostic protein biomarker, *Am*GSTF1.

Having established from previous literature that *Am*GSTF1 played a central role in eliciting MHR in both transgenic host plants and in black-grass, a novel antiserum was raised to the recombinant protein *Am*GSTF1. The anticipation was that the antiserum would have increased specificity and could be used in a quantitative analysis such as ELISA.

Firstly, a polyclonal antibody was raised to the recombinant protein *Am*GSTF1 (sequence (Figure 10)).The antiserum was used in a western blot to assess the protein levels in crude plant extracts from ten populations of black-grass as described in chapter 3. The antiserum indicated cross-reactivity to a polypeptide of 60 kDa which was not present in the pre-bleed so it is likely to be associated to the immunisation protocol. Despite this cross reactivity the anti-*Am*GSTF1-serum reacted with three polypeptides of Mr 25 kDa, 24 kDa and 22 kDa (Figure 29). The expected expression of *Am*GSTF1 in WTS and TSR was less than the constitutive expression in MHR populations of black-grass. Intriguingly, the relative abundance of the *Am*GSTF1 polypeptides of differing relative molecular mass varied between MHR populations, suggesting the presence of multiple component isoenzymes. However, having established that the anti-AmGSTF1-serum was reactive with increased sensitivity and identified novel patterns of polypeptides the results remained qualitative. When the biomarker was further screened against, rye-grass and wild-oat biotypes with varying degrees of herbicide resistance, the detection of *Am*GSTF1 orthologues was not as prominent. Due to the increased specificity of the biomarker, it was possible that detection in these biotypes wasn't possible due potentially to the lack of common epitopes.

Subsequently, the analysis of resistance focused on the introduction of a quantitative, quick, reliable and reproducible method of detecting resistance in biotypes of black-grass. Over recent years there has been an increased reliance within the agricultural sector on immunodiagnostic tests, especially enzyme-linked immunosorbent assay (ELISA) normally associated with the healthcare sector (Engvall and Perlmann, 1971, Engvall and Perlmann, 1972, Chirkov et al., 1984). The potential of such methods for the direct detection of infectious agents such as viruses or pathogens in plants has been proven successful and was a point of interest for this study (Kingsnorth et al., 2003, Bandte et al., 2016, Lacroix et al., 2016, Voller et al., 1976). This study introduced the use of an 'indirect' sandwich ELISA to quantify the relative expression of GSTF so as to correlate this to a resistance description MHR, TSR or WTS. Previously, Reade (2000) had developed an ELISA to detect the GST abundance in black-grass plants after a monoclonal was raised against the Peldon (*Am*GSTF1) polypeptide. This test assessed plants which survived herbicidal treatment, and the results provided an aid for the prediction of field performance at sites where black-grass control was poor. Using Reade's example, the affinity- purified polyclonal Anti-*Am*GSTF1-antibodies were implemented in an ELISA, as this system could readily be transformed for use in a lateral flow device (LFD), the production of which, offered the opportunity for a novel in-field testing of herbicide resistance in black-grass populations with the potential for further use in other wild grasses.

Therefore, the study focused on the detection of *Am*GSTF1 using the polyclonal Anti-*Am*GSTF1 serum and assessed its capacity in populations of wild grasses which included black-grass, wild oat and rye grass (Figure 31, Figure 32 and Figure 33). The assay results indicated that there was further potential for its use in an ELISA, but the method required further development to investigate the limitations and potential cross-reactants that were hindering the ELISA's reliability. The advantage of such an assay still stood, as it was relatively inexpensive, quick and provided a quantitative analysis of resistance. The overall intention of the assay was to aid the development of an LFD, as the quantitative absorbance values could have been used to assign thresholds which would correlate to the resistance traits associated with black-grass.

A parallel study investigated Federico Sabbadin's (Edwards Group) findings which identified novel virus markers AMPV1, AMPV2 and AMRV1 which had the potential to be coupled with *Am*GSTF1 to identify MHR black-grass populations. The virus markers were originally identified as being associated with MHR Peldon but absent in WTS biotypes following a

transcriptomic analysis. The RT-PCR screening used these viruses and identified that they were wide spread in black-grass populations across the UK, with varied abundance within and between populations of the same resistance traits. Subsequently, the sequences were not taken further forward for use in a protein study as they didn't prove discriminatory for MHR when the study was extended to include the ten characterised populations of black-grass. However, if the virus sequences had been found earlier during this study I would have carried out the Koch's postulates experiment which would have helped establish the inheritability of the viral traits, and whether this primed the plant against further abiotic / biotic stresses. These virus sequences remain a point of interest due to the lack of a clear caustic link between infection of black-grass with either AMPV or AMRV and the occurrence of herbicide resistance. I would hypothesise that there was a mutualistic link between the virus presence and the over-expression of xenome genes, resulting in subsequent generations with improved genetic pools enabling MHR populations to evolve.

The project moved into the second phase of addressing whether GSTF1 was the most appropriate marker for metabolic based herbicide resistance by implementing a genomic analysis of xenome genes in different biotypes of black-grass. *De novo* next generation sequencing (NGS) was carried out on MHR and WTS black-grass which enabled the virtual transcriptome of the xenome and associated genes to be assembled by fellow members of the Edwards group. The *de novo* transcriptome assembly, coupled with differential expression analysis, revealed a complex set of enzymes and pathways linked to multiple herbicide resistant (MHR) in black-grass. The up-regulation of distinct detoxification enzymes clearly indicated a key role of transferases, especially, Phi and Tau class GSTs and some UGTs, while the contribution of P450s and ABC transporters was only moderate (Table 10). Furthermore, as proof of concept for the development of a diagnostic tool, the expression of eight target genes was analysed in 10 black-grass populations. The eight xenome genes were identified as being differentially expressed namely a CYP, GSTU6, GSTF1, OPR1, UGTZ, TMT, ABC and MATE transporters. This identified *Am*OPR1 as a new powerful marker of MHR, which was instead un-perturbed in the herbicide sensitive and TSR population (Figure 36 and Figure 37). This genomic analysis had the capacity to be coupled with data from the proteomic screen to provide a two-tiered approach in order to detect and quantify herbicide resistance in a wide range of black-grass populations. There was also future potential to utilise the contig sequence of *Am*OPR1 to raise an antiserum and assess whether the expression at the protein level is as discriminatory for MHR, as it appears in the qPCR and

RNA-seq analysis. There was an expectation that the expression of xenome marker would alter depending on the associated metabolic resistance, so much like the aim of the ELISA, thresholds could then be implemented. These markers could then be used as a decision tool to aid the implementation of the appropriate alternative strategies when facing herbicide resistance.

To date the ALOMYbase (Gardin et al., 2015) is currently the largest comprehensive study of the black-grass transcriptome and proved a useful comparative tool in this study. As the results of the NGS study were based on a *de novo* transcriptome, identifying specific pathways and mechanisms for controlling MHR proved unclear despite using the ALOMYbase. It could be hypothesised that this was linked to the complex evolutionary processes in black-grass as it is an out-crossing species. Therefore, there still remain issues in understanding the plants genomic development which subsequently makes it harder to identify specific pathways which could help future mechanisms of MHR control.

To conclude, GSTF1 remains the most appropriate biomarker for metabolic based herbicide resistance. However, the studies in this thesis have established that the new anti-*Am*GSTF1-serum has greater specificity for *Am*GSTF1 homologues than its predecessor *Zm*GSTFI-II. This polyclonal antibody also appeared to have epitopes best suited for black-grass as opposed to wild-oat and rye-grass as there was no clear distinction between those that were MHR vs. WTS. This meant two things could be done with the antiserum: 1) it could be further developed and utilised in an ELISA and then subsequently used as an LFD developed to help determine whether a weed is not only resistant but whether the phenotype is black-grass, 2) the antiserum is limited to the use in black-grass so as an 'in-field test' it would become useless unless it is tested on black-grass, as the expression of GSTF1 remained unchanged irrelevant of resistance and therefore wouldn't provide the farmer with any viable information for strategies of control.

In contrast, the NGS analysis offered a detailed insight into potentially novel biomarkers for MHR as there was an expectation that the xenome markers expression would alter depending on the associated metabolic resistance. Therefore, there was potential for the biomarkers to be utilised on a 'microarray' which would mean that the quantitative analysis could also be performed as an 'in-field' test. These markers could then be used as a decision tool to aid the implementation of the appropriate alternative strategies when facing herbicide resistance, as they offered a more detailed insight into the genetic implications of MHR.

Therefore, although GSTF1 remains the best marker currently available there are potentially other markers eg. *Am*OPR1 which may be more insightful and potentially offer better distinctions between the different types of resistance displayed in the phenotype by better understanding the genetic mechanisms that confer them. There are also biomarkers like those of AMRV and AMPV which could potentially have a causation link between infection and the occurrence of herbicide resistance but further work is needed to address this. These studies have raised many more interesting questions regarding the role of xenome proteins in MHR, and offer exciting new avenues of study for both detection and control of metabolic based herbicide resistance.

6.2 Future plans

Following the development of the anti-*Am*GSTF1 serum collaborative work was undertaken with Mologic Ltd (Bedfordshire, UK) to develop an LFD. The work is currently in the preliminary stages as the antiserum has now been successfully bound to a gold-colloidal membrane of an LFD. However, the device needs further development to bring it to the commercial market. Currently, in Figure 39 the LFD indicates a visual difference in the expression of GSTF1, between MHR (denoted R) and WTS (denoted S). The test takes just 15 minutes to work, and a red band appears in the small window on the hand-held device if the protein *Am*GSTF1 is present. The device is sensitive enough to detect the molecule in the early stages of black-grass growth, therefore the device aims to help the farmers make management decision early in the crop cycle and ultimately prevent costly losses in the future. The prototype device was recently demonstrated at Cereals 2016, after development with the Edwards group and the diagnostics company Mologic, Bedfordshire. Currently there isn't a visual band present in-line with 'C' the control meaning that the results from the test remain somewhat tentative.

Figure 39 Prototype Lateral flow device (LFD) for GSTF1 detection in *A.myosuroides*.
The LFD displays visual results of GSTF1 detection in two populations Peldon (MHR) and Rothamsted (WTS) after 10 minutes exposure using only 10 cm of 'above ground' material. This LFDs can subsequently be read using a reader to enable a quantitative analysis based on absorbance.

Therefore, the membrane of the LFD has to be further optimised so that when the LFD is used as an 'in-field' diagnostic for the quantitative analysis of GSTF1 expression using absorbance which can be reliably reproduced. However, further work to standardise the protocol is currently scheduled to be carried out by the Edwards's group and Mologic Ltd in autumn/ winter 2016. The purpose of the study is to address a few points mainly, what is the minimum quantity of material needed for the test, can it be used after the use of a herbicide, how many times should a given field be tested before a comment on resistance is established, how vigorous should the extraction of crude protein from the material be and what are the thresholds for resistance traits MHR, TSR and WTS in relation to absorbance figures? It is hoped that following standardisation this will then enable the production of a commercially available LFD suitable for 'in-field' diagnostics, whereby science and decision making is returned to the end-user. Figure 40 illustrates the current prototype kit however this is potentially subject to change given the results from the large-scale field trials. The purpose of the 'tool-kit' is to aid decision making in the field, however there is further potential that the kit could be later combined with a genomic diagnostic. This kit could serve as the first step in identifying whether the sample is herbicide resistant, followed by a genomic analysis, using a micro-array designed with some of the genomic biomarkers identified in the study presented in this thesis. The combination of both a proteomic and genomic assay offered the potential to have a more targeted approach to diagnosing black-grass populations enabling an informed weed management strategy with the potential to counteract the phenomena.

Currently MHR is like a plague, the resistance is creeping up the country and our existing first line of defence, herbicides, are becoming less effective, just like antibiotics in medicine. Therefore this diagnostic tool marks an important step, using the latest research to deliver solutions in crop protection through a new approach known as ‘personalised agriculture’ representing a new way of dealing with resistance and emerging disease in the agricultural setting.

Figure 40 Prototype kit for *Alopecurus myosuroides* 'in-field' diagnostic.
Kit designed and produced by Mologic Ltd (Bedfordshire, UK) contains enough for 5 tests to be carried out, a reader is also included this is the red box shown in the kit.

Appendix

Table S 1 *Alopecurus myosuroides* populations tested for relative susceptibility to damage from commercial herbicide preparations on ALS (Atlantis) and ACCase (Cheetah), together with relative rates of herbicide detoxification based on GST-(fenoxaprop) and CYP- (chlorotoluron) mediated biotransformations.(Edwards group)

			Mutation rate (%)		Damage caused by application of herbicide 21 days after treatment (%)		Total metabolites produced five hours after treatment (nmol. gFW^{-1})		
							Fenoxaprop	Chlorotoluron	
Population	County	Location	ALS	ACCase	Atlantis	Cheetah	CBO-SG	Hydroxy-	N-demethy-
Roth 09**	Hertfordshire	Broadbalk	0	0	85	100	27	44	64
LongC 08*	Oxfordshire	Chalgrove	42	8.5	28	15	64	58	83
Notts 05*	Nottinghamshire	Notts	0	22.5	81	6	35	50	71
Kent1 02***	Kent	Kent-Survey	0	0	75	13	88	61	89
Hor 08*	Oxfordshire	Oxford	50	25	16	28	47	47	75
Suffolk 09***	Suffolk	Suffolk survey	0	27.5	33	17	105	91	94
Peldon 05**	Essex	Peld02 Suffolk survey	50	0	25	17	99	84	104
Velc 08*	Lincolnshire	Velcourt	32.5	31.5	25	10	72	96	101
Warren 09	Bedfordshire	Conts Atl G/Ha60	31.5	18.75	49	21	55	80	84
R30 08*	Cambridgeshire	Huntingdon	45	27.5	25	15	92	75	106

*Marshall et al. (2013), ** Moss et al.(2007), *** Moss et al. (2014), **** includes mutations at amino acids 197 and 574, *****includes mutations at amino acids 1781, 2027, 2078 and 2041.

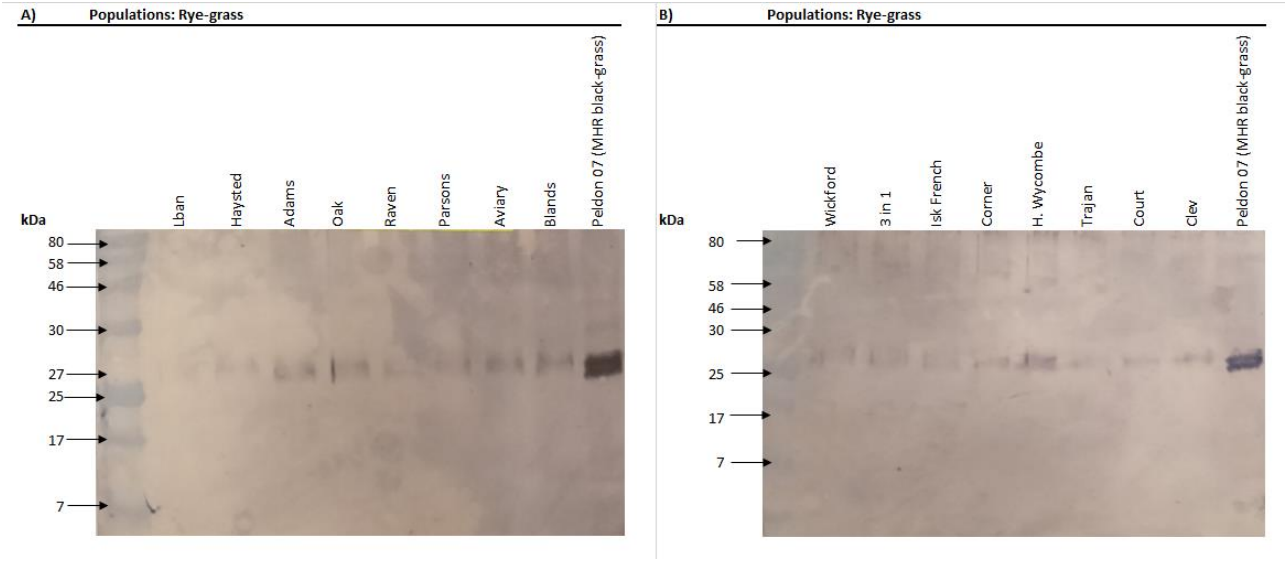


Figure S 1Western blot *Lolium rigidium* (rye-grass) populations following exposure to Anti-*Am*GSTF1-serum.
Trajan (Susceptible), Clev (ACCase NTSR std) and Lban (ACCase TSR Std). Included MHR Peldon black-grass as a positive control.

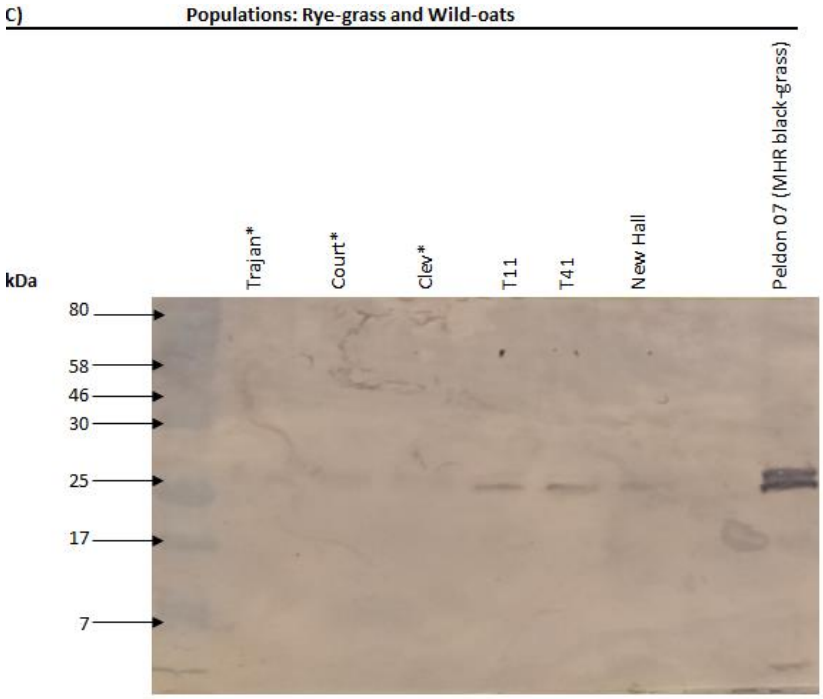


Figure S 2 Western blot Lolium rigidium (rye-grass) vs. Avena fatua (wild-oats) populations following exposure to Anti-*Am*GSTF1-sera.
Rye-grass population indicated by * Trajan-Susceptible, Court- ACCase 'Fops' TSR, Clev- ACCase NTSR std. Wild oats population; T11-NTSR std, T41-ACCase TSR (FOP) and New Hall NTSR

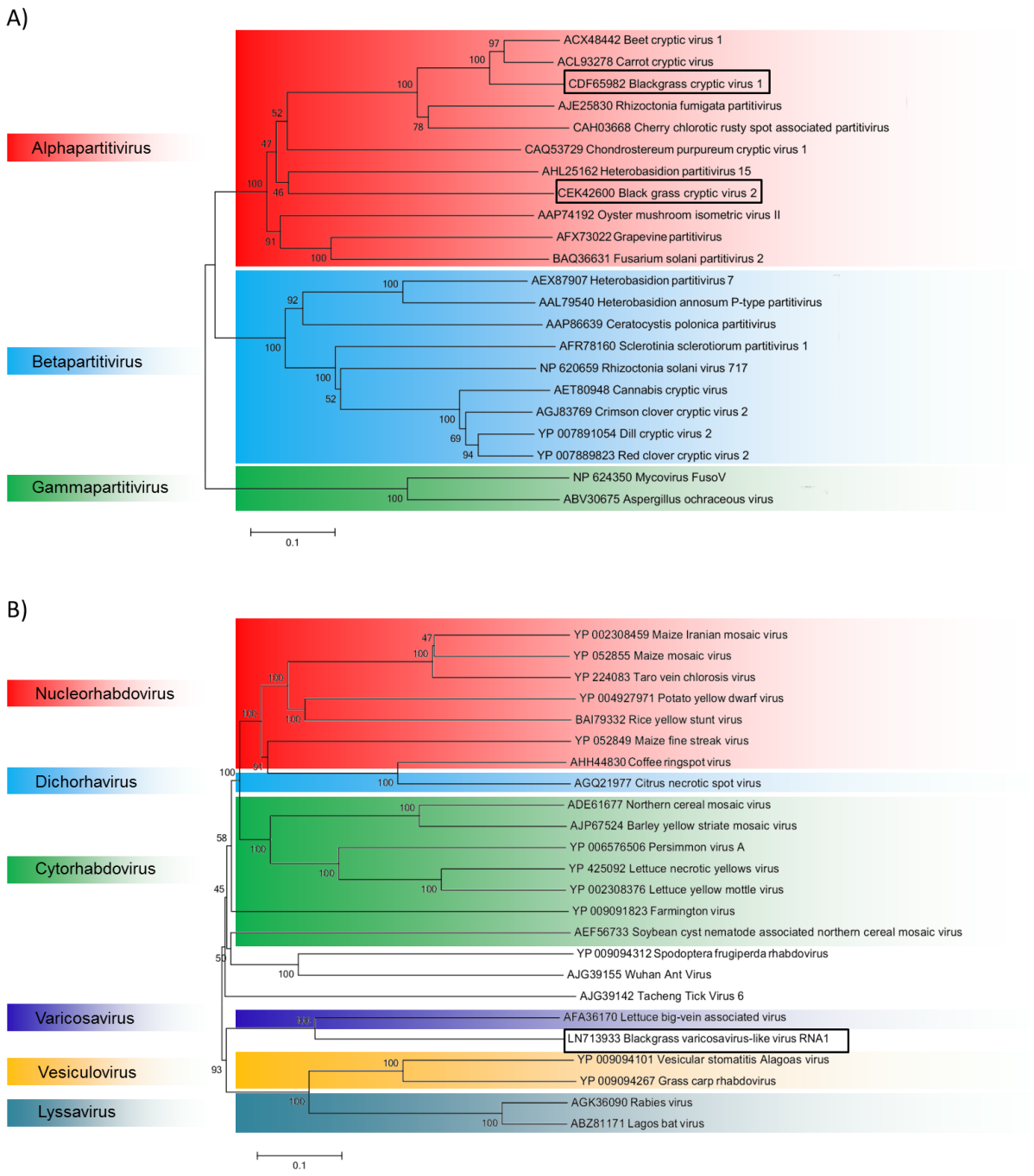


Figure S 3 Phylogenetic analysis of the RNA1 of the three assembled black-grass persistent viruses.
A) Protein alignment of the RdRp demonstrated that AMPV1 and AMPV2 belonged to the Alphapartitivirus genus within the Partitiviridae family. B) Protein alignment of the polyprotein of AMRV1 demonstrated that could be assigned to the Rhabdoviridae family (Sabbadin, 2017).

>Putative *Am*OPR1

MEPIPLLTPYKMGQINLAHRIVLAPLTRQRSYGNVPQPHAALYYSQRASAGGLLITEA
TGVSDTAQGYTYTPGIWTAEHVEAWKPIVAGVHEKGALIFCQIWHVGRVSTFELQP
GGKAPVSSTEKGVGPQISFDGHREEFSPPRRLAVEEIPGIVDDFRKAARNAIDAGFDG
VEIHGANGYLIEQFLKDSANDRTDEYGGSLENRCRFALEVVDAVVKEVGGHRVGIR
LSPFTDFMDCHDSDPHALALHMSTVLNDHDILYVHMIEPRMAIVDGRRVVPKRLLP
YREAFKGTFIANGGYDREEGGKVVTEGYTDLVAFGRLFLSNPDLPKRFEIGAELNKY
DRMTFYTSDPVVGYTDYPFLD

Figure S 4 Putative Amino Acid sequence *Am*OPR1

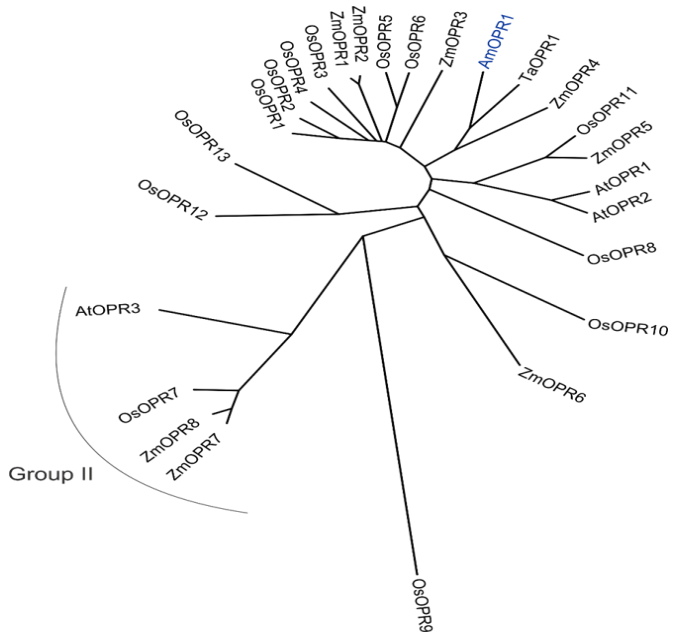


Figure S 5 Phylogenetic tree showing the relationship between the putative AmOPR1 (in blue) and other plant OPRs. Edwards group.
The majority of the OPRs belong to group I while those belonging to group II are labelled as such. The assession numbers are AtOPR1; NP_177794, AtOPR2; NP_177795, AtOPR3; NP_178662, OsOPR1; XP_015643915, OsOPR2; XP_015643914, OsOPR3; XP_015643918, OsOPR4; XP_015640930, OsOPR5; XP_015644547, OsOPR6; XP_015641952, OsOPR7; XP_015650810, OsOPR8; XP_015627739, OsOPR9; XP_015630712, OsOPR10; XP_015623263, OsOPR11; OPR11_ORYSJ, OsOPR12; XP_015620838, OsOPR13; XP_015637023; TaOPR1; AFC87832, ZmOPR1; NP_001105899, ZmOPR2; NP_001105905, ZmOPR3; NP_001105830, ZmOPR4; NP_001105831, ZmOPR5; NP_001105909, ZmOPR6; NP_001105832, ZmOPR7; NP_001105910, ZmOPR8; NP_001105833.

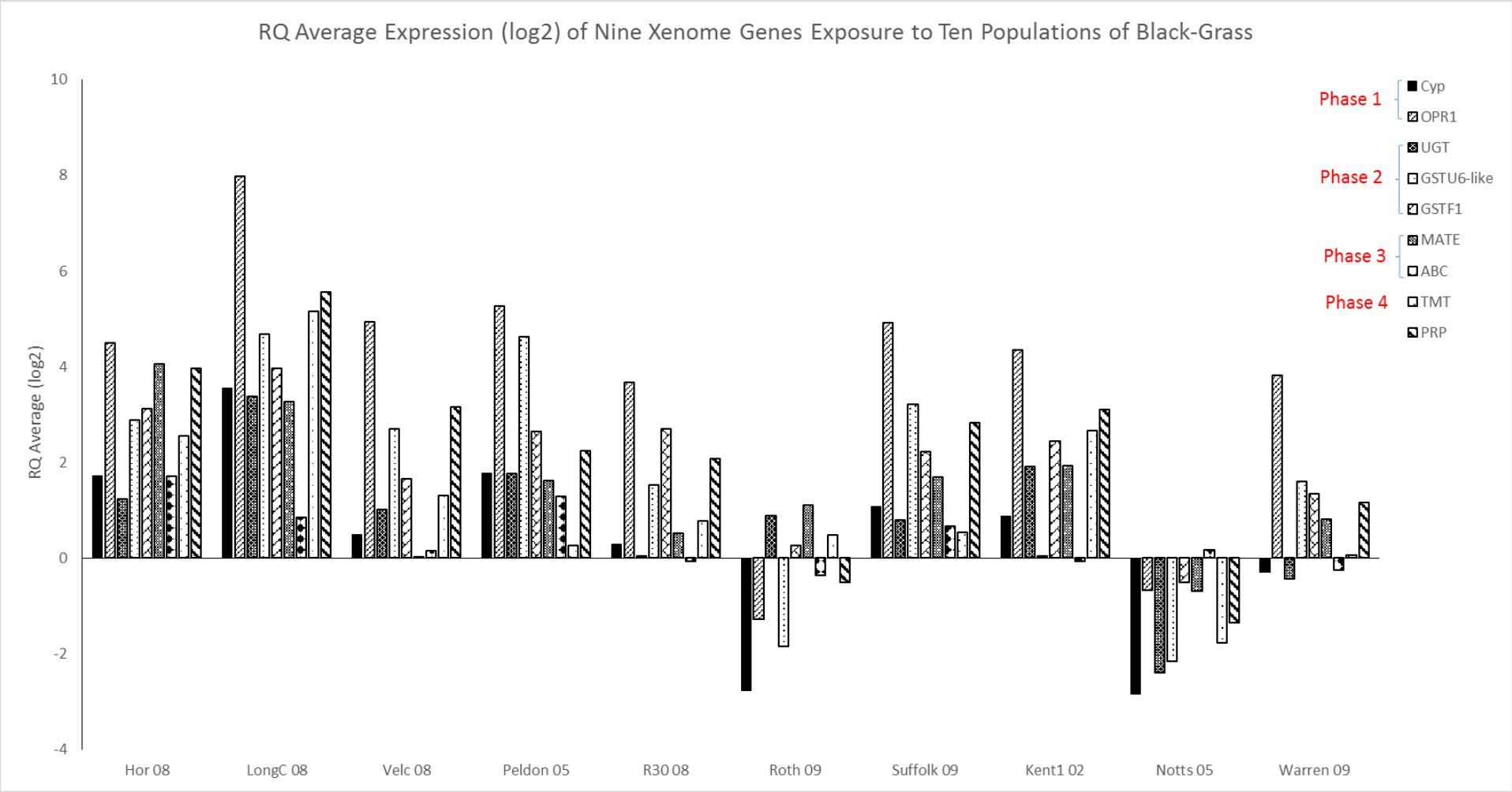


Figure S 6 Bar Chart graph indicating the relative expression of the nine xenome genes in the ten populations of *A.myosuroides*.
The relative expression levels were normalised in accordance to the expression of Broadbalk 09 (WTS) with the housekeeping gene (*G3PDH*) and then the expression is represented as log2. Each of the ten populations was exposed to the 9 xenome genes: CYP709, *Am*OPR1, ZUGT, *Am*GSTF1, UGT (GSTU-6 like), MATE, ABC, TMT and PRP.

Table S 2 Tukey HSD analysis of all wild-grass populations (black grass, Wild-oat and rye grass) indicating the significant differences.

ANOVA analysis followed by a Tukey HSD analysis on the populations expression of *Am*GSTF1 with significant difference indicated by the letters in the final column. (n = 3 replicates per population each containing 20 plants) Those with the same letters were not significantly different to each other (p value 0.05), those with a different letter are.

Population		N	Subset for alpha = 0.05									Sig Diff Indicator
			1	2	3	4	5	6	7	8	9	
Tukey HSD[a]	Roth	3	1.5170									A
	Notts	3		4.1928								B
	Hor	3			5.8790							C
	Trojan	3			6.3585	6.3585						C D
	Adams	3			6.5463	6.5463	6.5463					C D E
	Corner	3			6.6604	6.6604	6.6604	6.6604				C D E F
	H.wycombe	3			6.8865	6.8865	6.8865	6.8865	6.8865			C D E F G
	3 in 1	3			7.0800	7.0800	7.0800	7.0800	7.0800	7.0800		C D E F G H
	Blands	3			7.0904	7.0904	7.0904	7.0904	7.0904	7.0904		C D E F G H
	Wickford	3			7.1774	7.1774	7.1774	7.1774	7.1774	7.1774	7.1774	C D E F G H I
	T41	3			7.2418	7.2418	7.2418	7.2418	7.2418	7.2418	7.2418	C D E F G H I
	Raven	3			7.2998	7.2998	7.2998	7.2998	7.2998	7.2998	7.2998	C D E F G H I
	N.Hall	3			7.3517	7.3517	7.3517	7.3517	7.3517	7.3517	7.3517	C D E F G H I
	Court	3				7.5452	7.5452	7.5452	7.5452	7.5452	7.5452	D E F G H I
	Aviary	3				7.6341	7.6341	7.6341	7.6341	7.6341	7.6341	D E F G H I
	Lbans	3				7.6635	7.6635	7.6635	7.6635	7.6635	7.6635	D E F G H I
	Long	3				7.6812	7.6812	7.6812	7.6812	7.6812	7.6812	D E F G H I
	Parsons	3				7.6881	7.6881	7.6881	7.6881	7.6881	7.6881	D E F G H I
	Haysted	3				7.6933	7.6933	7.6933	7.6933	7.6933	7.6933	D E F G H I
	Clev	3					8.0537	8.0537	8.0537	8.0537	8.0537	E F G H I
	T11	3						8.1405	8.1405	8.1405	8.1405	F G H I
	Oak	3							8.2019	8.2019	8.2019	G H I
	Isk French	3							8.2305	8.2305	8.2305	G H I
	Kent	3							8.3297	8.3297	8.3297	G H I
	Warren	3								8.4429	8.4429	H I
	Suffolk	3								8.5073	8.5073	H I
	Peldon	3								8.5174	8.5174	H I
	Velc	3									8.6107	I
	R30	3									8.6627	I

List of Abbreviations and Symbols

A	Absorbance
ABC	Adenosine triphosphate-binding cassette
ACCase	Acetyl- coA carboxylase
Am	Alopecurus myosuroides
*Am*GSTF1	Alopecurus myosuroides glutathione transferase, phi class
AMPV	Alopecurus myosuroides partitiviridae
AMRV	***Alopecurus myosuroides varicosavirus***
AOPP	Aryloxyphenoxypropionate
APS	Ammonium persulfate
At	Arabidopsis thaliana
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CHDs	Cyclohexanediones
cm	Centimetre
CYP	Cytochrome P450 mixed-function oxidase
DAT	Days after treatment
DIM	Cyclohexanediones (Chemical family)

DMSO	Dimethyl sulfoixde
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E.Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
F1	Phi 1
FOPs	Aryloxyphenoxy propionate (chemical family)
FW	Fresh weight
FPLC	Fast-Protein Liquid Chromatography
g	Gram or relative centrifugal force (context specific)
gDNA	Genomic Deoxyribonucleic Acid
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
GPOX	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione transferase
GSTF	Phi-class glutathione transferase
GSTL	Lambda-class glutathione transferase
GSTP	Pi-class glutathione transferase
GSTU	Tau-class glutathione transferase
GSTZ	Zeta-class glutathione transferase
GT	Glycosy transferase

h	Hour
Ha	Hectare
HCl	Hydrochloric acid
HIC	Hydrophobic interaction column
HPLC	High-performance liquid chromatography
HRAC	Herbicide resistance action committee
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
L	litre
LBVaV	*Varicosavirus* Lettuce big-vein
LFD	Lateral flow device
M	Molar
MATE	Multidrug and Toxic compound Extrusion protein
MDR	Multiple Drug Resistance
mg	Milligram
MHR	Multiple Herbicide Resistance
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MoAs	Modes of Action
Na	Not assayed

NaCl	Sodium Chloride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
ND	Not detected
NGS	Next Generation Sequencing
NH_4SO_4	Ammonium sulphate
nkat	Nanokatal
NaN_3	Sodium azide
NTSR	Non-Target-site resistance
nmol	Nanomolar
NTSR	Non-target site resistance
OPR	Oxophytodienoic acid reductase
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + Tween20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pNPP	p-Nitrophenyl Phosphate
PPFD	Photosynthetic photon flux density
PPO	Protoporphyrinogen oxidase
PRP	Pathogenesis related protein

PTA	Plate trapped antigens
PVDF	Polyvinylidene difluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrollidone
qPCR	Quantitative Polymerase chain reaction
R	Resistant
RdRp	RNA-dependent RNA polymerase
Rh	Rhizobacteria
RNA	Ribonucleic acid
Roth	Rothamsted
RPM	Revolutions per minute
RRR	Rothamsted Rapid Resistance test
RT-PCR	Real-Time Polymerase Chain Reaction
S	Susceptible
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
Spec	Spectroscopy/ spectrum
Std	Standard
Sus	Susceptible
TaGSTU1-1	Wheat glutathione transferase tau class, homodimer
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TMT	Thiol methyltransferase

TSR	Target-site resistance
UDP	Uridine disphosphate
UGTZ	Zeatin glycosyltansferase
UV	Ultraviolet
v/v	Volume to Volume
w/v	Weight to volume
WTS	Wild-type sensitive
Zm	Zea mays
*Zm*GSTF1-2	Zea mays glutathione transferase phi class, heterodimer
℃	Degrees celsius
µg	Microgram
µL	Microlitre
µmol	Micromole
µM	Micromolar
ε	Molar extinction coefficient

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