# The role of glutathione transferases in herbicide detoxification - a genome-wide study on flufenacet resistant black-grass

Dissertation
for the award of the degree
“Doctor scientiarum agrariarum” (Dr. sc. agr.)
of the Georg-August-Universität Göttingen

Evlampia Parcharidou

Göttingen, 25 March 2024

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submitted by

Evlampia Parcharidou

from Thessaloniki (Greece)

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Thesis Committee

1. Prof. Dr. Andreas von Tiedemann
(Department of Crop Sciences / Plant Pathology and Crop Protection, University of Göttingen)

2. Dr. Roland Beffa
(Senior Scientist Consultant, Former Lab Leader of the Weed Resistance Competence Centre in Bayer AG, Crop Science Division)

3. Prof. Dr. Klaus Dittert
(Department of Crop Sciences / Plant Nutrition and Crop Physiology, University of Göttingen, Institute of Applied Plant Nutrition)

Members of the Examination Board

1. Reviewer: Prof. Dr. Andreas von Tiedemann
(Department of Crop Sciences / Plant Pathology and Crop Protection, University of Göttingen)

2. Second Reviewer: Prof. Dr. Klaus Dittert
(Department of Crop Sciences / Plant Nutrition and Crop Physiology, University of Göttingen, Institute of Applied Plant Nutrition)

3. Additional Reviewer: Dr. Roland Beffa
(Senior Scientist Consultant, Former Lab Leader of the Weed Resistance Competence Centre in Bayer AG, Crop Science Division)

4. Further members of the Examination Board: Dr. Rebecka Dücker
(Department of Crop Sciences / Plant Pathology and Crop Protection, University of Göttingen)

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Table of contents

# 1. List of abbreviations

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 2,4,5-T | 2,4,5-trichlorophenoxyacetic acid |
| ABC | ATP-Binding Cassette (EC 7.6.2.2) |
| ACCase | Acetyl-CoA carboxylase (EC 6.4.1.2), HRAC group 1 |
| a.i. | Active Ingredient |
| ALOMY | *Alopecurus myosuroides* Huds. |
| ALS | Acetolactate synthase (EC 2.2.1.6), HRAC group 2 |
| Beauv. | First described by Palisot de Beauvois |
| bZIP | Basic leucine zipper transcription factor |
| C18 | Fatty acids with chain of 18 carbon atoms |
| CDNB | 1-Chloro-2,4-dinitrobenzene |
| CREs | *Cis*-Regulatory Elements |
| CYP | Cytochrome P450 monooxygenase (E.C. 1.14) |
| DBDs | DNA-Binding Domains |
| DHAR | Dehydroascorbate reductase (EC 1.8.5.1) |
| Dinoseb | 6-sec-butyl-2,4-dinitrophenol |
| DNOC | Dinitro-ortho-cresol |
| Dof | DNA-binding one zinc finger transcription factor |
| E.C. | Enzyme Commission number |
| EDTA | Ethylendiaminetetraacetic acid |
| EF1Bγ | γ-subunit of the eukaryotic translation elongation factor 1B |
| Gaud. | First described by Charles Gaudichaud-Beaupré |
| GFP | Green fluorescent protein |
| GHR | Glutathionyl-hydroquinone reductase (EC 1.8.5.7) |
| GSH | Glutathione |
| GSP | Gene-specific primer |
| GST | Glutathione transferase (E.C. 2.5.1.18) |
| GSTF | Phi GST class |
| GSTH | Hemerythrin GST class |
| GSTI | Iota class GST class |
| GSTL | Lambda class GST class |
| GSTT | Theta class GST class |
| GSTU | Tau class GST class |
| GSTZ | Zeta class GST class |
| GT | Glucosyltransferase (E.C. 2.4) |

| | |
|---|---|
| HRAC | Herbicide Resistance Action Committee |
| Huds. | First described by William Hudson |
| IWM | Integrated Weed Management |
| KCS | 3-ketoacyl-CoA-synthase (EC.2.3.1.199) (preferred name: very-long-chain 3-oxoacyl-CoA synthase) |
| Lam. | First described by Jean-Baptiste Lamarck |
| LC-MS/MS | Liquid Chromatography Mass Spectrometry/Mass Spectrometry |
| LOLMU | *Lolium multiflorum* Lam. |
| LOLRI | *Lolium rigidum* Gaud. |
| mPGES2 | Microsomal prostaglandin E synthase type 2 (EC.5.3.99.3) |
| MCPA | 2-methyl-4-chlorophenoxyacetic acid |
| MoA | Mode of Action |
| Moq. | First described by Alfred Moquin-Tandon |
| MWCO | Molecular Weight Cut Off |
| NMWL | Nominal Molecular Weight Limit |
| NTSR | Non-Target-Site Resistance |
| PDS | Phytoene desaturase (EC 1.3.99.31), HRAC group 12 |
| RNA-Seq | RNA sequencing |
| TBPs | TATA-Binding Proteins |
| TCHQD | Tetrachlorohydroquinone dehalogenase |
| TEs | Transposable Elements |
| Tris | Tris(hydroxymethyl)-aminomethane |
| TSR | Target-Site Resistance |
| TSS | Transcription Start Site |
| VLCFAs | Very-Long-Chain Fatty Acids |
| WSSA | Weed Science Society of America |
| UDP-glucose | Uracil-Diphosphate glucose |
| UGT | UDP-glycosyltransferase |
| UTR | Untranslated Region |

# 2. General Introduction

## 2.1 Definition of a weed

Even in the earliest literature, including the Bible, weeds are mentioned as a threat to crops (Zimdahl, 2013). Besides pests and pathogens, weeds are the most prominent cause for crop losses with 34% (Oerke, 2006). According to the Weed Science Society of America (WSSA), a weed is “a plant that causes economic losses or ecological damage, creates health problems for humans or animals, or is undesirable where it is growing” (WSSAa, 2022).

According to Baker (1974), a plant needs to fulfil various requirements in order to be classified as “undesirable” and thus defined as “weed”, according to the following characteristics:

- Germination requirements met in many environments
- Discontinuous germination (internally controlled) and high seed longevity
- Rapid growth through the vegetative phase to flowering
- Continuous seed production for as long as growing conditions allow
- Self-compatible but not fully autogamous or apomictic
- Uses cross-pollination, unspecialised visitors or wind
- High seed production under favourable environmental conditions
- Produces seeds under a wide range of environmental conditions; tolerant and plastic
- Has adaptations for short- and long-distance dispersal
- If perennial, has strong vegetative reproduction or regeneration from fragments
- If perennial, has brittleness, so cannot be easily pulled from the ground
- Has the ability to compete interspecifically by special means (rosettes, smothering growth, allelochemicals)

Because weeds affect crop growth and yield, various control strategies have been used over the years.

## 2.2 History of weed control

Non-chemical weed management strategies have been used since the earliest days of agriculture until today, such as tillage, mowing, flooding/draining, hand-weeding, flaming, solarisation and mulching (Zimdahl, 2013). In addition to the above, temporal and spatial diversification is also developed, with farmers often preferring crop rotation to monoculture and intercropping to monoculture (Liebman & Dyck, 1993).

In the early 1930s, the first organic chemicals used to control weeds - called uncouplers - were introduced. The active ingredients (a.i.) dinitro-ortho-cresol (DNOC) and 6-sec-butyl-2,4-dinitrophenol (Dinoseb) belong to the chemical class of dinitrophenols. Their agronomic importance was immense, as their derivatives showed selectivity against broadleaf weeds

(Moreland, 1993). Later, in the 1940s (Figure 1), the first synthetic and systemic herbicides (2,4-D, 2,4,5-T, and MCPA) were launched into the market starting a new era in the weed control strategies (Troyer, 2001). As shown in Figure 1, between 1950 and 1980 was the era of discoveries and most of the currently known modes of action (MoA) were discovered and can be grouped into the following major categories: (i) light processes, (ii) cell metabolism, and (iii) growth and cell division. However, there was a tendency for farmers to use the same herbicides over and over again to control weed species, such as glyphosate (EPSPS, HRAC group 9), so there was no need for a new herbicide MoA. Besides, due to selection pressure through the use of specific herbicides, especially the post-emergent herbicides belonging to acetyl-CoA carboxylase (ACCase; HRAC Group 1) and acetolactate synthase (ALS; HRAC Group 2) inhibitors (Heap, 2023; Keshtkar et al., 2015; Peterson et al., 2018; Torra et al., 2021), herbicide-resistant weeds have increasingly become a problem in modern agriculture. Grass species particularly evolved herbicide resistance and one of these problematic species in Europe is black-grass (*Alopecurus myosuroides* Huds.) as described in §2.5 (Moss, 2017). However, the need for a new mode of action was essential (Selby et al., 2023) and the agrochemical community found an alternative herbicidal MoA, tetflupyrolimet (DHODH; HRAC Group 28) in 2021.

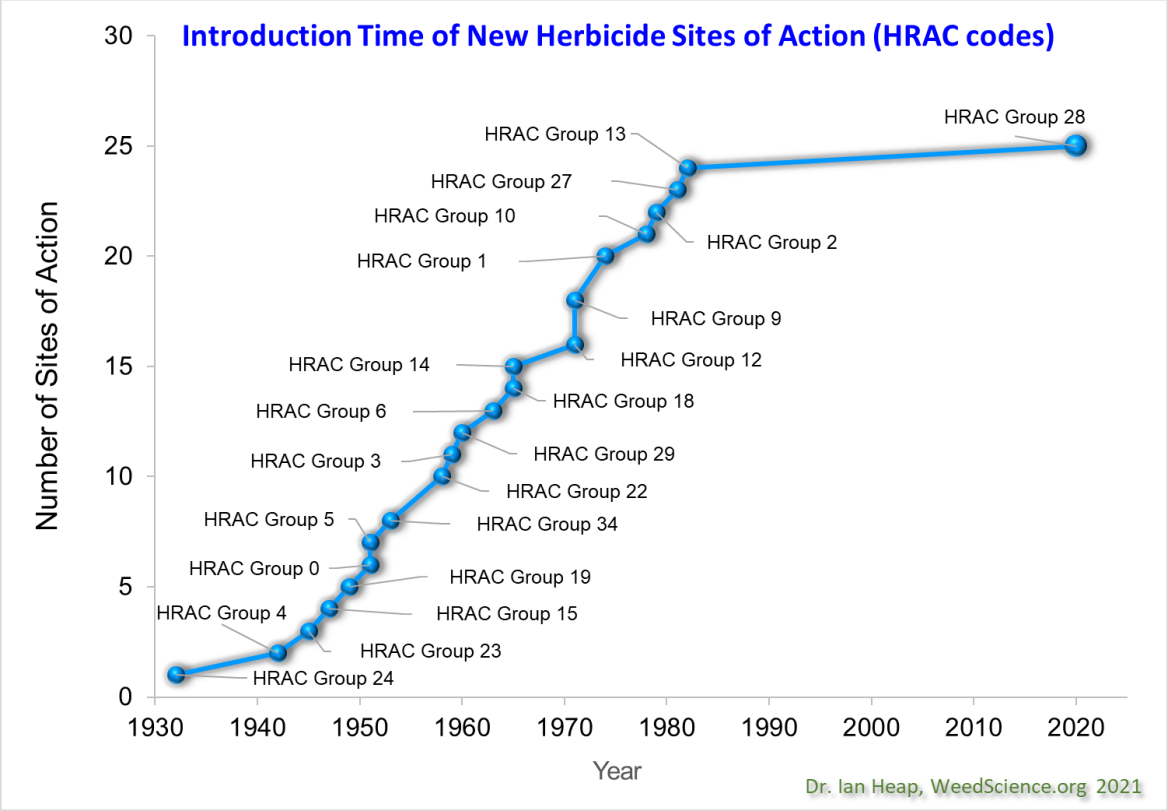



Figure 1. Introduction of different herbicides into the market. Figure from WeedScience.org (Heap, 2023; based on 2021 classification).

## 2.3 Herbicide resistance and detoxification pathways

According to the WSSA Terminology Committee (WSSAb, 2022), herbicide resistance is “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis”. However, herbicide tolerance is “the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant.”

In general, there are two types of herbicide resistance, the target-site resistance (TSR) and non-target-site resistance (NTSR) (Délye, Jasieniuk, et al., 2013; Powles & Yu, 2010), where specialist and generalist mechanisms respectively are involved (Comont et al., 2020a). These two types of resistance can co-exist (Comont et al., 2020a; Jugulam & Shyam, 2019).

TSR is caused due to changes at the molecular targets – which are typically proteins – thus decreasing the affinity for the a.i. In addition, overproduction of the target, resulting in the need for higher amounts of herbicide to achieve an adverse effect, falls into this category (Laforest et al., 2017; Gaines et al., 2010). Herbicide resistance typically evolves through selection from standing variation, rather than through *de novo* mutations in its target-site encoding genes (Hawkins et al., 2019), meaning that herbicide treatment itself cannot induce point mutations at the target site, but these are already present in weed populations. This, is in accordance with the following study where weed species with TSR-relevant mutations in herbaria (collected 1788-1975) were found before the commercial herbicide use (Délye, Deulvot, et al., 2013). Typically, TSR is the most common type of resistance to a.i. belonging to ACCase and ALS inhibitors (Powles & Yu, 2010).

NTSR, includes any mechanism that prevents all or part of the a.i. from reaching its molecular target. In that way, different mechanisms have been observed in plants, such as reduced penetration/absorption, altered translocation, sequestration/compartmentalisation, and metabolism of the a.i. (Jugulam & Shyam, 2019; Délye, Jasieniuk, et al., 2013).

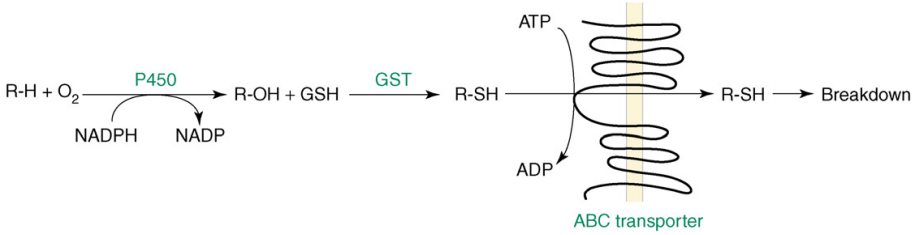



Figure 2. Detoxification pathway of xenobiotic compounds in plants (according to Yuan et al., 2007).

Herbicide resistance due to enhanced metabolism is the most frequent NTSR mechanism (Gaines et al., 2020; Torra & Alcántara-de La Cruz, 2022). The detoxification pathway of xenobiotic compounds in plants generally consists of four phases, although not all are necessarily involved in the detoxification of each xenobiotic (Figure 2). Phase I involves cytochrome P450 monooxygenases (CYPs, E.C. 1.14). These are oxygen- and NADPH-dependent proteins located in the endoplasmic reticulum that can be inhibited by carbon monoxide and then reversed by light. They are closely related biochemically to the P450 reductases and are dependent, as the former requires electrons produced by the latter. This is why CYPs can be inhibited by antibodies directed against P450 reductases. CYPs can carry out various reactions, such as isomerisation, dimerisation, dehydration and reduction, but in most cases they carry out mono-oxygenation (Werck-Reichhart et al., 2000). Typically, CYPs that detoxify herbicides belong to class II (eukaryotic microsomes) and have a 2-component system consisting of a NADPH:P450 reductase (FAD and FMN-containing flavoprotein) and a P450 oxidase (class II/class E) (Brazier-Hicks et al., 2022; Degtyarenko, 1995).

Glutathione transferases (GSTs, E.C. 2.5.1.18) and glucosyl transferases (GTs, E.C. 2.4), which catalyse the conjugation of xenobiotics with glutathione (GSH) or uridine diphosphate glucose (UDP-glucose) respectively, are commonly involved in phase II (Coleman et al., 1997). Although, there are known cases where the substrates are directly conjugated by GSTs or GTs without phase I reaction to take place. This holds true for flufenacet (Dücker, Zöllner, Parcharidou, et al., 2019), fenoxaprop-P-ethyl (Edwards & Cole, 1996; Tal et al., 1993), atrazine (Anderson & Gronwald, 1991), EPTC (Carringer et al., 1978) and pyroxasulfone (Busi et al., 2018; Goggin et al., 2021), which are detoxified by GSTs. These are proteins with wide spectrum of functions, including abiotic stress response and cell signalling (Frova, 2006; Labrou et al., 2015). GT genes form a large superfamily in higher plants, encoding enzymes that glycosylate a wide range of aglycones, including plant hormones and secondary metabolites, as well as xenobiotics such as herbicides. There are more than 100 genes encoding GTs in thale cress (*Arabidopsis thaliana* (L.) Heynh.) (Ross et al., 2001) and recently its UGT91C1 was found to detoxify sulcotrione (Huang et al., 2021).

The conjugated products are then transferred in phase III from the cytoplasm into the vacuole by specialised ATP binding cassette (ABC) transporter proteins (EC 7.6.2.2) (Bartholomew et al., 2002; Martinoia et al., 1993), which consist of two ABC domains (nucleotide-binding domains) and two transmembrane domains (TMDs) (Rees et al., 2009).

In phase IV, the conjugates are further degraded to non-phytotoxic metabolites and incorporated into the cell wall, bound to cellular constituents or partitioned into lipids (Edwards et al., 2011; Sterling, 1994).

Although the basic features of TSR and NTSR are now quite well understood, the regulation of the genes that lie behind the NTSR is still only rudimentary.

## 2.4 Plant gene regulation

### 2.4.1 Gene regulation in plants

Whether, and at which rate the transcription of a gene is occurring, depends on the *cis*-regulatory sequences (CREs) and trans-acting factors. The first are non-coding linear nucleotide fragments which serve as binding sites for the latter ones, which are coded by DNA regions called trans regulatory elements (Yamaguchi, 2018; Biłas et al., 2016).

First of all, many CREs are located in the promoter of a gene, which is comprised of the core (or basal), proximal and distal regions. The core promoter stretches approximately 100 bp upstream of the transcription start site (TSS) and elements such as the TATA box and – the animal counterpart of CAAT box in plants – AGGA box are confined there. Further upstream (up to 200 bp) the proximal promoter is located where various other CREs are found. Even more upstream, commonly up to 1 kb, most of the enhancer and silencer elements are located. These elements though can be found as well in the coding sequence of the gene and even downstream of it (Biłas et al., 2016; Porto et al., 2014; Bulger & Groudine, 2011). Once the basal (or general) transcription factors bind on the promoter, the RNA polymerase II then, in turn, can bind on the core promoter facilitating the transcription initiation. The binding of activators and repressors on their enhancer and silencer binding sites respectively, can regulate the expression rate of the corresponding gene accordingly (Heldt & Piechulla, 2015). However, the presence or absence of exons or introns can also affect the activity of TFs when they bind within the gene sequence.

### 2.4.2 Plant transcription factors

Based on (Yamaguchi, 2018) “Plant transcription factors (TFs) determine when and where plants’ genes are transcribed, how many proteins are synthesised, and what the plants look like.”

The classification of eukaryotic TFs is made based on the characteristics of their DNA-binding domains (DBDs) (L. Liu et al., 1999). For example, TFs which belong to the WRKY superfamily possess a WRKY domain, which is a conserved amino acid sequence WRKYGQK residue motif and functions as the region which binds on the DNA (Eulgem et al., 2000).

In general, TFs can bind in a forward or a reverse direction of the dsDNA (Lis & Walther, 2016). The majority of TFs bind on the major groove of the DNA double helix rather than the minor groove. This is more favourable because of the larger room for the TF to assess the sides of

the bases and the more distinct recognition pattern of the H-bond donors and acceptors between the bases (Pabo & Sauer, 1992). Only a few prefer the minor groove, for instance, the TATA-binding proteins (TBPs), which can recognise their binding site solely due to the distinctive architecture of the region and the DNA flanking regions (Wong & Bateman, 1994; Starr & Hawley, 1991).

For the binding of the TFs with the specific CREs, hydrogen bonds and van der Waals interactions are taking place. In addition, electrostatic interactions can play a role in stabilising the specific interactions of the TF residues and the DNA bases, although this is a most prominent interaction when it comes to nonspecific DNA-TF binding (Suter, 2020; Pabo & Sauer, 1992; Berg et al., 1981).

There are cases where interactions of two TFs regulate a plant gene expression. For instance, it has been shown that Dof proteins can interact in a specific manner with bZIP proteins, and this interaction triggers the latter to bind on the DNA target sequences in some plant promoters, such as *Arabidopsis thaliana* GST6 (Singh, 1998; Chen et al., 1996).

Online TFs databases exist, such as TRANSFAC (Wingender, 1996) but as well specific to plant TFs, as for example PlantTFDB (Guo et al., 2007). Moreover, plenty *in silico* tools containing TFs and their putative CREs can be found online in a friendly user interface, such as PlantPan (Chang et al., 2008), PlantRegMap (Tian et al., 2020; Jin et al., 2015, 2017), PROMO (Farré et al., 2003), etc.

## 2.5 Black-grass (*Alopecurus myosuroides* Huds.)

### 2.5.1 Biology of black-grass

The grass weed black-grass (*Alopecurus myosuroides* Huds. or ALOMY) - formerly also known as *Alopecurus agrestis* L. - was first described in 1762 (Linné & Salvius, 1762) and it has benefited from the practices of modern agriculture, as it is a winter annual weed well adapted to a high proportion of winter cereals in the crop rotation. Black-grass populations generally have two distinct emerging cohorts, a major in autumn, and a minor one in spring (Barralis, 1970); it is, therefore, less prevalent in spring cropping systems. As typical for Poaceae species (Naylor 1972), black-grass is wind pollinated and furthermore characterised by protogyny, and thus most of the times is cross-fertilised and typically classified as an obligate outcrosser (Moss, 1983; Johnsson, 1944).

Black-grass predominantly occurs on very heavy and wet soils (Moss, 2017). Its seedling can emerge before the crop or usually until a week after crop emergence. In Figure 3d a black-grass seedling is illustrated, and its first leaf is delicate and corkscrewed. Black-grass grows from 20 cm up to 80 cm tall (Figure 3a). The leaf blade is narrow and hairless, and the plant has erect stems and narrow sharp-edged bare ridged leaves. The ligule is long with rough and

irregular slits (Figure 3c), but there is no auricle. It has single-flowered spikelets, in a slender inflorescence (spike) up to 8 cm in length, which is often tinged with red (Figure 3b). The glumes of the seed are pointed and edged with short hairs and the lemma is awned (Figure 3e) (Naylor, 1972).

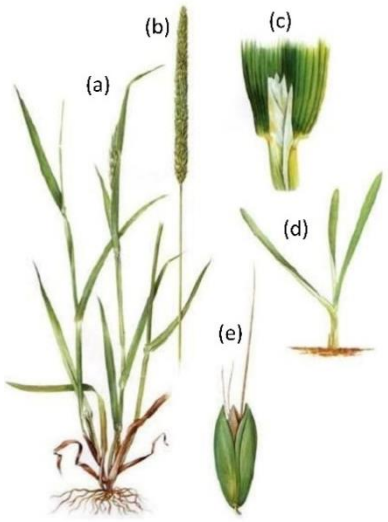



Figure 3. Black-grass (*Alopecurus myosuroides* Huds.) morphology (a) plant height varied from 20 to 80 cm, stem and non-hairy leaves, (b) panicle, (c) long ligule, generally irregularly dentate, (d) plant at three-leaf growth stage, first leaf rolled, (e) germinating seed (Geissel 2004).

### 2.5.2 Control of black-grass and resistance evolution

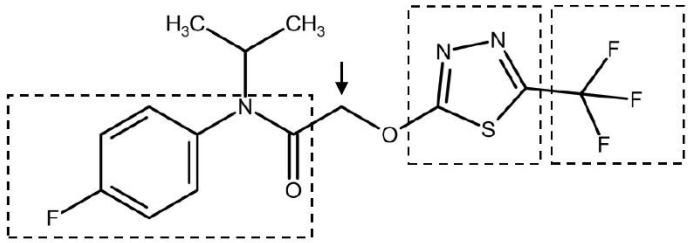
Black-grass in winter cereal fields in Western Europe has become more difficult to control over the past 17 years (Keshtkar et al., 2015; Moss et al., 2007). More specifically, resistance has evolved against up to five different herbicide modes of action (Heap, 2023). As resistance to the post-emergent herbicides inhibiting acetyl-CoA-carboxylase (ACCase, HRAC Group 1) and acetolactate synthase (ALS, HRAC Group 2) became more widespread, agronomic practices have been adapted to this situation and black-grass control gradually shifted to autumn treatments in winter crops (Bailly et al., 2012; Moss et al., 2007).

With increasing resistance, integrated weed management (IWM), which combines the use of chemical and non-chemical measures, is becoming increasingly important and is also used more frequently in practical agriculture. The most effective non-chemical tactic used for the control of black-grass is the ploughing. In addition, a rotation with spring cereals such as wheat and a delay in autumn sowing and a delay in drilling will result in a reduction in black-grass densities. Similarly, choosing more competitive varieties can reduce the percentage of black-grass heads (Lutman et al., 2013; Moss, 2017; Moss & Lutman, 2013). Today, however, pre-emergence herbicides such as flufenacet are used alone or in mixtures with diflufenican, pendimethalin, prosulfocarb and aclonifen (Bailly et al., 2012; BAYERa, 2022; BAYERb, 2022;

Klingenhagen, 2012). More recently, a newly registered a.i. in Europe – cinmethylin, belonging to the new mode of action (HRAC group 30) - is also an alternative for chemical control of this weed species (Messelhäuser et al., 2021). Such herbicides can be also applied in sequential applications.

Flufenacet is a herbicidal a.i. discovered by Bayer CropScience and it was launched on the German market 25 years ago (BVL, 2022; Krähmer et al., 2018). It is a pre-emergent herbicide belonging to the chemical class of α-oxyacetamides and is classified in HRAC Group 15 among other herbicides, which are generally less prone to evolve resistance (Moss et al., 2019). Particularly, flufenacet acts as cell division inhibitor by interfering with the first step of elongation of the biosynthesis of very-long-chain fatty acids (VLCFAs) (Haslam & Kunst, 2013; Trenkamp et al., 2004). VLCFAs are fatty acids longer than 18 carbons (C18) in length and are essential molecules produced by all plant cells. They are necessary for plant survival and have various roles dealing with the plant development (Haslam & Kunst, 2013; Krähmer et al., 2018). According to Bach et al. (2011), the VLCFAs are necessary for endomembrane dynamics during cytokinesis, thus after flufenacet application the cell division is inhibited. Flufenacet (Figure 4) exerts its activity by a nucleophilic attack of the thiol group of the conserved cysteine in the active centre of the target condensing enzyme, resulting in a separation of the acetanilide and trifluoromethyl-thiadiazol groups (Böger et al., 2000). In a similar approach, the same attack takes place *in vivo* in different weed and plant species while flufenacet is detoxified by plant enzymes i.e. GSTs (Dücker, Zöllner, Parcharidou, et al., 2019; Bieseler et al., 1997; Dücker, Zöllner, Lümmen, et al., 2019). Detoxification of flufenacet has been shown also *in vitro* by recombinant GSTs derived by black-grass populations with reduced flufenacet sensitivity, as well as ryegrass (Dücker, 2020; Parcharidou et al., 2023).

Although the metabolic pathway of flufenacet, which involves GST proteins, has been elucidated, the regulation of GST genes at the transcriptional level has not been investigated.




Flufenacet

Figure 4. Chemical structure of flufenacet [(4'-fluoro-*N*-isopropyl-2-(5-trifluoromethyl-1,3,4-thiadiazol-2-yloxy) acetanilide]. From left to the right, the 4'-fluoro-*N*-isopropyl-acetanilide, the thiadiazol and the trifluoromethyl groups are indicated in dashed frame boxes. The point where flufenacet is cleaved is indicated by the arrow.

## 2.6 Aim of the study

Resistance of black-grass to pre-emergent herbicides such as those belonging to Group 15 is evolving, among those flufenacet. Yet, the mechanisms at GST gene regulation level behind are not explicitly elucidated.

The aims of this study are to better characterise herbicide resistance in black-grass by (i) validation of candidate GSTs on flufenacet and other herbicides detoxification, (ii) investigation of cross-resistance at the individual protein level and (iii) investigation of their regulation at the transcriptional level. For that purpose, candidate GST genes found upregulated in flufenacet resistant black-grass populations were chosen for *in vitro* validation of their activity on flufenacet and other herbicides using analytical methods (HPLC, LC-MS/MS). Subsequently, all GSTs found in the black-grass genome were identified and their promoters were studied using *in silico* tools. Finally, a candidate CRE common to the promoters of three up-regulated GSTU genes were further investigated using an electrophoretic mobility shift assay (EMSA).

Knowledge of cross-resistance patterns between different GST proteins to different herbicides, may help farmers to establish a less favourable resistance evolution management system for black-grass. In addition, understanding the mechanism of flufenacet resistance at the molecular level in black-grass may allow companies to adjust their crop protection products and their use to prevent further resistance evolution.

Finally, the evolution, nomenclature and role of GSTs in herbicide detoxification and their regulation was reviewed as an extension of the General Introduction (§3).

# 3. A brief review of glutathione transferase proteins as key enzymes involved in herbicide detoxification.

## 3.1 The classification of the glutathione transferases (GST)

Glutathione transferases (GSTs; EC 2.5.1.18) are highly versatile enzymes encoded by a large and diverse family of genes in plants. The plant GSTome (Edwards et al., 2000; Frova, 2003, 2006; Labrou et al., 2015; Vaish et al., 2020) generally consists of three superfamilies: a cytosolic, a mitochondrial and a microsomal superfamily, each of which is subdivided into different classes. The former are located in the cytosol, as their name suggests, the latter would refer to microsomal GSTs, which are also known as the kappa class (GSTK) (Kumar & Trivedi, 2018), while the microsomal ones are known as Membrane Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEGs). Cytosolic plant GSTs are classified into 14 classes based on their protein sequence: Tau (GSTU), Phi (GSTF), Theta (GSTT), Zeta (GSTZ), dehydroascorbate reductase (DHAR), Lambda (GSTL), tetrachlorohydroquinone dehalogenase (TCHQD), γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ), Hemerythrin (GSTH), Iota (GSTI), glutathionylhydroquinone reductase (GHR) and ureidosuccinate transport 2 prion protein (Ure2p) (Bchini et al., 2021). The classes GSTU, GSTF, GSTL and DHAR are plant specific. GSTU was previously assigned to the former type III of GSTs with 2 exons, mainly described as auxin-induced GSTs. GSTF was previously assigned to the former type I with 3 exons, mainly involved in combating abiotic - including oxidative – stress (Marrs, 1996; Edwards et al., 2000; Labrou et al., 2015). Another GST is class the metaxins (MTXs) (Lister et al., 2007), which are cytosolic but mitochondrial-facing proteins, and enzymes classified as prostaglandin E synthase type 2 (mPGES2), which belong to the microsomal superfamily. Other proteins resemble the GST fold, such as 2-GSTN (Lallement et al., 2014), aminoacyl-tRNA synthetases (aaRS) (Nyamai & Tastan Bishop, 2019) and aminoacyl-tRNA synthetase cofactor 1 (Arc1p) (Simader et al., 2006; Frechin et al., 2010) and are therefore considered putative GST genes. The plant-specific GST classes GSTU, GSTF, GSTT, GSTZ and TCHQD contain a serine (Ser13) active site residue involved in GSH binding (Axarli et al., 2009), whereas the GSTI, GSTH, DHAR, GSTL, GHR, mPGES2 and MTX classes contain a cysteine (Cys) active site residue that facilitates a deglutathionylation reaction (Lallement et al., 2014). The nature of the catalytic residue in the EF1Bγ and Ure2p classes is not yet well understood. The cys-GSTs, DHAR and GSTL are monomeric, whereas the majority of GSTs are dimeric. Most cytoplasmic GSTs form dimers with a molecular mass of about 50 kDa and 25 kDa per monomer, catalysing a wide range of reactions including the conjugation of glutathione (GSH; g-Glu-Cys-Gly) to electrophilic compounds. Each monomer consists of an N-terminal and a C-terminal domain connected by a linker of about 10 residues in length. Monomers encoded by the same gene can form homodimers and monomers

encoded by different genes can form heterodimers, increasing the diversity of GST proteins. The first domain consists of β-sheets and α-helices, whereas the second domain consists of α-helices only (Reinemer et al., 1996). The N-terminal domain contains a hydrophilic G-site for GSH binding and the C-terminal domain contains a hydrophobic H-site for electrophilic substrate binding. In addition, there is a ligand binding site (L-site), the location of which can vary from protein to protein (Axarli et al., 2009; Sylvestre-Gonon et al., 2019).

## 3.2 The evolution of the GST genes

GST genes have been observed to follow a non-random distribution in plant genomes, which have been shaped by whole genome duplication (WGD), tandem duplication, segmental duplication and genomic rearrangements. WGD, or polyploidy, is a major force in plant genome evolution. During a WGD event, all functional elements (transcribed and regulatory) are included in the duplicated regions, and following such an event, all genes previously present once in the genome are present in duplicate. Studying the process and mechanism of loss/retention of duplicated genes resulting from polyploidy – the so called polyploidy-derived duplicate gene loss/retention – is particularly important for understanding the evolution of polyploidy (Liu et al., 2015). All these WGD events happened a million years ago (Ma) and through evolution many genes were lost, i.e., contracted, and other genes were retained, i.e., expanded, such as the GST genes of the black-grass (Cai et al., 2023). Despite undergoing WGD, black-grass remained diploid, probably by the same mechanism as some Arabidopsis species, through a diploidisation process involving massive genomic reorganisation and functional changes (Wolfe, 2001; Del Pozo & Ramirez-Parra, 2015; Li et al., 2021).

In general, gene duplications are necessary for new biological functions and widespread expansion of gene families, resulting in a broader substrate spectrum and a wide range of reactivity towards different substrates (Liu et al., 2013). The two dominant classes, GSTUs and GSTFs, are the result of recent, multiple duplication events and have undergone extensive duplication and divergence. The GSTTs and GSTZs are poorly represented in any organism, suggesting that these genes have undergone few duplications or that the duplicated copies have subsequently been lost. The high sequence similarity of clustered genes indicates recent multiple duplication events, confirming a general clustering tendency of GST genes (Frova, 2003). Interestingly, despite overall sequence and gene structural similarities, even the most recently duplicated and diverged GST homologs differ significantly in their gene expression patterns, their response to various biotic and abiotic stresses, and their substrate specificity and kinetic properties (Labrou et al., 2015). GSTs evolved from a thioredoxin fold (Martin, 1995) and through evolution, have undergone several differentiation steps involving exon shuffling and recombination (Mannervik et al., 1988) to the classes that exist today (Frova,

2006; Öztetik, 2008). Exon shuffling has been a driving factor in conferring GSTs the activity they have today, but in some cases it has had the opposite effect, leading to the loss of GST activity, as in the case of the S-crystallin genes (Tomarev et al., 1992, 1995). In many different plant species, GST genes are arranged in clusters, each containing genes belonging to the same GST class. Clusters have been observed in rice (*Oryza sativa* L.; (Jain et al., 2010; Soranzo et al., 2004), carnation (*Dianthus caryophyllus* L.; (Itzhaki & Woodson, 1993), pink shepherd's-purse (*Capsella rubella* Reut; He et al. 2016), barley (*Hordeum vulgare* L.; Rezaei et al. 2013), thale cress (*Arabidopsis thaliana* (L.) Heynh.;(Dixon et al., 2002; Dixon & Edwards, 2010; Edwards et al., 2000), California poplar (*Populus trichocarpa* Torr. & A.Gray ex. Hook.; Lan et al. 2010) oilseed rape (*Brassica napus* L. and *Brassica rapa* L.; Wei et al. 2019) soybean (*Glycine max* (L.) Merr.; Liu et al. 2015) and black-grass (*Alopecurus myosuroides* Huds.; Cai et al. 2023).

In pink shepherd's-purse and barley (Rezaei et al., 2013) the GST genes arose from tandem gene duplication, especially for the clusters of GSTU and GSTF (He et al., 2016). Also in thale cress (Dixon et al., 2002; Dixon & Edwards, 2010; Edwards et al., 2000), GST genes arose from tandem gene duplication events. In Californian poplar (Lan et al., 2010) a WGD combined with a more recent large-scale duplication event was the driving force. Speciation and whole-genome triplication (WGT) played important roles in the divergence of the GST-duplicated genes in oilseed rape (*Brassica napus* L.), GST duplicated genes (Wei et al., 2019) and the WGT in mustard (*Brassica rapa* L.), but duplication analysis showed that 45.33% of the genes occurred mainly by tandem duplication, especially of GSTU, and in the classes GSTU and GSTF, a high degree of gene retention was observed after the last WGD. Segmental duplication also contributed to the expansion of the BraGSTs family (Khan et al., 2018). In soybean, the duplicated GST genes were formed by WGD (Wei et al., 2019). Segmental duplication appears to contribute more than tandem duplication to the expansion of the GSTs gene family in pepper (*Capsicum annuum* L.; Islam et al. 2019). Overall, it has been found that 101 GSTs genes exist in soybean (Liu et al., 2015), 81 in poplar (Lan et al., 2010), 48 in thale cress (Dixon et al., 2002), 42 in potato (Ding et al., 2017), 99 in sorghum (Chi et al., 2011), 75 in mustard (Khan et al., 2018), 65 in *Brassica oleracea* (Vijayakumar et al., 2016) and 115 in black-grass (Cai et al., 2023).

## 3.3 The role of GST proteins in non-target-site herbicide resistance (NTSR)

As stated above, GSTs are enzymes involved in phase II of the xenobiotic detoxification pathway. They can act as scavengers (glutathione peroxidases) (Cummins et al., 1999), be involved in plant secondary metabolism (Cummins et al., 2013) or conjugate electrophilic substances with reduced GSH, thus deactivating them (Bieseler et al., 1997). Some of these

are herbicides, either pre- or post-emergent herbicides (Powles & Yu, 2010; Yuan et al., 2007). For example, a VLCFA inhibitor, flufenacet, is metabolised by GSTs *in vivo* in black-grass, and an increased rate of metabolism in the flufenacet resistant populations was correlated with reduced efficacy of the formulated product in the greenhouse (Dücker, Zöllner, Parcharidou, et al., 2019). *In vitro* studies have also shown that recombinant GST proteins can detoxify flufenacet and produce flufenacet-GSH conjugates and, in some cases, flufenacet-alcohol conjugates (Parcharidou et al., 2023). In the same study, a GSTF was able to metabolise another VLCFA inhibitor, pyroxasulfone. *In vitro* metabolism of this active ingredient was associated with high levels of GST expression and GSH-pyroxasulfone metabolites in annual rye grass (*Lolium rigidum* Gaud.; Busi et al. 2018; Goggin et al. 2021). Furthermore, *S*-metolachlor tolerance has been associated with GST activity in maize (*Zea mays* L.; Cottingham et al., 1993) and GST involvement in waterhemp (*Amaranthus tuberculatus;* Strom et al. 2020) and Palmer amaranth (*Amaranthus palmeri*; Brabham et al. 2019). There is also the case of another VLCFA, EPTC, which is GSH conjugated (Lay & Casida, 1976) but not necessarily enzymatically (Carringer et al., 1978). Furthermore, atrazine resistance in velvetleaf (*Abutilon theophrasti* Medik.) was controlled by GST activity via GSH conjugation (Anderson & Gronwald, 1991; Gray et al., 1996; Plaisance & Gronwald, 1999). The association of GSTs with the post-emergent prodrug fenoxaprop-P-ethyl and its active ingredient fenoxaprop-P-acid has been studied *in vivo* and *in vitro*, and GSH-conjugation has been shown to occur either enzymatically or non-enzymatically (Edwards & Cole, 1996; Parcharidou et al., 2023; Tal et al., 1993). In addition, detoxification of the herbicide fomesafen via GSH-conjugation was observed (Skipsey et al., 2005). Analytical studies *in vivo* have confirmed that several herbicides previously mentioned, undergo conjugation in the presence of reduced GSH, such as flufenacet in ryegrass (Dücker, Zöllner, Lümmen, et al., 2019), black-grass (Dücker, Zöllner, Parcharidou, et al., 2019), pyroxasulfone in maize (Shimabukuro et al., 1970) and ryegrass (Busi et al., 2018; Goggin et al., 2021).

Table 1. *In vitro* activity of recombinant glutathione transferase proteins on different pre- and post-emergent herbicides

| Chemical Class (MoA) | Herbicide | GST Gene | GST Class | Reference |
|---|---|---|---|---|
| α-chloroacetamides (VLCFAs inhibitors) | Acetochlor | LrGSTF | phi | Georgakis et al. 2021; Ioannou et al., 2022 |
| | | AmGSTF1[1] | phi | Georgakis et al. 2021; Ioannou et al. 2022 ; Parcharidou et al. 2023 |
| | | ALOMY5G35766 | phi | Parcharidou et al. 2023 |
| | | ALOMY3G13667 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13668 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13670 | tau | Parcharidou et al. 2023 |
| | Alachlor | ZmGSTI | phi | Mozer et al., 1983 |
| | | ZmGSTII | phi | Mozer et al., 1983 |
| | | ZmGSTIII | phi | Moore & Purugganan, 2003 |
| | | LrGSTF | phi | Georgakis et al. 2021; Ioannou et al. 2022 |
| | | AmGSTF | phi | Georgakis et al. 2021; Ioannou et al. 2022 |
| | Butachlor | LrGSTF | phi | Georgakis et al. 2021; Ioannou et al. 2022 |
| | | AmGSTF | phi | Georgakis et al. 2021; Ioannou et al. 2022 |
| | | HvGSTF | phi | Georgakis et al. 2021; Ioannou et al. 2022 |
| | | TaGSTF | phi | Georgakis et al. 2021 |
| | | TdGSTF | phi | Ioannou et al. 2022 |
| | *S*-metolachlor | LOLSSGST1A | tau | Dücker 2020 |
| α-Oxyacetamides (VLCFAs inhibitors) | Flufenacet | LOLSSGST1A | tau | Dücker 2020 |
| | | LOLSSGST3 | phi | Dücker 2020 |
| | | ALOMY5G35766 | phi | Parcharidou et al. 2023 |
| | | AmGSTF1 | phi | Parcharidou et al. 2023 |
| | | ALOMY3G13667 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13668 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13670 | tau | Parcharidou et al. 2023 |
| Isoxazolines (VLCFAs inhibitors) | Pyroxasulfone | ALOMY5G35766 | phi | Parcharidou et al. 2023 |
| Triazines (D1 Serine 264 | Atrazine | hGSTP1-1[2] | pi | Abel 2004 |
| | | mGSTP1-1[2] | | |

| | | | | |
|---|---|---|---|---|
| binders (and other non-histidine 215 binders; PS II inhibitors) | | | | |
| Diphenyl ethers (PPO inhibitors) | Fomesafen | GmGSTU21 | tau | Skipsey et al. 2005 |
| Aryloxphenoxy-propionates (FOPs; ACCase inhibitors) | Fenoxaprop-P-ethyl (prodrug) | ALOMY5G35766 | phi | Parcharidou et al. 2023 |
| | | AmGSTF1 | phi | Parcharidou et al. 2023 |
| | | ALOMY3G13667 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13668 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13670 | tau | Parcharidou et al. 2023 |
| | Fenoxaprop-P-acid (active herbicide) | ALOMY5G35766 | phi | Parcharidou et al. 2023 |
| | | AmGSTF1 | phi | Parcharidou et al. 2023 |
| | | ALOMY3G13667 | tau | Parcharidou et al. 2023 |
| | | ALOMY3G13670 | tau | Parcharidou et al. 2023 |

[1]In (Parcharidou et al., 2023) the gene was annotated as AmGSTF1/ALOMY3G11300 and the accession number was AJ010453.1, which is the same isoform studied in (Georgakis et al., 2021) and (Ioannou et al., 2022) called as AmGSTF (accession number: CAA09192.1).

[2]Data are derived by human (hGSTP1-1) and mouse (mGSTP1-1) studies. Direct *in vitro* validation with GST proteins from plant and weed species has not yet been carried out, only as extracts (see Egaas et al. 1993; Nakka et al. 2017).

## 3.4 Restoration of herbicide sensitivity using inhibitors of GST proteins

The partial restoration of reduced herbicide sensitivity in weed species has already been discussed in the scientific community and examples related to GST enzymes have been described. The most prominent substances are ethacrynic acid and tridiphane. In studies on plant species, ethacrynic acid was able to reduce the metabolism of *S*-metolachlor in maize (Li et al., 2017) and interacted with the GmGST1-1 of soybean (Skipsey et al., 1997). Tridiphane has been used as a synergist for different herbicides in maize and proso millet (*Panicum miliaceum* L.; Ezra et al., 1985). It has been used as a synergist for atrazine in various plant species at the molecular level (Lamoureux & Rusness, 1986) and also in atrazine mixtures (Ahrens & Ehr, 1991). Both compounds slowed the degradation of flufenacet in flufenacet resistant black-grass (Dücker et al., 2020). In the same study, flufenacet degradation rates were also reduced by the addition of malathion, which is typically used as a cytochrome P450 monooxygenase inhibitor (CYP; Kreuz & Fonné-Pfister, 1992).

However, the use of this type of inhibitors for field application is somewhat problematic due to the inhibition of human GST classes and the potential risk to human health (Yang et al., 2010; Yu et al., 2015), and restoration of full sensitivity is generally not feasible.

## 3.5 Regulation of transcription of GST genes associated with abiotic stresses, including herbicide resistance

The promoters and regions where the *cis*-elements of the GST genes are located are quite divergent and differ even within the same class as found in black-grass (Parcharidou et al., 2024), rice (Soranzo et al., 2004), *Arabidopsis* and *Brassica* (Wang & Adams, 2015). In several plant GST promoters (Marrs, 1996) and promoters of pathogenesis related (PR) genes (Kong et al., 2018) the *ocs* (octopine synthase) element is found. This is a 20-bp DNA region, comprising a tandem core sequence of ACGT serving as binding site for dimeric bZIP transcription factors, as OCSBF-1 and ASF1 (Chen et al., 1996; Lam & Lam, 1995; B. Zhang & Singh, 1994) and is triggered under stress conditions, hormones and chemical agents (Marrs, 1996; Ulmasov et al., 1994). This is found in thale cress (Zhang & Singh, 1994), soybean, wheat (*Triticum aestivum* L.) and tobacco (*Nicotiana benthamiana* Domin) (Frova, 2003; Marrs, 1996). A weighted gene co-expression network analysis (WGCNA) in *Brassica napus* revealed several TFs such as NAC, MYB, WRKY and bZIP, which might play a role in the expression of various GST genes belonging to GSTU, GSTF, DHAR and EF1Bγ classes (Wei et al., 2019). In the tea plant (*Camellia sinensis* (L.) Kuntze), a WRKY protein had the ability to act as transcriptional activator by binding on the promoter of the CsGSTU8 under drought stress and ABA treatment (Zhang et al., 2021). The presence of various *cis*-elements on the promoter of GST genes found in pepper was correlated with stress-response and hormones; however, without further functional validation (Islam et al., 2019). Combinatorial interactions between Dof and bZIP proteins has been observed regarding the regulation of the GST genes, specifically in thale cress. These two proteins interact and the last one binds on the ocs element of AtGST6 (Chen et al., 1996; Singh, 1998). An ethylene responsive factor in thale cress, the AtEBP has been shown to interact *in vitro* with OBF4, which is a bZIP factor, although the functional importance of this interaction has not been investigated regarding the expression of the GST or PR-related genes (Büttner & Singh, 1997). Yet, studies targeted on the regulation of the GST genes related to herbicide NTSR have not been explicitly performed. In the first study contacted recently (Parcharidou et al., 2024), it showed constitutive upregulation of AP2/ERF TFs based on transcriptome studies between flufenacet sensitive and resistant black-grass populations, as well, the role of E2F/DP factors in an upregulated cluster of GSTUs was investigated.

## 3.6 References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# 4. Recombinant glutathione transferases from flufenacet-resistant black-grass (*Alopecurus myosuroides* Huds.) form different flufenacet metabolites and differ in their interaction with pre- and post-emergence herbicides

Evlampia Parcharidou[a*], Rebecka Dücker[a], Peter Zöllner[b], Susanne Ries[b], Roberto Orru[b], and Roland Beffa[c*]

[*] *Correspondence to: E Parcharidou, Georg-August University Göttingen, Division of Plant Pathology and Crop Protection, Göttingen, Germany, E-mail: e.parcharidou@stud.uni-goettingen.de and R Beffa, Senior Scientist Consultant, Liederbach am Taunus, Germany, E-mail: roland.beffa@t-online.de*

[a] *Georg-August University Göttingen, Division of Plant Pathology and Crop Protection, Göttingen, Germany*

[b] *Bayer AG, CropScience Division, Frankfurt am Main, Germany*

[c] *Senior Scientist Consultant, Liederbach am Taunus, Germany*

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*See Annex (§10.1) for the supporting information section of this paper.*

Abstract

BACKGROUND: Black-grass (*Alopecurus myosuroides* Huds.) has become a problematic weed in cereals in Europe. Besides resistance to post-emergent herbicides becoming increasingly widespread, enhanced metabolism of inhibitors of the synthesis of very-long-chain fatty acids (VLCFAs), such as flufenacet, is evolving. Yet, cross-resistance patterns and evolution of this resistance remains poorly understood.

RESULTS: The cDNA sequences of five glutathione transferases (GSTs) upregulated in flufenacet resistant black-grass were identified and used for recombinant protein expression. Moderate to slow detoxification of flufenacet was verified for all candidate GSTs expressed in *E.coli*, and the most active protein produced flufenacet-alcohol instead of a glutathione conjugate, in presence of reduced glutathione (GSH). Moreover, cross-resistance to other VLCFA-inhibitors e.g. acetochlor and pyroxasulfone and the ACCase inhibitor fenoxaprop was verified *in vitro*. Various other herbicides of different modes of action including VLCFA-inhibitors were not detoxified by the candidate GSTs.

CONCLUSIONS: As several *in planta* upregulated GSTs detoxified flufenacet *in vitro*, the shift in sensitivity observed in black-grass populations, is likely a result of an additive effect. The polygenic character and the relatively low turnover rate of the individual GSTs may explain the

slow evolution of flufenacet resistance. In addition, flufenacet resistance was accompanied by cross-resistance with some, but not all, herbicides of the same mode of action, and furthermore to the ACCase inhibitor fenoxaprop-ethyl. Hence, not only the rotation of herbicide modes of action, but also of individual active ingredients is important for resistance management.

Keywords: cross-resistance; fenoxaprop; flufenacet-alcohol; HRAC Group 15; metabolic resistance; VLCFAs

## 4.1 Introduction

Black-grass (*Alopecurus myosuroides* Huds.) has become one of the most difficult weed species to handle in Western Europe, especially in cereals.(Keshtkar et al., 2015; Moss et al., 2007) This grass weed has evolved resistance against up to five different herbicide modes of action.(Heap, 2023) As resistance to the post-emergent herbicides inhibiting acetyl-CoA-carboxylase (ACCase, HRAC Group 1) and acetolactate synthase (ALS, HRAC Group 2) is becoming more widespread (Heap, 2022), agronomic practices have been adapted to this situation and black-grass control gradually shifted to autumn treatments in winter crops. Herbicides, such as flufenacet, which are classified as inhibitors of the synthesis of very-long-chain fatty acids (VLCFAs, HRAC Group 15) are most relied on for that purpose. As the use of flufenacet has gradually increased since the market introduction in 1998 in Germany (BVL, 2022) selection pressure by this herbicide is also enhanced. Although this mode of action is classified as less prone to evolve resistance (Moss et al., 2019), cases of reduced sensitivity have been reported worldwide to two dicotyledons, tall waterhemp (*Amaranthus tuberculatus* (Moq.) JD Sauer; Strom et al., 2019) and palmer amaranth (*Amaranthus palmeri* S. Watson; Brabham et al., 2019) and various monocotyledonous species (Heap, 2023). The affected grass weeds include wild oat (*Avena fatua* L.;Mangin et al., 2017) barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.; Juliano et al., 2010) Italian ryegrass (*Lolium multiflorum* (Lam.); Dücker, Zöllner, Lümmen, et al., 2019; Rauch et al., 2010) and rigid ryegrass (*Lolium rigidum* Gaud.; Busi et al., 2018; Busi & Powles, 2013). Furthermore, black-grass (*A. myosuroides*) is affected by evolving resistance against several VLCFA-inhibitors including the oxyacetamide flufenacet (Dücker, Zöllner, Parcharidou, et al., 2019; Rosenhauer & Petersen, 2015). Target site-resistance has so far not been described as a cause of resistance and is unlikely for two main reasons: On the one hand the putative binding site of flufenacet, the highly conserved cysteine residue in the active centres of β-keto-acyl-CoA synthases (KCSs), is crucial for the catalytic function (Böger et al., 2000; Krähmer et al., 2018). On the other hand, redundancy was observed among this enzyme family (Tanetani et al., 2013; Todd et al., 1999). Thus, a single mutation is unlikely to have a significant effect on the resistance level. Instead, reduced herbicide efficacy of inhibitors of VLCFAs was linked to metabolic herbicide resistance, mostly in already multiple resistant populations.

Analytical studies in black-grass revealed that glutathione transferases (GSTs; EC 2.5.1.18) are the enzymes catalysing the first step of flufenacet detoxification *in vivo* (Dücker, Zöllner, Parcharidou, et al., 2019). The same pathway was described for other grass weeds such as ryegrass (Dücker, Zöllner, Lümmen, et al., 2019) and several crops (Bayer AG, 2017; Bieseler et al., 1997; Gould & Lemke, 2002). Analytical studies conducted with maize, soybeans, sunflower, and wheat (Gould & Lemke, 2002) suggest two distinct pathways, with either a flufenacet-glutathione conjugate or flufenacet-alcohol as the first occurring metabolite. While the reaction leading to the formation of flufenacet-alcohol was not further specified, it is known that conjugation of the tripeptide glutathione (GSH) to flufenacet and various herbicides of different chemical classes such as fenoxaprop-ethyl (Edwards & Cole, 1996; Tal et al., 1993), atrazine (Anderson & Gronwald, 1991), EPTC (Carringer et al., 1978) or pyroxasulfone (Busi et al., 2018; Goggin et al., 2021) is catalyzed by GSTs.

Due to the large quantity of GST isoforms and the wide range of substrates, the chemical interactions between herbicides and the detoxifying GSTs have only been studied to a minor extent. However, the increasing availability of omics technologies in plant science has led to the identification of several GSTs of the classes tau and phi as candidate genes in weeds such as rigid ryegrass (Goggin et al., 2021) or black-grass (Dücker et al., 2020; Gardin et al., 2015; Tetard-Jones et al., 2018). Cross-resistance studies, however, are often conducted using greenhouse-assays, but rarely at the candidate gene-level, which allows differentiation between different detoxification mechanisms and can eventually lead to conclusions on resistance evolution and resistance management.

The functions of GSTs range from GSH conjugation, via ligandin and signaling functions to peroxidase activity and do not necessarily include the detoxification of a given compound (Labrou et al., 2015). AmGSTF1, for instance, which was shown to be upregulated in different black-grass populations with different herbicide resistance patterns, was shown to have peroxidase activity and was suggested to play a signaling role in metabolic herbicide resistance (Cummins et al., 1999, 2013).

As the herbicide substrate spectra of GSTs in herbicide resistant weeds are poorly described, the full-length cDNA sequences and respective recombinant proteins of cytosolic GSTs upregulated in flufenacet resistant black-grass were obtained. The goal was to characterise their activity on flufenacet and various other herbicides leading to gain of knowledge regarding resistance evolution and management. Moreover, one of the GSTs was additionally mutated at a putative GSH binding site as it formed a different metabolite. The activity of these GSTs and the herbicide resistance-associated AmGSTF1 on flufenacet and various pre- and post-emergent herbicides was tested *in vitro*. Finally, the identity of the metabolites was validated by liquid chromatography-mass spectrometry (LC-MS/MS) and an assay to exclude metabolite deconjugation was conducted.

## 4.2 Materials and Methods

### 4.2.1 Characterization of the cDNA sequences of four candidate GSTs

After an RNA-Seq approach (Dücker et al., 2020), four candidate GSTs (GST1, GST2, GST4 and GST5), which are differentially expressed in flufenacet resistant black-grass, were chosen for cloning and thus a 3' and 5' RACE PCR technique was employed. Once the black-grass genome (Cai et al., 2022) was available, those candidate genes were identified as ALOMY3G13667 (GST1), ALOMY3G13668 (GST2), ALOMY3G13670 (GST8; derived by the realignment of the RNA-Seq reads against the black-grass genome using STAR aligner; version 2.6.1d) and ALOMY5G35766 (GST4 and GST5 contigs correspond to the same gene), respectively. Prior to genome availability, the total RNA of pooled untreated samples of the flufenacet resistant Kehdingen2 population (Dücker, Zöllner, Parcharidou, et al., 2019) was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in order to obtain the full length sequences of the candidate GSTs. The RNA of the samples was transcribed into cDNA using the Invitrogen GeneRacer™ Kit using oligo(dT) primers and the SuperScript™ III reverse transcriptase according to the instructions of the manufacturer (Invitrogen™, Darmstadt, Germany). 3'- and 5'-RACE PCR were performed using the Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Schwerte, Germany), 3'- or 5'-GeneRacer™ RACE primers included in the kit and gene-specific primers with modified annealing temperatures (Table 1). After gel electrophoresis, PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) prior to Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The sequencing results were analysed using the Vector NTI® software version 10.3.0 (Invitrogen, Carlsbad, USA).

Table 1. Primer sequences used for 3'- and 5'-RACE PCR.

| Gene | Class of protein | Genome ID | Primer probe | Sequence (5´-3´) | Annealing temperature (°C) |
|---|---|---|---|---|---|
| GST1 | tau | ALOMY3G13667 | Forward 5' | Gene Racer 5´ | 68.0 |
| | | | Reverse 5' | CTCCACCCACGCCGCTAGGAGCGGAGTC | |
| | | | Forward 3' | CGCAGGAGATGACCTGAAGCTGCTCGG | 66.0 |
| | | | Reverse 3' | Gene Racer 3´ | |
| GST2 | tau | ALOMY3G13668 | Forward 5' | Gene Racer 5´ | 68.0 |
| | | | Reverse 5' | CATCTTTCCAAACTCGATCACCCTGTCGACGTCCG | |
| | | | Forward 3' | CTTCTGGGCCGCCTACATCGACGACAAG | 66.0 |
| | | | Reverse 3' | Gene Racer 3´ | |
| GST8 | tau | ALOMY3G13670 | Forward 5' | Gene Racer 5´ | 68.8 |
| | | | Reverse 5' | GAATGGGACTTACGATCCCCGTGC | |
| | | | Forward 3' | CAAGGGCCTGAGCTTCGAGAAC | 67.1 |
| | | | Reverse 3' | Gene Racer 3´ | |
| GST4, GST5 | phi | ALOMY5G35766 | Forward 5' | Gene Racer 5´ | 68.0 |
| | | | Reverse 5' | CACGGAGCGGGACGACGATGCAC | |
| | | | Forward 3' | GTAGCCAAGCAGCAACTCAACTCAATAG | 66.0 |
| | | | Reverse 3' | Gene Racer 3´ | |

Gene Racer 5´: 5´-CGACTGGAGCACGAGGACACTGA-3´

Gene Racer 3´: 5´-GCTGTCAACGATACGCTACGTAACG-3´

### 4.2.2 Expression of *in planta* significantly upregulated GSTs as recombinant proteins in *Escherichia coli*

The obtained sequences of the candidate genes ALOMY3G13667, ALOMY3G13668, ALOMY3G13670 and ALOMY5G35766 (Figure S1) were used for the construction of plasmids suitable for the production of recombinant proteins in *Escherichia coli* and were commercially cloned (GeneArt Gene Synthesis, Thermo Fisher Scientific, Schwerte, Germany). The plasmids pRSET for EmGFP, pET302 for N-terminal His-tagged GSTs, and pET303 for C-terminal His-tagged GSTs were used. In order to investigate the formation of different metabolites, a mutated sequence of ALOMY3G13668 (ALOMY3G13668m) with a substitution of aspartic to glutamic acid at position 68 (Asp68Glu, Figure 5a) was cloned into pET303, as this position possibly affects GSH binding.

Plasmids containing *AmGstF1* (corresponding to ALOMY3G11300), which was previously shown to play a key role in metabolic herbicide resistance in black-grass(Cummins et al., 1999; Tetard-Jones et al., 2018) (NCBI sequence ID no. AJ010453.1) and emerald green fluorescent protein (*EmGFP*) were produced. These constructs were transformed into One Shot™ BL21(DE3) chemically competent *E. coli* cells (Thermo Fisher Scientific, Schwerte, Germany) for recombinant protein expression according to the manufacturer's protocol. The cells were harvested by centrifugation (4000 x *g*; Avanti J-26 XP, JLA 10.500 rotor, Beckman Coulter) for 20 min at 4°C. The His-tagged proteins were purified under native conditions using the QIAexpress Ni-NTA Fast Start Kit (Qiagen, Hilden, Germany) at 8°C according to the manufacturer's instructions. Subsequently they were desalted on PD-10 desalting columns (GE Healthcare, Freiburg, Germany) using the gravity protocol and eluted in 3.5 mL 100 mM sodium phosphate buffer containing 250 µM EDTA (pH 7.0). The purified GST and GFP proteins were concentrated by diafiltration (4000 x *g*; Avanti J-26 XP, JS 5.3 swing bucket, Beckman Coulter) using 10 kDa molecular weight cut-off (MWCO) Amicon® Ultra-15, PLGC Ultracel-PL Membrane filter units (Merck Millipore, Darmstadt, Germany) for 25 min at 4°C. Protein aliquots were stored at -80°C.

### 4.2.3 *In vitro* activity of recombinant GSTs towards different herbicides

The activity of the purified recombinant GST proteins was verified photometrically with the model substrate CDNB (1-Chloro-2,4-dinitrobenzene; Sigma-Aldrich, Darmstadt, Germany). Reactions were performed in 200 µL reaction mixtures containing 100 mM sodium phosphate buffer (pH 6.5), 1 mM reduced GSH (Sigma-Aldrich, Darmstadt, Germany), 250 µM EDTA (Sigma-Aldrich, Darmstadt, Germany) and 1 mM CDNB (Sigma-Aldrich, Darmstadt, Germany) in duplicates with each protein at a concentration of 250 µg $mL^{-1}$. The absorbance was measured at 340 nm during 8 min (17 cycles; 25°C) with a CLARIOStar® microplate reader

(BMG Labtech, Ortenberg, Germany). Subsequently, the enzymatic activity was determined based on the absorbance and the extinction coefficient of CDNB using the Beer-Lambert law (Table S1; Swinehart, 1962). GSTs with activity towards CDNB and EmGFP were selected for further *in vitro* assays (ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m, and ALOMY3G13670 with C-terminal His-tag and ALOMY5G35766, ALOMY3G11300, and EmGFP with N-terminal His-tag).

The activity of ALOMY3G13667, ALOMY3G13668, ALOMY3G13670, ALOMY5G35766, ALOMY3G11300 and EmGFP for several $^{14}$C-radiolabelled herbicides (flufenacet, *S*-metolachlor, diflufenican, fenoxaprop-ethyl, fenoxaprop-acid, clodinafop-propargyl, clodinafop-acid, mesosulfuron-methyl; Table S2) and unlabelled herbicides (acetochlor, pyroxasulfone, prosulfocarb, cinmethylin, pendimethalin; Table S2) was examined. Esterase from porcine liver (7.91 units; Sigma-Aldrich, Darmstadt, Germany) was added to a final concentration of 50 µM of $^{14}$C-clodinafop-propargyl and $^{14}$C-fenoxaprop-P-ethyl respectively and incubated for 30 min (room temperature) in the reaction buffer in order to produce their radiolabelled active metabolites; $^{14}$C-clodinafop-acid and $^{14}$C-fenoxaprop-acid. The activity of ALOMY3G13668m was tested solely on $^{14}$C-radiolabelled flufenacet. Each herbicide was assayed at final concentration of 50 µM in a total volume of 100 µL containing 100 mM sodium phosphate buffer, 1 mM reduced GSH and 250 µM EDTA (pH 6.5) and the selected proteins at a concentration of 250 µg mL$^{-1}$. The reactions were set up in duplicate and stopped after 2 h by addition of an equal amount of acetonitrile. Chromatographic separation of the samples (injection volume 20 µL) was performed at 35°C and a flow rate of 0.5 mL min$^{-1}$ on a reverse-phase XLC system (Jasco, Pfungstadt, Germany) using a Kinetex® 2.6 µm C18 100 Å 150 x 3 mm (Phenomenex, Aschaffenburg, Germany), except for clodinafop-propargyl and -acid, which was run on a Synergi™ 4 µm Hydro-RP 80Å 250 x 4.6 mm (Phenomenex Inc., Aschaffenburg, Germany). The different gradients are depicted in Table S3. The radiolabelled compounds were quantified using a radio flow detector Berthold FlowStar LB 513/cell YG40-S6M (Berthold Technologies, Bad Wildbad, Germany), and the unlabelled compounds via a MD-4015 PDA (Photometric Diode Array) detector and JASCO ChromPass chromatography software (Jasco, Pfungstadt, Germany). The unlabelled compounds were quantified at different specific wavelengths: acetochlor at 226 nm, pyroxasulfone at 224 nm, prosulfocarb at 233 nm, cinmethylin at 215 nm, and pendimethalin at 240 nm. Enzyme activities of the unlabelled compounds were calculated based on standard measurements with dilution series of each parent compound. The sampling interval for the unlabelled compounds was fixed at 20 points sec$^{-1}$ and for the radiolabelled compounds at 5 points sec$^{-1}$. The experiment was repeated.

For the identification of the metabolites of $^{14}$C-radiolabelled flufenacet, $^{14}$C-fenoxaprop-ethyl and -acid liquid chromatography-mass spectrometry (LC-MS/MS) analyses were performed

on a QTOF Premier MS system (Waters, Milford, MA, USA), an Alliance 2795 Separation Module (Waters, Milford, MA, USA) and a radio detector (LB 513 Flow Star, Berthold Technologies GmbH, Bad Wildbad, Germany). Instrument control and data evaluation was done with MassLynx 4.1 (Waters, Milford, MA, USA). Chromatographic separation of the samples (injection volume 20 µL) was achieved on a 150 x 3 mm inner diameter Kinetex® C18 80Å, 2.6 µm (Phenomenex, Aschaffenburg, Germany) column at a flow rate of 0.5 mL $min^{-1}$ at 35 °C. The LC gradients of each compound are depicted in Table S4. Electrospray ionization was achieved in the positive and negative full scan ion mode (mass range, positive ion mode: m/z 100 - m/z 1000; mass range, negative ion mode: m/z 115 - m/z 1000). Compound identities were confirmed by high-resolution mass spectrometry (determination of the elemental composition of molecular ions and fragment ions) in the MS and MS/MS mode (product ion scan). Argon was used as collisional gas.

### 4.2.4 Deconjugation assay

As flufenacet-alcohol instead of flufenacet-glutathione conjugates was detected as a metabolite of flufenacet formed by ALOMY3G13668 (shown in §3.2), the methods described in §2.3 were modified to exclude deconjugation activity. The reaction volume was scaled up to 300 µL and the experiment took place in two subsequent reactions. As shown in Figure 1, in the first reaction ALOMY3G13667 protein was incubated for 2 h at room temperature in order to produce the flufenacet-glutathione conjugate. Then by size exclusion filtration ALOMY3G13667 was eliminated through a 10 kDa nominal molecular weight limit (NMWL) Amicon® Ultra-0.5 Centrifugal Filter Unit at 11,750 x *g* for 30 min and the free of protein eluent (containing GSH buffer, flufenacet-glutathione conjugate and flufenacet) was used for the further experiment. Ninety-five µL of the eluent were incubated with the ALOMY3G13668 protein (250 µg $mL^{-1}$) and the other 95 µL with EmGFP protein as a negative control (250 µg $mL^{-1}$) in a final volume of 100 µL for each reaction. After 2 h at room temperature the reaction was stopped with 100 µL acetonitrile and measured on the XLC-system described above. Same methods and eluents were applied as described for the measurement of *in vitro* activity of recombinant GSTs towards different herbicides. The experiment was conducted with two replicates.

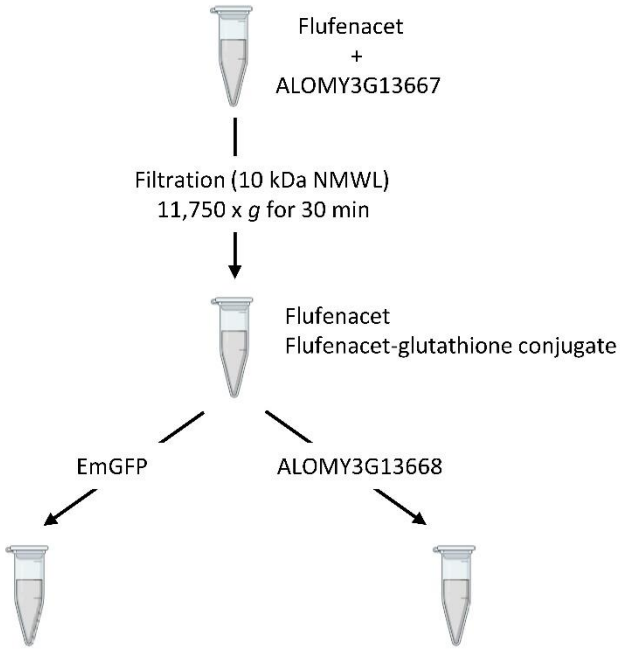



Figure 1. Workflow for the investigation of potential deconjugation of flufenacet conjugates by ALOMY3G13668.

### 4.2.5 Alignment of GST protein sequences

The protein sequences (Figure S2) of the GSTs ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m and ALOMY3G13670 were aligned using the software CLC Main Workbench 20.0.4 (Qiagen, Hilden, Germany) with a gap open cost of 10.0 and gap extension cost of 1.0 in the very accurate (slow) mode.

Proteins sequence identity among ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m and ALOMY3G13670 was calculated with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

### 4.2.6 Protein modelling

A protein model of ALOM3G13668 was prepared starting from the Alphafold prediction(Jumper et al., 2021; Varadi et al., 2022) using GSTU2 protein (https://alphafold.ebi.ac.uk/entry/A0A3Q8C1B8, Uniprot A0A3Q8C1B8, 96 % identity and 97% similarity to ALOMY3G13668, Figure S3). Point mutations found in the experimental ALOM3G13668 sequence were introduced in the model with PyMol Molecular Graphics System (Version 2.0; Schrödinger & DeLano, 2020).

## 4.3 Results

### 4.3.1 *In vitro* activity of recombinant GSTs

The activity of the GSTs, whose genes are upregulated in flufenacet resistant black-grass (Dücker et al., 2020), was tested using the respective recombinant proteins on various substrates (i.e. active ingredients) and evaluated using liquid chromatography (Figure 2). All of the GSTs tested showed activity on the VLCFA-inhibitor flufenacet with ALOMY3G13668 having the highest activity (1.50 nmol product $min^{-1}$ mg $protein^{-1}$). Among the other herbicides of the same mode of action, acetochlor was degraded by all tested GSTs, pyroxasulfone only by the phi class GST ALOMY5G35766 (≥ 1.67 nmol product $min^{-1}$ $mg^{-1}$ protein, 100% of the substrate), and *S*-metolachlor and prosulforcarb by none of the GSTs. None of the other pre-emergent herbicides tested (cinmethylin, diflufenican, pendimethalin) were degraded by the recombinant GSTs tested in the *in vitro* reactions.

Among the post-emergent herbicides, the ALS inhibitor mesosulfuron-methyl and the ACCase inhibitor clodinafop-propargyl and its active metabolite clodinafop-acid were not degraded by any of the GSTs tested. However, the ACCase inhibitor fenoxaprop-ethyl was detoxified by all the GSTs. The degradation rates of the active metabolite fenoxaprop-acid were in all cases lower than for the prodrug fenoxaprop-ethyl. ALOMY3G13668, which had the lowest activity on fenoxaprop-ethyl did not detoxify fenoxaprop-acid.

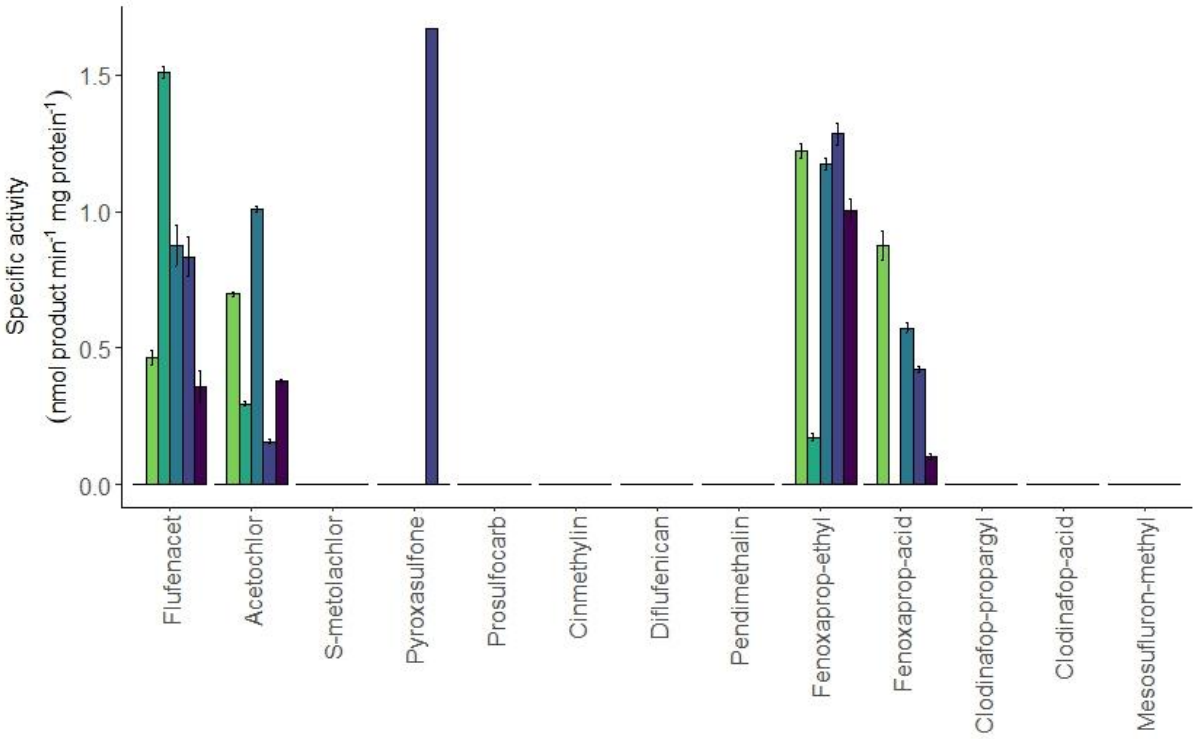



Figure 2. Enzyme activity of recombinant EmGFP (■), ALOMY3G13667 (■), ALOMY3G13668 (■), ALOMY3G13670 (■), ALOMY5G35766 (■), AmGSTF1/ALOMY3G11300 (■) on different

substrates (flufenacet, acetochlor, *S*-metolachlor, pyroxasulfone, prosulfocarb, cinmethylin, diflufenican, pendimethalin, fenoxaprop-ethyl, fenoxaprop-acid, clodinafop-propargyl, clodinafop-acid, mesosulfuron-methyl). The error bars represent the standard error of the mean (n=4).

### 4.3.2 Metabolites produced by recombinant GSTs

Due to their different activity patterns, the detoxification pathways of flufenacet and fenoxaprop-ethyl, as well as its active metabolite fenoxaprop acid, were investigated using $^{14}$C-radiolabelled compounds. The detected mass spectra showed that flufenacet was conjugated to GSH by ALOMY3G13667, ALOMY3G13670, ALOMY5G35766, and ALOMY3G11300, whereas a flufenacet-alcohol was formed in presence of the ALOMY3G13668 and reduced GSH (Figure 3a). When fenoxaprop-ethyl was used as a substrate all the tested recombinant GSTs (ALOMY3G13667, ALOMY3G13668, ALOMY3G13670, ALOMY5G35766 and ALOMY3G11300) produced GSH conjugates (Figure 3b). Furthermore, the metabolites chlorobenzoxazolone (CDHB) and hydroxyphenoxy-propionic-ethyl ester (HOPP-ethyl) were detected. Fenoxaprop-acid followed the same detoxification pathway, with the difference that ALOMY3G13668 showed no activity for this substrate (Figure 3b).

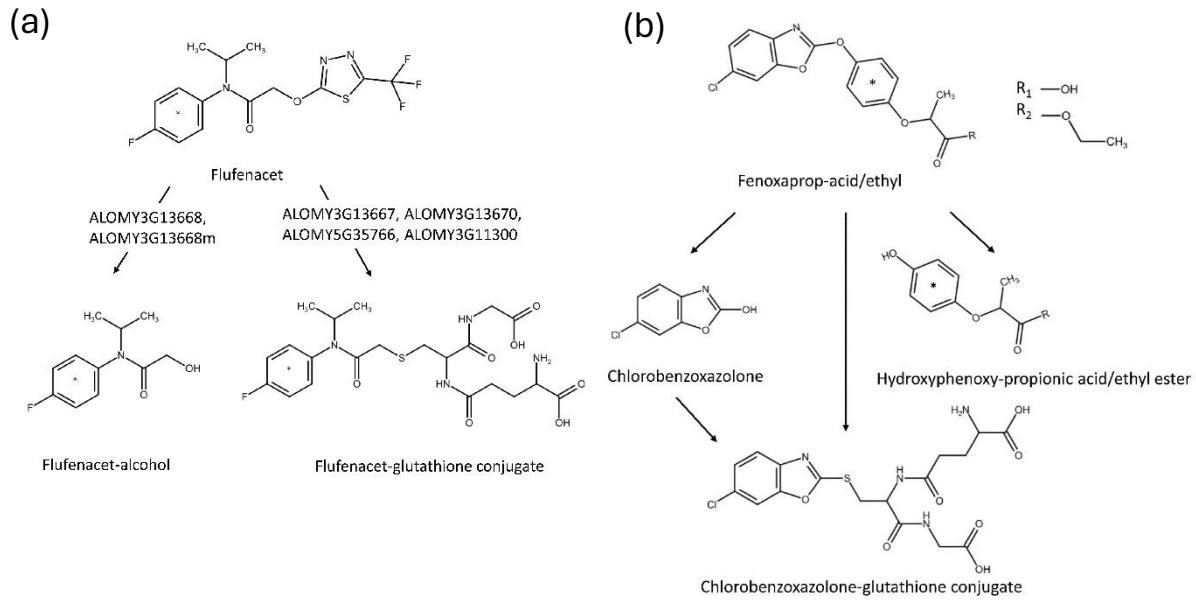



Figure 3. (a) Detoxification of flufenacet *in vitro* by the black-grass GSTs ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m, ALOMY3G13670, ALOMY5G35766, AmGSTF1/ALOMY3G11300 proposed based on mass spectrometry data (b) Detoxification of fenoxaprop-acid and -ethyl *in vitro* by the black-grass GSTs ALOMY3G13667, ALOMY3G13668, ALOMY3G13670, ALOMY5G35766, AmGSTF1/ALOMY3G11300 proposed based on mass spectrometry data. The asterisks indicate the radiolabeled carbon rings.

### 4.3.3 Deconjugation assay with ALOMY3G13668

To investigate if flufenacet-alcohol is formed by deconjugation of flufenacet-glutathione adducts in presence of ALOMY3G13668, reaction mixtures containing purified ALOMYG13668 or EmGFP as well as the substrates flufenacet and flufenacet-glutathione conjugate were analysed by HPLC and LC-MS/MS. After two hours no more flufenacet could be detected in presence of the GST (Figure 4). Instead, flufenacet-alcohol was present. The amount of flufenacet-glutathione conjugate (66%) was similar to the quantity detected in the control reaction with the negative control EmGFP (65%).

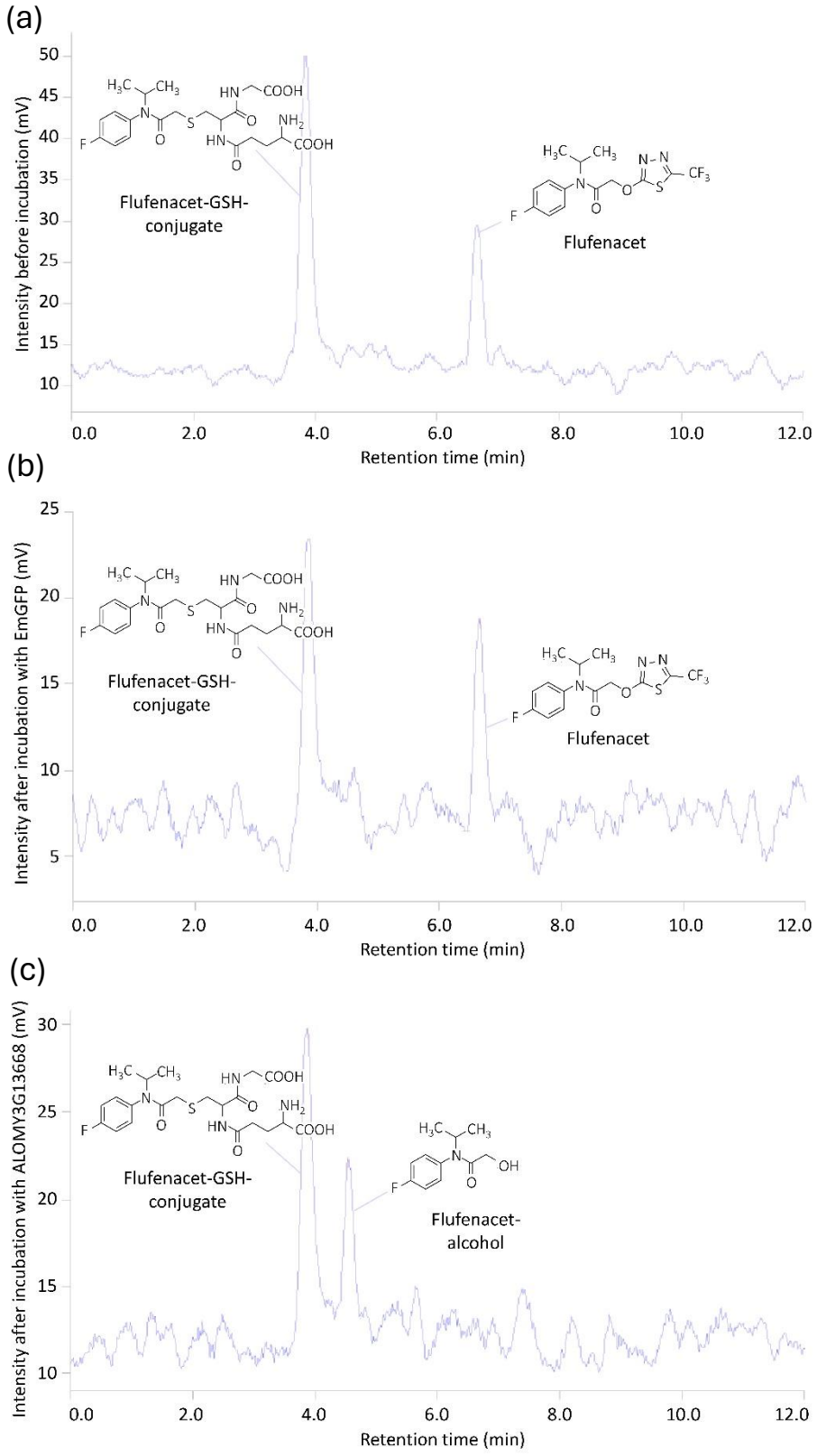
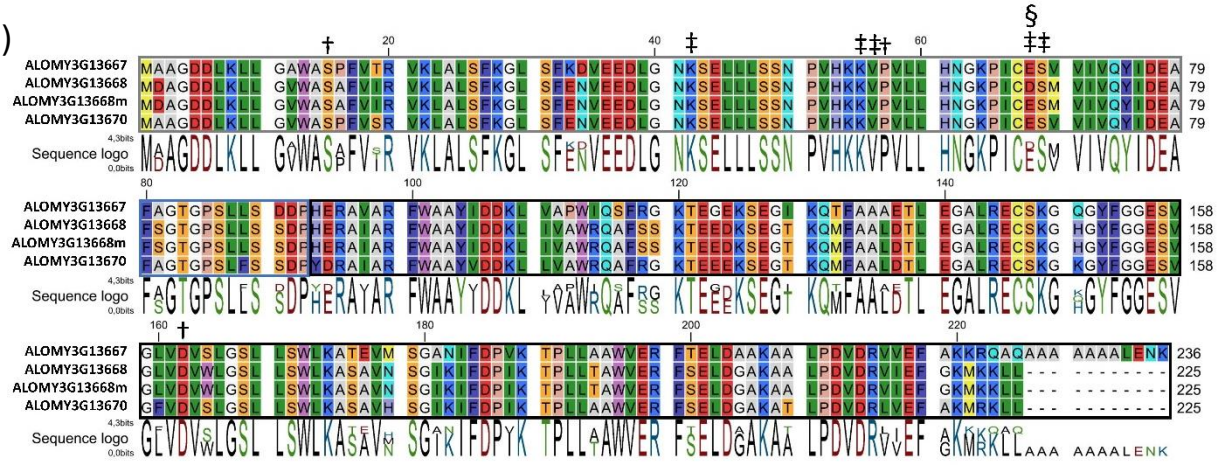

(a)
Intensity before incubation (mV)
Flufenacet-GSH-conjugate
Flufenacet
Retention time (min)
(b)
Intensity after incubation with EmGFP (mV)
Flufenacet-GSH-conjugate
Flufenacet
Retention time (min)
(c)
Intensity after incubation with ALOMY3G13668 (mV)
Flufenacet-GSH-conjugate
Flufenacet-alcohol
Retention time (min)

Figure 4. Radiochromatograms of a reaction mixture without enzymes added (a), a reaction mixture with EmGFP (b) and a reaction mixture with ALOMY3G13668 (c) of a deconjugation assay. Metabolites were verified by LC-MS/MS.

### 4.3.4 Comparison of the GST proteins belonging to class tau (U)

The coding genes of the tau (U) class GST proteins are located next to each other on the chromosome 3 of the black-grass genome and the corresponding proteins share highly similar amino acid sequences, with sequence identity of more than 80% (Table S5). All of them possess a serine residue on the active site (Ser15), as well as a proline (Pro57) and aspartic acid (Asp162), typically conserved for GSTU proteins.(Sylvestre-Gonon et al., 2019) Moreover, residues involved in GSH binding expanding in the G-site (N-terminus) are conserved among the proteins such as the residues 42, 55, 56 and 69. Despite the high identity percentage, some conserved residues in ALOMY3G13667 and ALOMY3G13670 are different in ALOMY3G13668 (Figure 5). In Figure 5a, the ALOMY3G13668m protein sequence is depicted, and the site mutation (Asp68Glu) is pointed out (§), which is a position possessing important role in GSH binding (Sylvestre-Gonon et al., 2019). Despite the targeted point mutation, ALOMY3G13668m catalyses the formation of flufenacet-alcohol as product detected by HPLC, as in the case of wild type protein ALOMY3G13668 (data not shown).

(a)



(b)

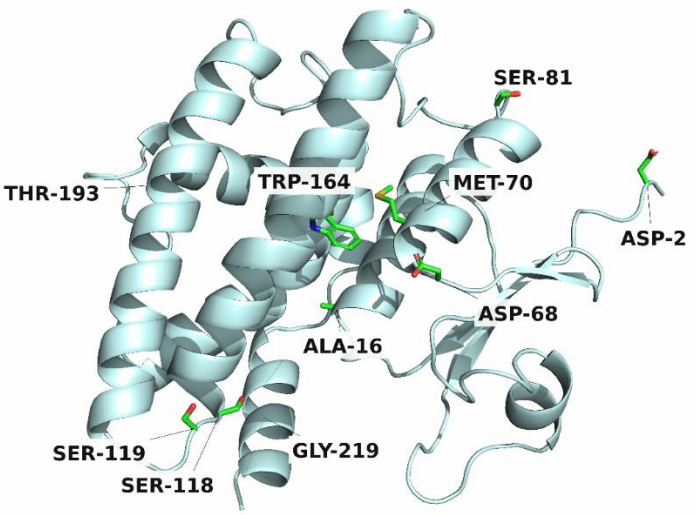



Figure 5. (a) Alignment of tau class (ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m, ALOMY3G13670) glutathione transferases. The active site serine, the invariant proline and the quasi-invariant aspartic acid are marked with † and the residues involved in binding glutathione (G-site)(Sylvestre-Gonon et al., 2019) are marked with ‡. The § symbol indicates a mutation conferring an amino acid substitution at position 68 of ALOMY3G13668. The N-terminus of the proteins is marked with a grey frame, the C-terminal with a black frame and the linker residues between the two terminals with a blue frame (Valenzuela-Chavira et al., 2017). (b) Model of ALOMY3G13668. Residues which differ among tau class GSTs (Ala2Asp, Pro16Ala, Glu68Asp, Val70Met, Ala81Ser, Arg118Ser, Gly119Ser, Ser164Trp, Ala193Thr, Ala219Gly) are depicted in stick (green) and labelled.

## 4.4 Discussion

During the last decades pre-emergent herbicides such as flufenacet are used more frequently for the control of black-grass, particularly in cereals. However, some black-grass populations have evolved resistance to this herbicide, which was shown to be associated with an upregulation of several GSTs (Dücker et al., 2020). Nevertheless, many studies do not proceed to functionally validate these candidate genes and the actual cause for resistance remains undetermined. In order to validate the function of the computationally predicted genes, the full-length cDNA sequences of three tau and one phi class GST were obtained and later also identified in the recently assembled black-grass genome (Cai et al., 2022). The three tau class GSTs ALOMY3G13667, ALOMY3G13668, and ALOMY3G13670 had a high sequence identity of > 80%. Interestingly, those three upregulated GSTs were located next to each other on the same chromosome, only separated by a pseudogene (ALOMY3G13669; Cai et al., 2022). The sequence similarity and their location in the genome suggests that these genes may be coregulated in the same signalling pathway, as previously described in eukaryotic organisms (Arnone et al., 2012).

After expression of the recombinant proteins *in E.coli* and the purification of the four candidate GSTs and the previously described AmGSTF1 (ALOMY3G11300;Cummins et al., 1999, 2013), detoxification of flufenacet by all these GSTs was validated *in vitro*. The relatively low detoxification rates ranged from 0.47 to 1.5 nmol product $min^{-1}$ mg $protein^{-1}$ and suggest an additive effect of the different GSTs. The involvement of various genes is consistent with the slow resistance evolution and the low resistance level described in the field (Hull & Moss, 2012). While the observed additive effect of different GSTs suggests polygenic (Hawkins et al., 2019) and generalist (Comont et al., 2020) resistance, it is not clear how many of these genes are coregulated or how complex the signalling chains are.

The present study further elucidated substrate specificity and metabolite formation by upregulated GSTs. The GSTs ALOMY3G13667, ALOMY3G13670, ALOMY5G35766, and ALOMY3G11300 conjugated flufenacet with GSH**,** likely due to a nucleophilic attack of the thiol group of the GSH on the methylene group of flufenacet leading to a loss of the thiadiazole moiety, as previously described for several crops (Bayer AG, 2017; Bieseler et al., 1997; Gould & Lemke, 2002), black-grass (Dücker, Zöllner, Parcharidou, et al., 2019) and ryegrass (Dücker, Zöllner, Lümmen, et al., 2019) *in vivo*. Nevertheless, despite the high sequence similarity, the GST with the highest specific activity towards flufenacet (ALOMY3G13668) catalysed a reaction leading to the formation of flufenacet-alcohol and only traces of the flufenacet-glutathione conjugate. The formation of flufenacet-alcohol and the subsequent metabolite flufenacet-oxalate were previously described as an additional pathway in crops (maize, soybeans, sunflower, wheat), besides GSH conjugation (Bayer AG, 2017; Gould & Lemke, 2002). However, flufenacet-alcohol was neither described in pulse experiments with black-grass, nor in other weeds and therefore likely does not occur in large quantities *in vivo*. Differences between *in vitro* and *in vivo* studies may be due to rapid formation of further metabolites such as flufenacet-oxalate and the observation that the majority of GSTs active on flufenacet produce a glutathione conjugate. Furthermore, factors such as differences in protein folding between *E. coli* and plants altering enzyme activity, although rare, cannot be completely excluded.

To investigate that the formation of flufenacet-alcohol is not the product of the deconjugation of the GSH-conjugate of flufenacet, a specific assay was performed with ALOMY3G13668. Glutathione deconjugation of metabolites was previously described for some GSTs, such as cysteine-containing GSTs (Cooper & Hanigan, 2018; Lallement et al., 2014). In our assay, the flufenacet-glutathione conjugates were neither deconjugated nor decreased in concentration in the reaction mixture which confirmed that only flufenacet was used as substrate by ALOMY3G13668. Besides deconjugation, steric hindrance for GSH binding due to changes in the amino acid sequence may be a cause for flufenacet-alcohol, as it has been observed for cysteine residues in a pi (P) class GST (Shen et al., 1993). In comparison to the GSTs which

were closely located on the chromosome, ALOMY3G13668 exhibited 10 amino acids substitutions (Figure 5). Particularly, the residue on position 68, where the glutamic acid is exchanged by an aspartic acid is critical for GSH binding (Sylvestre-Gonon et al., 2019). While both negatively charged amino acids share similar physicochemical properties, the side chain of aspartic acid is one carbon atom shorter and may lead to spatial preferences (Jonson & Petersen, 2001). Similarly, a substitution of glutamic acid by aspartic acid at position 65 of a prokaryotic GST (PmGSTB1-1) led to loss of enzyme activity (Allocati et al., 2002) and the replacement of glutamic acid with non-functionally conserved amino acids of the *Anopheles dirus* GST D3-3 at position 64 had impact on the folding and structural maintenance of the protein (Winayanuwattikun & Ketterman, 2007). For that reason, a mutated version of ALOMY3G13668 (ALOMY3G13668m) was tested *in vitro*, which also produced a flufenacet-alcohol metabolite. Consequently, the formation of flufenacet-alcohol by ALOMY3G13668 does not or not solely result from aspartic acid substitution on position 68, but instead it may depend on several of the ten amino acids substitutions (Figure 5b), which can eventually affect enzyme activity (Figure 2) and metabolite formation (Figure 3a).

The investigated GSTs also differed partially in their substrate spectra, besides the formation of flufenacet metabolites. The experiments with recombinant expressed black-grass GSTs have shown that different cross-resistance patterns exist within and outside the mode of action of flufenacet. The ALS inhibitor mesosulfuron-methyl, the microtubule assembly inhibitor pendimethalin, the phytoene desaturase inhibitor diflufenican, and the fatty acid thioesterase inhibitor cinmethylin were not affected by cross-resistance. Resistance to all of these herbicides is associated with the activity of cytochrome P450 monooxygenases (CYPs) (Abdollahi et al., 2021; Duhoux & Délye, 2013; Goggin et al., 2022; Lu et al., 2020; Tardif & Powles, 1999; Zhao et al., 2022), which catalyse phase I reactions in the detoxification pathway of xenobiotics, and in some cases additionally with other non-target-site mechanisms (Haynes & Kirkwood, 1992).

It has to be stressed that some herbicides can be directly conjugated by phase II enzymes, without previous formation of more polar intermediates in phase II. This was observed for the VLCFA-inhibitors flufenacet, acetochlor and pyroxasulfone as well as the ACCase inhibitor fenoxaprop-ethyl and its active metabolite fenoxaprop-acid *in vitro*. While these herbicides were detoxified by the tested GSTs, other herbicides of the same modes of action were not affected. This applies to the ACCase inhibitor clodinafop-propargyl and its active metabolite clodinafop-acid, which more frequently lose efficacy due to target site resistance e.g. in American sloughgrass (*Beckmannia syzigachne*; Wang et al., 2021) and in black-grass (Petit et al., 2010). Fenoxaprop-ethyl, on the other hand, was also previously described to be conjugated to GSH in black-grass (Cummins et al., 1997), late watergrass (*Echinochloa phyllopogon*; Bakkali et al., 2007) and in monocotyledonous crops (Tal et al., 1993). The

degradation rates of the active metabolite fenoxaprop-acid were in all cases lower than for the prodrug fenoxaprop-ethyl. This is in accordance with the findings of Edwards & Cole (1996) and particularly true for ALOMY3G13668. This enzyme detoxified flufenacet fastest by formation of flufenacet-alcohol, had little activity on fenoxaprop-ethyl and no activity on fenoxaprop-acid. *In planta* though, the direct conjugation of fenoxaprop-ethyl competes with esterases which activate aryloxyphenoxypropionate (AOPP) herbicides and with CYPs, which produce CDHB (Zhao et al., 2022). In this study, it was shown by mass spectrometry that all recombinant GSTs produced CDHB-glutathione conjugates (Figure 3b). Furthermore, the metabolites CDHB, HOPP-ester and HOPP-acid were detected. This supports an enzymatical GSH conjugation either on the substrates fenoxaprop-ethyl and -acid (Hoagland & Zablotowicz, 1998) directly or via a CDHB intermediate (Zhao et al., 2022).

Similarly to the ACCase inhibitors tested, the VLCFA-inhibitors showed varied resistance patterns, as the thiocarbamate prosulfocarb and the chloroacetamide *S*-metolachlor were also not detoxified by the candidate GSTs. Differences in the inhibition of various phi class GSTs by different chloroacetamide herbicides, such as higher inhibition rates of acetochlor in comparison with *S*-metolachlor, were recently described.(Ioannou et al., 2022) Furthermore, the absence of or only weak cross-resistance between the chloroacetamides *S*-metolachlor and acetochlor was previously observed *in planta* in palmer amaranth (*A. palmeri*;Brabham et al., 2019) and waterhemp (*A. tuberculatus*; Strom et al., 2019). The resistance mechanism against both herbicides was shown to be caused by enhanced GST activity in different plant species (Dücker, 2020; Cottingham et al., 1993; Brabham et al., 2019; Strom et al., 2020). However, in a recent study with resistant waterhemp it was also demonstrated that previous *O*-demethylation by CYPs played a central role in the expression of the resistance phenotype (Strom et al., 2021). In contrast, thiocarbamates such as prosulfocarb (former HRAC class N) in general require initial oxidase-based sulfoxidation (Lay & Casida, 1976) before conjugation to the GSH tripeptide. Beyond that, glucosylated metabolites of prosulfocarb or prosulfobarb sulfoxide were mostly described in previous studies (Bellisai et al., 2022; EFSA, 2007), even though cross-resistance with pyroxasulfone was observed in resistant rigid ryegrass (*L. rigidum*; Busi & Powles, 2013). The isoxazoline pyroxasulfone, was previously shown to be detoxified by GSTs (Busi et al., 2018; Goggin et al., 2021; Tanetani et al., 2013), and although this herbicide was never used in the European Union, a single candidate GSTs (ALOMY5G35766) was able to detoxify this herbicide. This observation is in accordance with the low shift in sensitivity previously observed in flufenacet resistant black-grass (Dücker, 2020). All of the tested candidate GSTs, on the other hand, detoxified the chloroacetamide acetochlor and the oxyacetamide flufenacet, which suggests a stronger cross-resistance, even though acetochlor was never used in Germany. The application of the other herbicides found to be cross-resistant *in vitro* may have contributed to evolution of flufenacet resistance.

Overall, the resistance patterns were largely independent of modes of action and chemical classes of the herbicides and specific activities varied in all cases.

## 4.5 Conclusions

Three tau and two phi class GSTs, which are differentially expressed in flufenacet resistant black-grass were tested *in vitro* on various herbicide substrates. Detoxification of the VLCFA-inhibitor flufenacet was verified for all five differentially expressed GSTs, underlying the fact that the slowly evolving resistance to flufenacet in black-grass is a polygenic trait. It was caused by an increased GST activity *in planta*[16] due to an additive effect of at least four upregulated GSTs (ALOMY3G13667, ALOMY3G13668, and ALOMY3G13670, ALOMY5G35766). However, it cannot be totally ruled out that other GST encoding genes contribute to the detoxification. Furthermore, three coding genes of these proteins are located next to each other on the genome and are highly similar, which suggests co-regulation of these genes and therefore requires further investigations. Despite the sequence similarity, different flufenacet metabolites were produced. The formation of flufenacet-glutathione conjugates was observed by four candidate GSTs and the isoform with the highest detoxification rate, formed flufenacet-alcohol as a metabolite. The different metabolite formation is likely caused by a conformational change due to several amino acid substitutions. In conclusion, GSTs produce the first metabolites of two different detoxification pathways.

All tested GSTs were able to detoxify the VLCFA-inhibitor acetochlor and the ACCase inhibitor fenoxaprop-ethyl and one GST detoxified the VLCFA-inhibitor pyroxasulfone. Other herbicides of the same mode of action and selected herbicides of four other modes of action were not affected. Thus, metabolic resistance is complex and does not necessarily confer strong resistance to a wide spectrum of herbicides. It was shown that the same enzyme can confer cross-resistance with other modes of action, while other active ingredients of the same mode of action or even chemical class may not be affected.

In that regard, herbicides such as diflufenican, pendimethalin, prosulfocarb, cinmethylin or mesosulfuron-methyl are particularly suitable compounds for an herbicide management to delay flufenacet resistance evolution in black-grass population showing such resistance pattern. Moreover, alternation of compounds of the same class that are not affected by cross-resistance such as, flufenacet and *S*-metolachlor, is preferable to repetitive use of the same compound. A sustainable alternation of active ingredients to slow down resistance evolution can therefore not only be based on the classification of herbicides by their mode of action but rather on a classification by their resistance mechanisms, detoxification pathways and observed resistance patterns.

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# 5. Genome-wide study of glutathione transferases and their regulation in flufenacet susceptible and resistant black-grass (*Alopecurus myosuroides* Huds.)

Evlampia Parcharidou[a*], Rebecka Dücker[a], Roland Beffa[b*]

*Correspondence to: E. Parcharidou, Georg-August University Göttingen, Division of Plant Pathology and Crop Protection, Göttingen, Germany, E-mail: e.parcharidou@stud.uni-goettingen.de; R. Beffa, Senior Scientist Consultant, Liederbach am Taunus, Germany, roland.beffa@t-online.de

[a]Georg-August University Göttingen, Division of Plant Pathology and Crop Protection, Göttingen, Germany

[b]Senior Scientist Consultant, Liederbach am Taunus, Germany

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*See Annex (§10.2) for the supporting information section of this paper.*

Abstract

BACKGROUND: Glutathione transferases (GSTs) are enzymes with a wide range of functions, including herbicide detoxification. Upregulation of GSTs and their detoxification activity enables the grass weed black-grass (*Alopecurus myosuroides* Huds.) to metabolise the very-long-chain fatty acid synthesis inhibitor flufenacet and other herbicides leading to multiple herbicide resistance. However, the genomic organization and regulation of GSTs genes is still poorly understood.

RESULTS: In this genome-wide study the location and expression of 115 GSTs were investigated using a recently published black-grass genome. Particularly, the most abundant GSTs of class tau and phi were typically clustered and often followed similar expression patterns but possessed divergent upstream regulatory regions. Similarities were found in the promoters of the most upregulated GSTs, which are located next to each other in a cluster. The binding motif of the E2F/DP transcription factor complex in the promoter of an upregulated GST was identical in susceptible and resistant plants, however, adjacent sequences differed. This led to a stronger binding of proteins to the motif of the susceptible plant, indicating repressor activity.

CONCLUSIONS: This study constitutes the first analysis dealing with the genomic investigation of GST genes found in black-grass and their transcriptional regulation. It highlights the complexity of the evolution of GSTs in black-grass, their duplication and divergence over time. The large number of GSTs allows weeds to detoxify a broad spectrum of herbicides. Ultimately, more research is needed to fully elucidate the regulatory mechanisms of GST expression.

Keywords: black-grass GSTome, duplication, E2F/DP, electrophoretic mobility assay (EMSA), herbicide resistance, transcription factor binding sites (TFBSs)

## 5.1 Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) are involved in a broad range of functions, such as detoxification of xenobiotics, stress tolerance, and cell signalling (Frova, 2006; Labrou et al., 2015). Moreover, they are key proteins in metabolic herbicide resistance (Rigon et al., 2020), an increasing threat for weed control impacting food security and quality in modern agriculture. A multitude of different GSTs have evolved from a common thioredoxin-like ancestor (Martin, 1995). The resultant plant GSTome consists of the mitochondrial, microsomal and cytosolic superfamilies. Plant GSTs are divided into 14 different classes as follows (Nianiou-Obeidat et al., 2017): tau (GSTU), phi (GSTF), theta (GSTT), zeta (GSTZ), lambda (GSTL), γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ), dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase (TCHQD), ureidosuccinate transport 2 prion protein (Ure2p), hemerythrin (GSTH), iota (GSTI), glutathionyl-hydroquinone reductase (GHR), metaxin (MTX), microsomal prostaglandin E synthase type 2 (mPGES2). Further putative GSTs, which lack characteristic domains include class kappa (GSTK), membrane associated proteins in eicosanoid and glutathione metabolism (MAPEGs), two repeated N-terminal thioredoxin domains (GST2N) (Lallement et al., 2014), aminoacyl-tRNA synthetases (aaRS) (Nyamai & Tastan Bishop, 2019) and aminoacyl-tRNA synthetase cofactor I (Arc1p) (Frechin et al., 2010; Simader et al., 2006).

Most of the plant GSTs are cytosolic, active as dimers and possess a serine residue in their active center. Exceptions are classes e.g. DHAR, GSTL and TCHQD, which are monomeric and possess a cysteine residue on the active center (Kumar & Trivedi, 2018; Lallement et al., 2014; Warner & Copley, 2007). The cytosolic GSTUs and GSTFs are particularly relevant for the detoxification of herbicides and the resulting metabolic resistance (Ioannou et al., 2022). These classes are most abundant in higher plants and typically the most highly expressed classes under abiotic stress conditions (Frova, 2006; Ioannou et al., 2022; McGonigle et al., 2000).

The herbicides flufenacet, acetochlor and fenoxaprop were shown to be detoxified *in vitro* by recombinant GSTUs and GSTFs deriving from the weed species black-grass (*Alopecurus myosuroides* Huds.), and pyroxasulfone only by a GSTF (Parcharidou et al., 2023). Detoxification of fenoxaprop in crops (Tal et al., 1993) and pyroxasulfone in annual ryegrass (*Lolium rigidum* Gaud.), possibly by a phi class GST (Goggin et al., 2021) was supported in previous *in vivo* studies. Similar studies have shown that detoxification of atrazine in velvetleaf (*Abutilon theophrasti*; Anderson & Gronwald, 1991) and maize (*Zea mays* L.; Shimabukuro et al., 1970) and EPTC also in maize (Carringer et al., 1978) correlates with GST activity.

The well-characterized AmGSTF1, a phi class GST typically upregulated in multiple-herbicide resistant black-grass can act as GST (Parcharidou et al., 2023) but most commonly exerts its function as peroxidase (Cummins et al., 1999). AmGSTF1 could have a key role in secondary metabolism and in signalling (Cummins et al., 2013). Grass weeds e.g. black-grass and ryegrass (*Lolium* spp.) are particularly prone to evolve metabolic resistance to herbicides of different chemical classes (Dücker, 2020; Yu & Powles, 2014). Resistance to leaf-applied acetyl-CoA synthase (ACCase) (HRAC group 1) or acetolactate synthase (ALS) inhibitors (HRAC group 2) is typically first observed. As a result, soil-active herbicides e.g. inhibitors of the synthesis of very-long-chain fatty acids (VLCFAs) (HRAC group 15) have been relied on increasingly to control affected grass weed populations. Particularly, flufenacet has become a key herbicide to control grass weeds in the major crop winter wheat in Western Europe (Bailly et al., 2012). This herbicide inhibits the elongation of fatty acids with chain length of ≥ 18 carbons by binding to several condensing enzymes (very-long-chain 3-oxoacyl-CoA synthase, synonym: keto-acyl-CoA-synthase) of the VLCFA elongation complex (Lechelt-Kunze et al., 2003; Trenkamp et al., 2004; Haslam & Kunst, 2013). However, resistance to this herbicide class is evolving: two of the most noxious grass weeds in Europe, black-grass (Dücker, Zöllner, Parcharidou, et al., 2019) and ryegrass (Dücker, Zöllner, Lümmen, et al., 2019) have become resistant to flufenacet via upregulation of tau or tau and phi class GSTs (Dücker, 2020; Dücker et al., 2020; Parcharidou et al., 2023).

Particularly, these two most abundant GST classes are frequently found in clusters on the chromosomes (Martin, 1995). Gene duplications can result either from whole-genome, tandem, proximal, transposed, or dispersed duplication (Lallemand et al., 2020; Leister, 2004). However, studies on GST gene duplication show that the large numbers of GSTs in plants have arisen from whole-genome duplication (WGD), often followed by tandem gene duplications (Khan et al., 2018; Liu et al., 2015; Nebert & Vasiliou, 2004; Wei et al., 2019). Moreover, the location of GSTs on the chromosomes might be affected by transposable elements (TEs), which can move within the genome and affect the transcription of genes, cause mutations and even modify regulatory networks (Bourque et al., 2018; Fedoroff, 2000). They account for more than 80% of the genomes of plant species like wheat (*Triticum aestivum*

L.), barley (*Hordeum vulgare* L.) and black-grass (Cai et al., 2023; Mascher et al., 2017; Wicker et al., 2018). TEs have chiseled the architecture of plant genomes, making it even more difficult to track GST duplication events.

The expression of GSTs is mainly regulated at the transcriptional level by the presence of specific *cis*-regulatory elements (CREs) and bearing mutations within them (Frova, 2003; Marrs, 1996). In GST promoters of thale cress (*Arabidopsis thaliana* (L.) Heynh.; Zhang & Singh, 1994), soybean (*Glycine max* (L.) Merr.), wheat and benth (*Nicotiana benthamiana* Domin; Marrs, 1996) the *ocs* (octopine synthase) element was identified. This 20-bp DNA region comprises of a tandem core sequence of ACGT and acts as a DNA binding motif triggered under stress conditions, by hormones and chemical agents (Marrs, 1996; Ulmasov et al., 1994). *Ocs* elements are binding sites for dimeric bZIP transcription factors (TFs), as OCSBF-1 and ASF1 (Chen et al., 1996; Lam & Lam, 1995; Zhang & Singh, 1994). Besides bZIP, further TFs e.g. NAC, MYB and WRKY are associated with GST regulation (Wei et al., 2019). The expression and activation of TFs is strongly influenced by environmental conditions, such as secondary metabolites, and subsequently affects the expression of stress-responsive genes for instance GSTs, resulting in complex regulatory pathways (Marrs, 1996; Meraj et al., 2020). Nevertheless, there is evidence from animal studies that GST expression is not only controlled at the transcriptional level, but can also be affected by post-transcriptional modifications (Moriya et al., 2012; Uchida et al., 2013; Zhang et al., 2012).

However, neither expression regulation nor post-transcriptional modification of GSTs associated with herbicide resistance has been explicitly investigated in weeds. For that reason, a genome wide study of GST expression in flufenacet susceptible and resistant black-grass was conducted using the black-grass genome recently published by Cai et al. (2023). Additionally, the number of GST genes in the black-grass genome, their location on the chromosomes and within clusters were studied *in silico*. Candidate promoter sequences were investigated *in silico* and *in vitro*.

## 5.2 Materials and Methods

### 5.2.1 Transcriptome (RNA-Seq) and differential gene expression analysis

RNA-Seq reads (Dücker et al., 2020) derived from a previous study with flufenacet susceptible (Herbiseed abbreviated as H and Appel abbreviated as A) and resistant black-grass populations (Kehdingen1 abbreviated as K1 and Kehdingen2 abbreviated as K2) were aligned against a black-grass genome (Cai et al., 2023) using the STAR aligner (version 2.6.1d) using default settings. Subsequently, a differential gene expression (DGE) analysis was conducted using edgeR (Robinson et al., 2010) in RStudio (version 3.32.1). Resulting *p*-values were adjusted for false discovery rate (5%) using Benjamini and Hochberg. Only genes with logFC

values ≥ 1.5 were considered. The commonly differentially expressed genes (DEGs) were filtered using Venny 2.1.0 (Oliveros, 2007) by extracting the genes overlapping area of the Venn diagram (Figure S2).

### 5.2.2 Phylogenetic tree of the GST proteins found in the black-grass (*Alopecurus myosuroides* Huds.) genome and their gene expression heatmap

The protein sequences of all the GSTs found in the black-grass genome were aligned using the MUSCLE (Edgar, 2004) algorithm and the respective phylogenetic tree was created using the neighbor-joining (NJ) method (p-distance model) with 1000 bootstrap replicates in MEGA11 (version 11.0.13) (Tamura et al., 2021). The average raw counts of each gene were extracted and log10-normalized for each population. A phylogenetic tree combined with a heatmap was produced by Tbtools-II (version 1.120) (Chen et al., 2020).

### 5.2.3 Investigation of GSTs and other differentially expressed genes

In order to explore the influence of the genome structure on the resistance level, neighbouring genes of all GSTs and all genes differentially expressed in at least one resistant population were investigated. Genes of the same family found in chromosomal proximity were illustrated individually or in clusters on the seven black-grass chromosomes. Furthermore, the number of differentially expressed genes belonging to other protein families, as well as the total number of genes belonging to these families were displayed based on Interpro annotations (including Pfam) in WeedPedia (Cai et al., 2023; WeedPedia, 2023). For protein kinases (PKs) and protein phosphatase (PPs), the eggNOG database annotations (Cai et al., 2023) were used in order to consider all PKs and PPs. The predicted GST with ID ALOMY5G35749 (GSTF35749) is likely an exon of ALOMY5G35750 (GSTF35750; IGV data not shown). Therefore, a new gene was created and further used with the ID ALOMY5G35750 (GSTF35750). In addition, the number of KCS (Trenkamp et al., 2004) isoforms, the putative target enzymes of flufenacet and other inhibitors of the synthesis of VLCFAs was determined, as well as the number of differentially expressed genes belonging to this family. In order to obtain only correctly annotated genes, *Arabidopsis thaliana* KCSs (TAIR, 2023) were identified using blastp search in WeedPedia (2023). Hereinbelow, the term ‘DEG cluster’ refers to a region on the chromosome in which two or more genes potentially related to flufenacet resistance, are assigned to the same gene family, but which may be interrupted by otherwise annotated genes, insufficiently annotated genes (indicated by 'NA') or pseudogenes (indicated by 'PG').

### 5.2.4 Investigation of the 5’ upstream regulatory regions of GST genes and their putative transcription factor binding sites (TFBSs)

The 5’ upstream regulatory regions (5’URRs) of all 115 GST genes found in the black-grass genome, were extracted with a length of 2 kbp and explored in the Integrative Genome Viewer (version 2.12.2) (Robinson et al., 2011) regardless their respective gene expression. A phylogenetic tree (Figure S3) was created with the 5’URRs using the NJ method with 1000 bootstrap replicates using the p-distance model in MEGA11 (version 11.0.13) (Tamura et al., 2021).

For the investigation of putative transcription factor binding sites (TFBSs) and the corresponding TFs 2 kbp (or less in case the previous upstream gene was encountered) of the 5’URRs upstream of the putative translation start site (ATG) of all 115 GSTs were extracted. Binding sites were predicted using PlantRegMap (Tian et al., 2020; Jin et al., 2017) with barley as plant species matrix and a cut-off threshold of $p \leq$ 1e-4.

Since the differentially expressed GSTs GSTU13667, GSTU13668 and GSTU13670 were found in direct proximity on chromosome 3, share similar promoter sequences (e.g. two E2F/DP binding motifs on opposite strands – see Figure 5a), and their *in vitro* activity on flufenacet was validated (Parcharidou et al., 2023), their cluster (Chr3_CL2) was chosen for further investigation.

#### 5.2.4.1 Genomic DNA extraction (gDNA) and upstream PCR for promoter investigation of GSTU13670

The functionally validated (Parcharidou et al., 2023) and most upregulated GSTU13670 in the cluster Chr3_CL2 was chosen for upstream PCR. In total, 100 mg of pooled leaves of the most susceptible black-grass population H and the most resistant one K2 (Dücker et al., 2020) were cut and milled with tungsten carbide beads at 30 Hz for 2 min using a swing mill (Tissue Lyser II, Qiagen, Hilden, Germany). Genomic DNA was extracted using the NucleoSpin Plant II, Mini kit for DNA from plants (Macherey-Nagel™, Düren, Germany). The PL1 lysis buffer was used according to the protocol and the DNA was eluted with 50 µL PE buffer.

The promoters of H (susceptible) and K2 (resistant) were amplified using the Phusion Hot-Start II High-Fidelity DNA-Polymerase (Thermo Fisher Scientific, Schwerte, Germany) in combination with a forward (5’-3’) TAAGTAGTTTGGGATCCAATGC and a reverse (5’-3’) primer ATCAGTAAATCGCAAATTTCAATGC at an annealing temperature of 63°C. After gel electrophoresis, PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) prior to Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The sequencing results were analysed using the Qiagen CLC Main Workbench 20.0.4 (Qiagen, Hilden, Germany).

### 5.2.4.2 Total protein extraction and quantification

To investigate TF binding on the promoter of GSTU13670 an electrophoretic mobility shift assay (EMSA) was conducted. As the E2F/DP candidate TFs were not differentially expressed, 90 mg of leaf material of H and K2 were mixed and total protein was extracted with the Pierce™ Plant Total Protein Extraction Kit (Thermo Fisher Scientific, Schwerte, Germany) using the native lysis buffer with protease inhibitors cOmplete ULTRA EDTA-free tablets (1 tablet per 50mL buffer; Roche, Mannheim, Germany). The extraction took place according to the manufacturer's instructions. The last step was extended to 60 min at 16,000 x *g* (4°C), to eliminate the plant debris completely.

Subsequently, the total extract was quantified using the Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Schwerte, Germany) according to the room temperature protocol for test-tubes.

### 5.2.4.3 Oligonucleotide synthesis and annealing

Based on the promoter sequences of GSTU13670 of the susceptible H and the resistant K2 population (see Figure 4b), two pairs of complementary biotinylated and unbiotinylated 22 bp oligonucleotides were designed for EMSA (Table S1) by Thermo Fisher Scientific (UK) containing a E2F/DP motif which was found in forward (motif1) and once in reverse direction (motif2).

The oligonucleotides were dissolved in a Tris-EDTA buffer (pH 8.0) at a concentration of 100 pmol $\mu L^{-1}$. For the buffer the following reagents were used: 10 mM Trizma® base (Tris (hydroxymethyl) aminomethane, Sigma-Aldrich, Germany), Tris-HCl [Tris (hydroxymethyl) aminomethane hydrochloride, Serva, Heidelberg, Germany], 1 mM EDTA (ethylenedinitrilotetraacetic acid, Sigma-Aldrich, Germany), 50 mM NaCl (sodium chloride, Sigma-Aldrich, Germany).

The oligonucleotides then were diluted to 1 pmol $\mu L^{-1}$ for each oligonucleotide and annealed using a thermocycler (Biometra TAdvanced, Analytik Jena, Germany) as indicated in Table 1 in order to obtain biotinylated and unbiotinylated dsDNA for EMSA. Annealed biotinylated oligonucleotides were diluted to 10 fmol $\mu L^{-1}$ with Tris-EDTA buffer just prior to EMSA performance.

Table 1. Oligonucleotide annealing programme for dsDNA used for EMSA.

| Step | Temperature (°C) | Duration | ΔT °C/s |
|---|---|---|---|
| 1 | 95 | 5 min | 8.0 |
| 2 | 25 | 12 min and 7 s | 0.1 |
| 3 | 4 | ∞ | 8.0 |

### 5.2.4.4 Investigation of E2F/DP binding motifs

For H and K2 each three reactions were set up for the two E2F/DP motifs using the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific, Pierce). One control reaction containing only the biotinylated dsDNA, one reaction containing biotinylated dsDNA and protein extract and one reaction containing biotinylated dsDNA, protein extract and unbiotinylated dsDNA. An additional control was included in the experiment using scrambled E2F/DP motifs (see Table S1) to avoid binding due to random electrostatic DNA forces. Protein extract was added at a concentration of 13 µg and unbiotinylated dsDNA was added at a concentration of 4 pmol per 20 µL reaction to the respective reaction tubes.

To improve binding, 1 µL of 50% glycerol, of 1% NP-40 and of 100 mM $MgCl_2$ were added according to the manufacturer's protocol. Pre-incubation took place for 2-3 min on ice before addition of 20 fmol of biotinylated dsDNA. Eventually the reactions were incubated for 20 min at room temperature and split in two aliquots. Two mini-PROTEAN® TGX™ Precast polyacrylamide gels (Any kDa, Bio-Rad, Feldkirchen, Germany) were run in parallel under native conditions at 8°C with 0.5x TBE (Bio-Rad, Feldkirchen, Germany) as running buffer at 100 V for 90 min (Bio-Rad PowerPac HC, version 1.07). Directly afterwards, one gel was blotted on a positively charged nylon membrane (Hybond-N+, GE Healthcare, Solingen, Germany) in a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) (10 V, 30 min, room temperature). Then, the DNA/protein complex was crosslinked on the nylon membrane at 120 mJ $cm^{-2}$ using a commercial UV-light crosslinking instrument (UVP Crosslinker CL-3000, Analytik Jena, Jena, Germany) for 60 s with the auto crosslink function with the DNA-side facing up. Then, the membrane was activated by chemiluminescence and visualised using a CCD camera (ChemiDoc™ Imaging System, Bio-Rad).

The other gel was directly stained with 20 mL PageBlue™ Protein Staining Solution (Thermo Scientific, Darmstadt Germany) according to manufacturer 's instructions. After overnight agitation at 50 rpm (Labnet, Rocker 25, Labnet International Inc.) the gel was destained twice within 45 min using ultrapure water and folded Kimwipes™ Tissues (Kimtech, Muggensturm, Germany) at room temperature. The gel bands were cut out and placed in reaction tubes with a droplet of ultrapure water and stored at -20°C.

Control reactions of the kit were performed according to the manufacturer 's instructions (Figure S4).

## 5.3 Results

### 5.3.1 Abundance, clustering and expression of black-grass GSTs in flufenacet susceptible and resistant black-grass populations

As the first detoxification step of the herbicide flufenacet is catalysed by GSTs, this superfamily of genes was investigated at the genome level. The black-grass genome contains 115 protein-coding genes which are annotated as GST (Figure 1 & S1). These belong to 11 different classes and are distributed over all seven chromosomes, with some of them assigned to the unscaffolded chromosome 0 (Figure 2). While a large fraction of GSTs has a higher expression rate in the flufenacet resistant populations, this difference was significant with a logFC cut-off threshold of ≥ 1.5 for only four genes (GSTU13667, GSTU13668, GSTU13670 and GSTF35765). The majority of the GST genes are assigned to the largest plant specific classes GSTU and GSTF with 65 and 35 isoforms, respectively. The rest of them are classified to various other classes, as follows: 3 GSTL, 3 GSTZ, 2 GSTT, 2 DHAR, 1 TCHQD, 1 GHR, 1 GSTH, 1 MTX and 1 mPGES2. However, there are additional genes assigned to families resembling the GST structure (Table S2), such as 2 GST2N, 2 aaRS, 2 Arc1p.

While the GSTs of the different classes are found on all of the seven chromosomes, they are most represented on the chromosomes 1,2,3,5 and 6, where the clusters of genes belonging either to class phi, tau or lambda are found. Genes belonging to the same cluster typically had similar protein sequences (Figure 1), which was also the case for the four differentially expressed GSTs. Expression patterns, however, were not always, but mostly independent from the cluster, e.g. analogous expression patterns can be observed in the first tau clusters on chromosome 2 (Chr2_CL1) or on chromosome 5 (Chr5_CL1). Yet, their 5'URR sequences don't cluster together (Figure S3).

In another example, the two phi clusters Chr5_CL3 and Chr5_CL4 consisted of each three genes. Each of these genes had a corresponding highly similar gene in the other cluster which was more similar in expression than the directly neighboring genes in the following manner: GSTF35750 and GSTF35764, GSTF35751 and the differentially expressed GSTF35765, GSTF35752 and GSTF35766. The genes GSTF35751 and GSTF35765 had 100% protein similarity (Figure S1). However, none of the 5'URR sequences clustered together. In case of the differentially expressed genes GSTU13667, GSTU13668 and GSTU13670 (Chr3_CL2), these three genes had similar expression patterns while the other two genes in the cluster, GSTU13665 and GSTU13666 had different expression patterns (Figure 1). Despite the different expression patterns, the 5'URRs of GSTU13666 and GSTU13667 were most similar (Figure S3). As GSTU13667, GSTU13668 and GSTU13670 share the same E2F/DP binding motifs, this cluster was further investigated.

Coregulation due to sharing the same 5’ URR, as it could be the case e.g. with the gene pair GSTU01909 and GSTU01910 (Figure 2) was excluded.

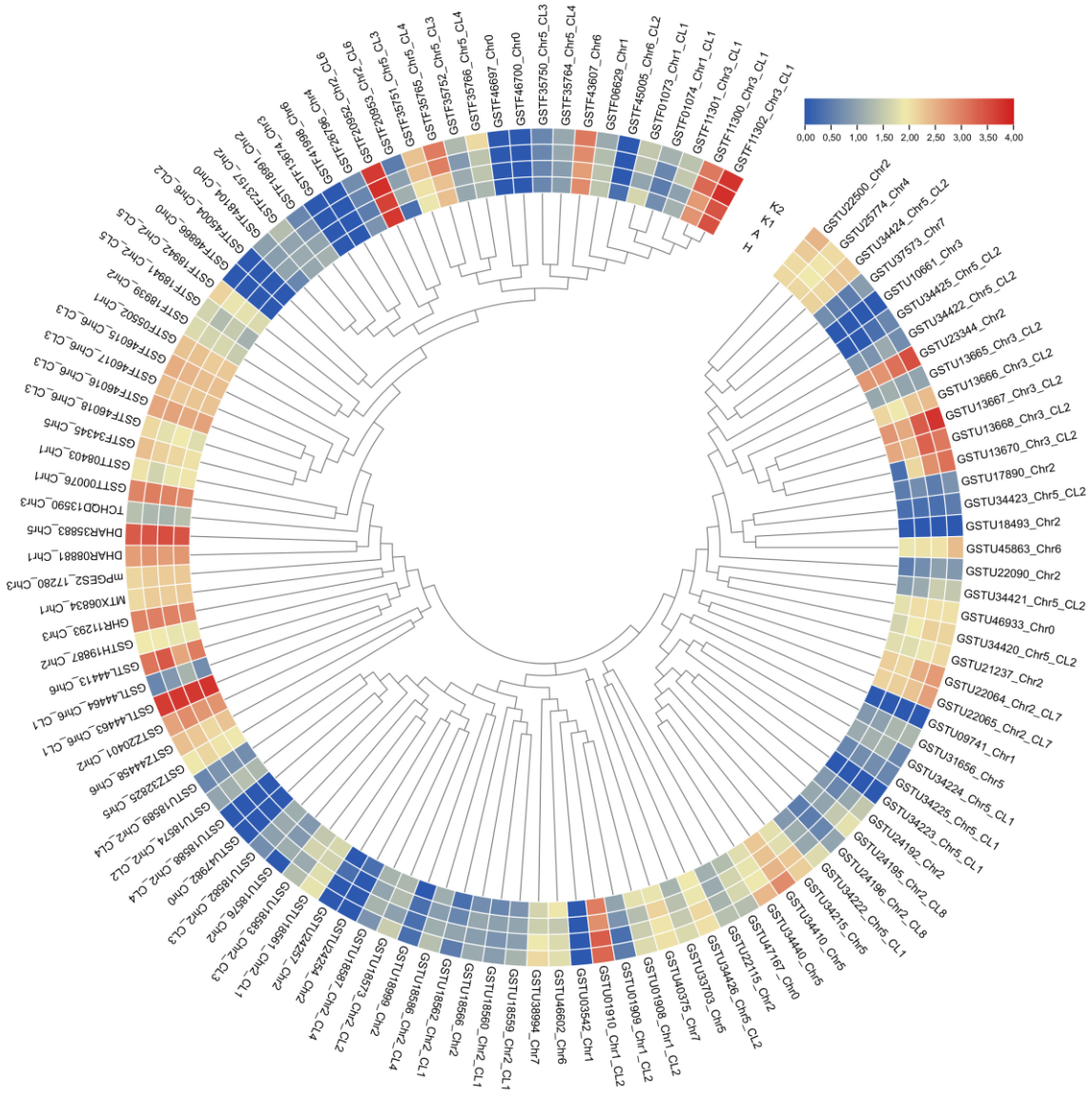


Figure 1. Circular phylogenetic tree of glutathione transferase (GST) proteins in the black-grass genome(Cai et al., 2023) in combination with a heatmap showing gene expression in flufenacet susceptible (H and A) and resistant (K1 and K2) populations. Chromosome (Chr) and cluster number (CL) are indicated (see Figure 2).

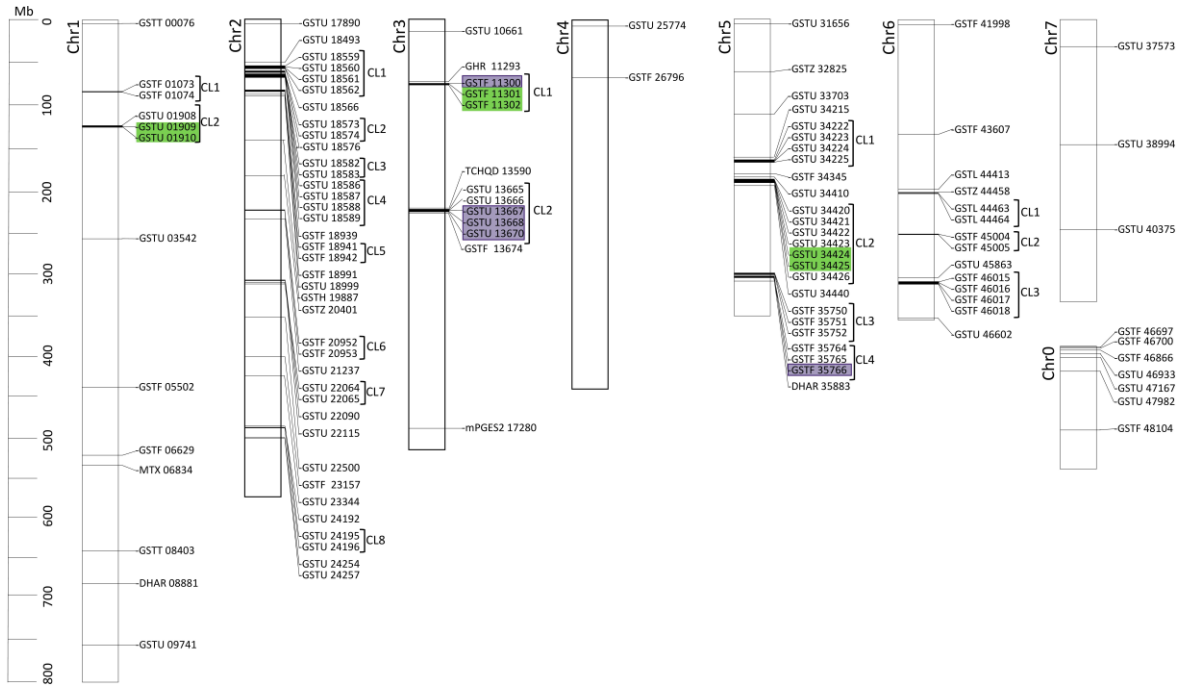


Figure 2. Location of the glutathione transferases (GST)on the black-grass chromosomes: tau (GSTU), phi (GSTF), theta (GSTT), zeta (GSTZ), lambda (GSTL), dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase (TCHQD), hemerythrin (GSTH), glutathionyl-hydroquinone reductase (GHR), metaxin (MTX) and microsomal prostaglandin E synthase type 2 (mPGES2) (Chronopoulou et al., 2017; Labrou et al., 2015). The cluster (CL) is indicated with a respective by a bracket. The green box indicates genes sharing a common 5’ upstream regulatory region. The purple boxes indicate genes which have been biochemically validated (Parcharidou et al., 2023).

### 5.3.2 Differentially expressed genes and associated clusters

Not only genes belonging to the glutathione transferase superfamily were found in clusters, but also genes of other families, as shown in Figure 3. The differential gene expression analysis revealed 134 significantly differentially expressed genes when both flufenacet resistant black-grass populations (K1 and K2) were compared to both susceptible populations (H and A). Among them 86 (64%) were upregulation in the resistant populations and the remaining 48 (36%) were downregulated (Figure S2). Among the DEGs, several genes code for proteins associated with the detoxification of xenobiotics. With 8, 5 and 4 DEGs, respectively, genes coding for UDP-glucosyl transferases (UGTs), cytochrome P450s (CYPs), and glutathione transferase (GSTs) were most frequently identified. However, no gene corresponding to an ATP-binding cassette (ABC) transporter was found among the common DEGs. Noteworthy, NADPH-dependent FMN reductases (FMNs), which are associated with the CYP proteins and are essential for their function, were also found among the genes

differentially expressed in the flufenacet resistant black-grass populations. With 10% of all FMNs in the genome differentially expressed, these genes were stronger correlated with the resistance phenotype than other enzyme classes e.g. the CYPs (less than 1%). In addition, the resistant population K2 exhibited the most prominent differential expression of genes coding for TFs, with classes belonging to AP2/ERF and bHLH. Proteins modulating the activity of TFs, such as protein kinases (PKs) and protein phosphatases (PPs) were found in small quantities among the DEGs. Furthermore, genes coding for proteins related to oxidative stress response, such as aldo/keto reductases (AKRs) and peroxidases (PODs) were listed among the common DEGs. The former ones were only found differentially expressed in K2, whereas the latter were found differentially expressed in both populations but also in each one exclusively, with K1 to possess the more DEGs with higher statistically significantly logFC values.

Moreover, the putative target enzyme of flufenacet, the family of KCSs wasn't found among the commonly DEGs. In total, more DEGs (801) were found in the more resistant population K2 compared to K1 (367).

Table 2. Differentially expressed genes (DEGs) corresponding to enzymes involved in pathways related to detoxification of xenobiotics, oxidative stress, signalling and TFs found in flufenacet resistant (K1 and K2) compared to the susceptible (H and A) black-grass populations. In addition, genes corresponding to the putative target enzymes of flufenacet are included.

| Gene family | Total number | DEGs | UP | DOWN | Population |
|---|---|---|---|---|---|
| Cytochrome P450s (CYPs) | 595 | 5 | 3 | 2 | K1∩K2 |
| | | 5 | 2 | 3 | K1 |
| | | 23 | 18 | 5 | K2 |
| UDP-glucuronosyl/ | 298 | 8 | 7 | 1 | K1∩K2 |
| UDP-glucosyl transferases | | 0 | 0 | 0 | K1 |
| (UGTs) | | 20 | 18 | 2 | K2 |
| Glutathione transferases | 115 | 4 | 4 | 0 | K1∩K2 |
| (GSTs) | | 3 | 3 | 0 | K1 |
| | | 11 | 10 | 1 | K2 |
| ATP-binding cassette (ABC) | 212 | 0 | 0 | 0 | K1∩K2 |
| transporters | | 2 | 0 | 2 | K1 |
| | | 0 | 0 | 0 | K2 |
| NADPH-dependent FMN | 30 | 3 | 3 | 0 | K1∩K2 |
| reductases (FMNs) | | 1 | 1 | 0 | K1 |
| | | 1 | 1 | 0 | K2 |

| | | | | | |
|---|---|---|---|---|---|
| Aldo/keto reductases (AKRs) | 46 | 0 | 0 | 0 | K1∩K2 |
| | | 0 | 0 | 0 | K1 |
| | | 5 | 3 | 2 | K2 |
| Peroxidases (PODs) | 243 | 3 | 3 | 0 | K1∩K2 |
| | | 12 | 11 | 1 | K1 |
| | | 6 | 4 | 2 | K2 |
| Peptidase family A1 (PEPs) | 111 | 0 | 0 | 0 | K1∩K2 |
| | | 2 | 0 | 2 | K1 |
| | | 7 | 5 | 2 | K2 |
| Protein kinases (PKs) | 427 | 3 | 0 | 3 | K1∩K2 |
| | | 3 | 1 | 2 | K1 |
| | | 7 | 2 | 5 | K2 |
| Protein phosphatases (PPs) | 130 | 0 | 0 | 0 | K1∩K2 |
| | | 0 | 0 | 0 | K1 |
| | | 1 | 1 | 0 | K2 |
| APETALA2/ethylene | 156 | 1 | 1 | 0 | K1∩K2 |
| responsive factors | | 1 | 1 | 0 | K1 |
| (AP2/ERFs) | | 6 | 6 | 0 | K2 |
| Myc-type/basic helix-loop- | 179 | 0 | 0 | 0 | K1∩K2 |
| helix (bHLH) | | 0 | 0 | 0 | K1 |
| | | 5 | 2 | 3 | K2 |
| 3-ketoacyl-CoA synthase | 47 | 0 | 0 | 0 | K1∩K2 |
| (KCSs) | | 0 | 0 | 0 | K1 |
| | | 1 | 1 | 0 | K2 |

Some of the differentially expressed genes were found in close proximity on the chromosomes, forming clusters as shown in Figure 3. In most cases genes within these clusters follow the same trend or are not expressed or expressed at a low level, with a few exceptions (e.g. CYP cluster on Chr5). Differences in the expression between the two resistant populations were particularly pronounced in the clusters of AP2/ERFs and PKs on chromosome 6.

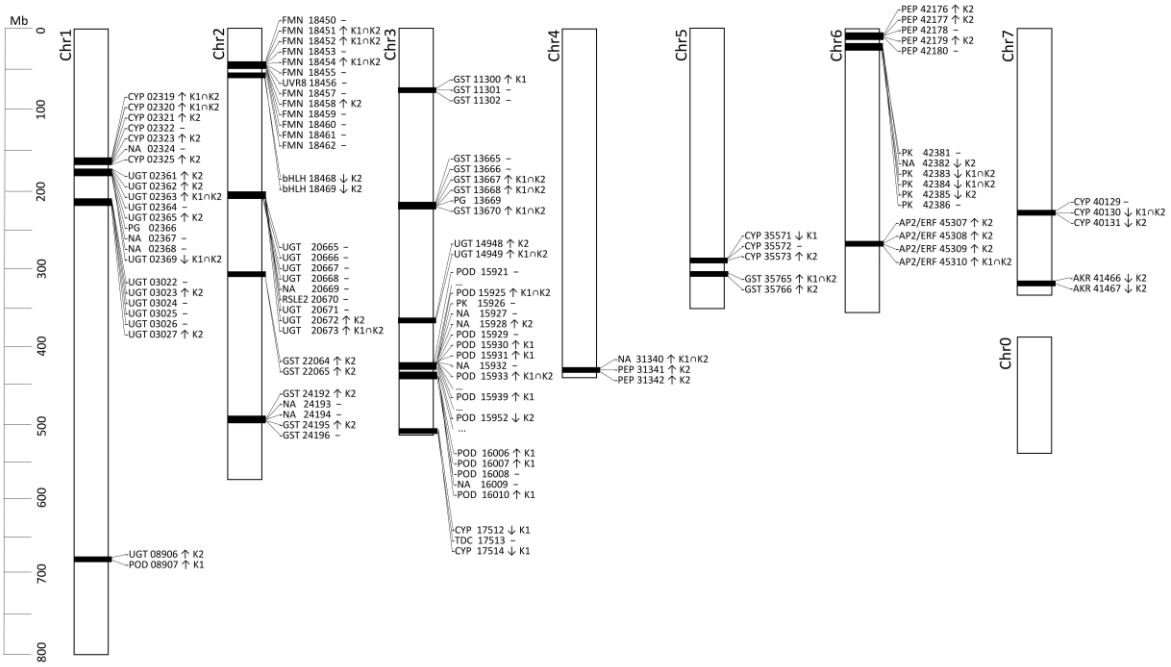


Figure 3. Clusters of significantly upregulated (↑) and significantly downregulated (↓) genes in flufenacet resistant black-grass populations (K1 and K2) compared to two susceptible populations (H and A). No differential gene expression (-), classification as pseudogene (PG), no annotation (NA), and occurrence in one or both resistant populations (K1 and K2) are additionally indicated next to the gene name. The gene families described are cytochrome P450s (CYPs), UDP-glucuronosyl/UDP-glucosyl transferases (UGTs), glutathione transferases (GSTs), ATP-binding cassette (ABC) transporters, NADPH-dependent FMN reductases (FMNs), aldo/keto reductases (AKRs), peroxidases (PODs), peptidases A1 (PEPs), protein kinases (PKs), protein phosphatases (PPs), APETALA2/ethylene responsive factors (AP2/ERFs), myc-type/basic helix-loop-helix (bHLH), 3-ketoacyl-CoA synthase (KCSs), ultraviolet-B receptor (UVR8), RICESLEEPER 2 (RSLE2), tyrosine decarboxylase (TDC).

### 5.3.3 Investigation of binding E2F/DP binding motifs

To validate differences in promoter sequences in the candidate GST cluster, the promoter sequences of the GSTU genes belonging to the cluster Chr3_CL2 were investigated and the functionally validated(Parcharidou et al., 2023) and most upregulated gene GSTU13670 was chosen as candidate for upstream. By performing upstream PCR using gDNA of the flufenacet most susceptible (H) and most resistant black-grass (K2) populations, the promoter sequences were revealed (Figure 4b). The sequence of the motifs resembles the genomic sequence, with only few insertions or deletions (Cai et al., 2023). The sequences of motif1 and motif2 are identical in both populations but the regions adjacent to the motifs have dissimilarities.

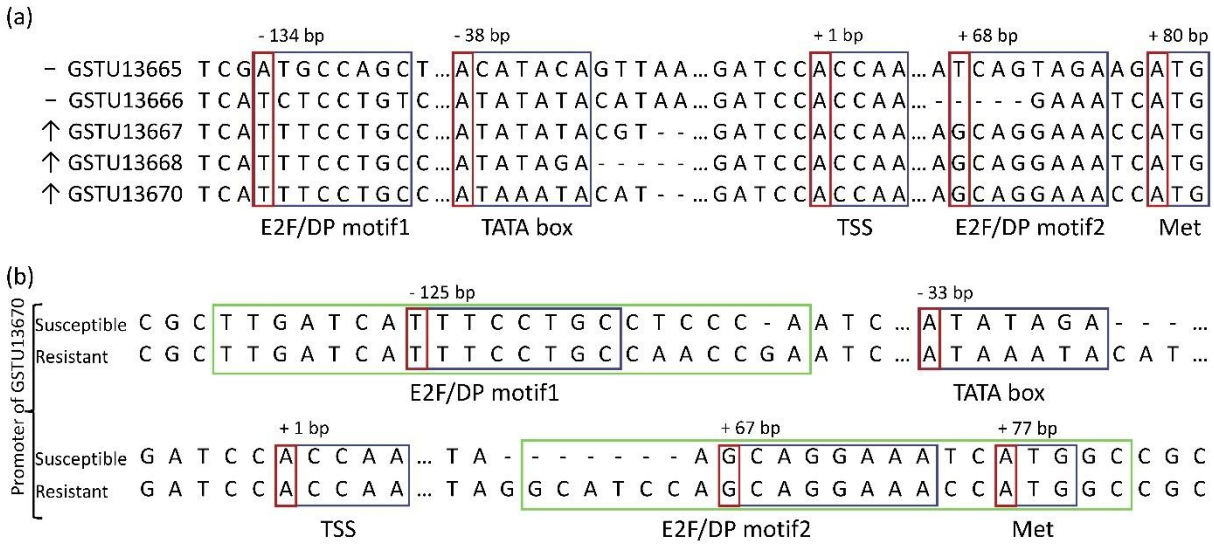


Figure 4. (a) Core and proximal promoter regions of tau class glutathione transferases (GSTs) (GSTU13665, GSTU13666, GSTU13667, GSTU13668, GSTU13670) located on the same cluster, based on black-grass genome sequences (Cai et al., 2023). The E2F/DP binding sites (in forward and reverse complement directions), the TATA box and the putative ATG start site (methionine) are indicated by numbers in relation to transcription start site (TSS; + 1) and highlighted with a purple box. The first nucleotide of each one of the features is indicated with a red box. (b) Core and proximal promoter regions of GSTU13670 gene based on upstream region PCR using genomic DNA template of the susceptible (H) and the resistant (K2) black-grass populations. All features are highlighted as in Figure 4a. The DNA sequences used for the electrophoretic mobility assay (EMSA) are highlighted with green boxes.

To investigate the effect of these sequence dissimilarities on TF binding at the putative E2F/DP binding site, an electrophoretic mobility shift (EMSA) was used. The visualised gel (Figure 5a) shows a shift of the DNA/protein complex for both, the sequence of motif1 of the susceptible (H) and the resistant (K2) population. However, the band is considerably more pronounced in the case of the susceptible population. Since these bands are not visible in the control reaction with unbiotinylated DNA, specific binding, e.g. motif recognition, can be assumed. Random binding due to electrostatic forces can furthermore be excluded as no additional bands were visible in the control reactions with scrambled version of motif1.

In case of motif2, significant shifts were observed neither for the probe deriving from the susceptible population (H) nor for the probe deriving from the resistant population (K2), as shown in Figure 5b.

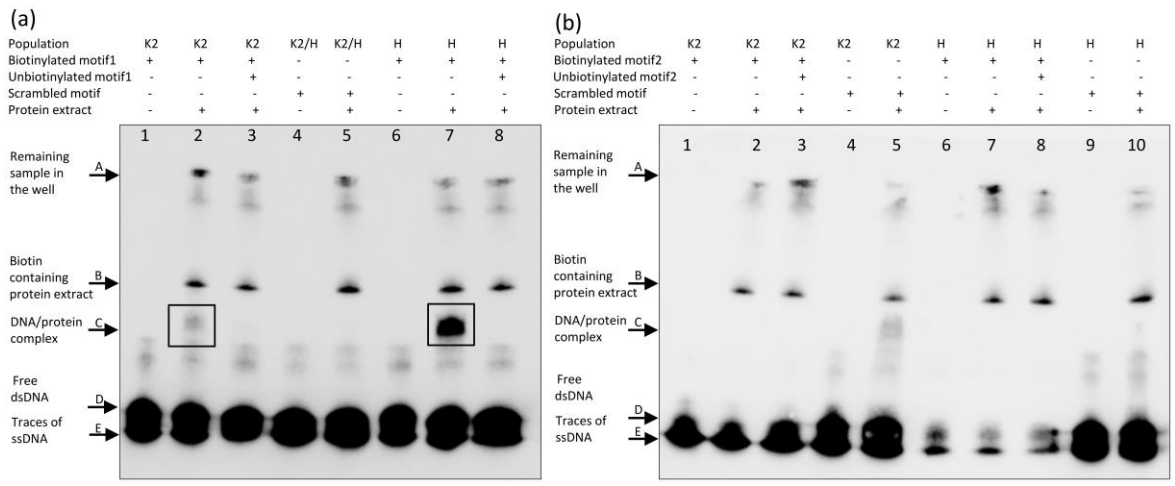


Figure 5. Nylon H+ membranes of electrophoretic mobility assay (EMSA) visualised by chemiluminescence with reactions of E2F/DP binding site motif1 (a) and motif2 (b) of the promoter of GSTU13670 deriving from the resistant population K2 and the motif of the susceptible population H. Complexes between the oligonucleotides and the protein are marked with black squares.

## 5.4 Discussion

Multiple herbicide resistant black-grass is a major challenge in Western European agriculture. The VLCFA synthesis inhibitor flufenacet is a key herbicide to control resistant black-grass, however resistance to this herbicide is also evolving (Dücker, Zöllner, Parcharidou, et al., 2019; Heap, 2023). As in the present study, where the plants also detoxify mesosulfuron-methyl, iodosulfuron-methyl-sodium and clodinafop-propargyl faster (Figure S6), flufenacet resistance often co-occurs with metabolic resistance to other herbicides, which lies under the umbrella of the non-target-site resistance (NTSR) (Coleman et al., 1997). Metabolic resistance to flufenacet in black-grass in particular, was shown to be caused by enhanced GST activity (Dücker, 2020; Dücker et al., 2020; Dücker, Zöllner, Parcharidou, et al., 2019). Recently, it was proven that some of these genes are able to detoxify flufenacet *in vitro*, by forming a flufenacet-glutathione conjugate or in unique cases a flufenacet-alcohol metabolite (Parcharidou et al., 2023). With the recent black-grass genome assemblies (Cai et al., 2023; Kersten et al., 2023) it was possible to explore GST families and their abundance, and to investigate the regulation of specific GST genes. This approach was followed using RNA-Seq reads from susceptible and flufenacet-resistant black-grass populations with differential gene expression analysis and the genome published by Cai et al. (2023) as a reference.

Out of 45,263 protein-coding genes found in the black-grass genome, 134 genes with logFC values ≥ 1.5 were constitutively differentially expressed (FDR ≤ 0.05) in both flufenacet resistant (K1 and K2) compared to both susceptible (H and A) populations. The majority of

those genes showed upregulation. Nevertheless, a particularly large number of additionally upregulated (445) and downregulated (222) genes was found in the more resistant population K2. Genes coding for enzymes involved in metabolic resistance – 8 UGTs, 5 CYPs, 4 GSTs – were observed among the upregulated genes, which was also found independently in various metabolically resistant weed species (Gaines et al., 2014). These results are in accordance with a previous transcriptomic analysis (Dücker et al., 2020) however led to lower amounts of identified DEGs in each enzyme class.

The fact that resistance-related genes were not only found differentially expressed in both resistant populations, but that some of these genes were exclusively differentially expressed in either of the resistant populations, goes along with the hypothesis that resistance evolution is driven by both parallel and non-parallel evolution. This phenomenon is supported also by other studies on black-grass (Cai et al., 2023), common waterhemp (*Amaranthus tuberculatus* (Moq.) J.D.Sauer; Kreiner et al., 2021) and common morning-glory (*Ipomoea purpurea* (L.) Roth) (Van Etten et al., 2020).

FMNs which are partner proteins necessary for the function of CYPs (Werck-Reichhart et al., 2000) were found among the commonly and exclusively differentially expressed DEGs of the flufenacet resistant black-grass populations. They showed the same trend as most CYPs, which is in accordance with other studies on rat liver, where gene expression followed same pattern (Buchmann et al., 1985). Meanwhile, genes belonging to the target site enzyme family of flufenacet, the very-long-chain 3-oxoacyl-CoA synthases (synonym: keto-acyl-CoA-synthase - KCS) were not differentially expressed. This observation is expected and well supported by the findings that GST upregulation is the main mechanism causing flufenacet resistance (Dücker et al., 2020; Dücker, Zöllner, Parcharidou, et al., 2019).

Nevertheless, the well-described AmGSTF1 (GSTF11300), which was frequently upregulated in multiple herbicide resistant black-grass (Cummins et al., 2013; Goldberg-Cavalleri et al., 2023), was found only significantly upregulated in K1 (multi-resistant to ALS-, ACCase-, and VLCFAs-inhibitors), even if K2 is also similarly multi-resistant. Only 4 GST genes (GSTU13667, GSTU13668, GSTU13670, GSTF35765) were found commonly significantly upregulated in the flufenacet resistant populations, which are active on flufenacet (Parcharidou et al., 2023). As all candidate GSTs detoxified flufenacet at low to moderate levels, thus resistance is likely caused by an additive effect of different GSTs (Parcharidou et al., 2023).

To better understand the complex interplay of GSTs in flufenacet resistance, a genome-wide GST analysis was performed. In total 115 GST genes were identified, which is disproportionately large compared to other diploid species, such as GST genes in thale cress (55) (Sappl et al., 2009), rice (79) (Jain et al., 2010), pink shepherd's-purse (*Capsella rubella* Reut.) (49) (He et al., 2016), barley (84) (Rezaei et al., 2013), cotton (*Gossypium raimondii* Ulbr. (59) and *Gossypium arboreum* L. (49)) (Dong et al., 2016). A large number of functionally

distinct GSTs is likely to be a major fitness advantage in the setting of selection pressure from repeated herbicide use.

It was shown that GSTU and GSTF are most abundant among the 11 GST classes found in the black-grass genome (GSTU, GSTF, GSTL, GSTZ, GSTT, DHAR, TCHQD, GHR, GSTH, MTX, mPGES2).

Particularly, the most abundant classes GSTU and GSTF were typically arranged in clusters. The phenomenon of GST clustering is well described in other plant species (Dixon et al., 2002; He et al., 2016; Soranzo et al., 2004). WGD was likely the force of soybean GSTs expansion (Liu et al., 2015), whereas in pink shepherd's-purse and oilseed rape the force was the tandem gene duplication (Khan et al., 2018; Wei et al., 2019). The black-grass genome has most likely been chiselled by a WGD followed by diploidization and small-scale local duplication events, with GST family to be one of the most expanded gene families (Cai et al., 2023; Li et al., 2021; Wolfe, 2001). Furthermore, the similarity of the two clusters CL3 and CL4 on Chr5, and the fact that each protein in the first cluster corresponds to its counterpart in the second cluster indicates a segmental gene duplication.

The fact that GSTUs and GSTFs have expanded most and have not been lost during the millions of years of evolution can be explained by fitness advantages e.g. due their major role in detoxification of xenobiotics and defence responses to biotic and abiotic stress (Benekos et al., 2010; Cummins et al., 2013; Gullner et al., 2018; Hasan et al., 2021; Jha et al., 2011; Karavangeli et al., 2005; Loyall et al., 2000), such as herbicide resistance.

Plant genomes have evolved not only through changes in protein-coding genes but also in regulatory sequences, resulting in altered genome architecture (Choudhuri, 2014; Lan et al., 2010). This is confirmed by the recent studies on the black-grass genome, where it was described that the TEs, which actually play major role in evolution of CREs (Maeso & Tena, 2016), constitute 81.7% of the genome (Cai et al., 2023). Similar proportions of TEs were already observed in barley (Mascher et al., 2017) and wheat (Wicker et al., 2018) with 80.8% and 84.7%, respectively. CREs can be also a target of divergence leading to expansion or loss of TFBSs and thus influence the expression of duplicated genes (Choudhuri, 2014; Force et al., 1999). For example, in soybean it was observed that the regulatory regions of duplicated genes are most prone to high mutational rates (Liu et al., 2015) and in oilseed rape (*Brassica napus* L.) duplicated GST genes were described to have divergent expression patterns (Wei et al., 2019). Overall, it was shown that black-grass GST genes coding for highly similar proteins and proteins positioned in clusters, exhibited high differences in their 5' URRs and respective CREs (Figure S3 & S5).

In the promoters of the three upregulated clustered GSTUs a conserved putative motif of the E2F/DP TF complex was found. The DNA motif was present twice on opposite strands at about 130 bp upstream and 67 bp downstream of the TSS, respectively (Figure 5b). The E2F TF

typically forms heterodimeric complexes with the DP as both TFs interact with the promoter DNA. The complex can affect the transcription of the downstream genes, either by activating or repressing it.(van den Heuvel & Dyson, 2008) Studies of the E2F/DP TFs have been conducted in wheat(Ramirez-Parra & Gutierrez, 2000) and thale cress (Heckmann et al., 2011; Shen, 2002). So far, it was not described in promoters of a GST gene, but in a promoter of a UGT (Ramirez-Parra et al., 2004). The E2F/DP binding motifs were fully conserved and only present in the upregulated GST genes of the cluster Chr3_CL2 and mutated or even absent in the non-DEGs. Therefore, the promoter of GSTU13670, the GST which was confirmed to detoxify flufenacet at a comparably high rate *in vivo*, was further investigated in susceptible and resistant plants. The binding motif was conserved between the H and K2 populations. However, the flanking sequence downstream of motif 1 was characterized by three mismatches within the next six bases, while deletions were identified in the sequence flanking motif 2, which TF flanking sequences can significantly affect TFs binding (Atchley et al., 1999; Fisher & Goding, 1992). Therefore, an EMSA assay was performed with both motifs. A shift was observed when using the K2/1 and H1 biotinylated-dsDNA probes of motif 1 (Figure 5a), which was more intense and represented by a thicker band in case of the susceptible (H1) motif. This suggests that the E2F/DP complex might repress the transcription of GSTU13670. Thus, its repression can lead to a lower detoxification rate in the susceptible populations.

Although the EMSA method is capable of resolving complexes with various conformations using whole-cell extracts, it's important to note that dissociation can occur during electrophoresis, which can hinder the detection of these complexes. Furthermore, it cannot be excluded that additional TFs bind to the promoter and affect the resistance level.

However, TFs belonging to the families of AP2/ERF and Myc-bHLH were found differentially expressed. Members of the AP2/ERF family are found in higher plants (Guo et al., 2007; PlantTFDB, 2023) with functions involved in physiological processes. Interestingly, AP2 was found upregulated in flufenacet treated Arabidopsis plants (Lechelt-Kunze et al., 2003). Furthermore, AtEBP (AtERF072) has been shown to interact *in vitro* with the bZIP TF OBF4, which binds on *ocs* elements (Nakano et al., 2006). Although the latter are typically found in plant GST promoters (Marrs, 1996), they were not only associated with the differentially expressed GST genes. Myc-bHLH genes, which were downregulated in K2 are known to be involved in many plant abiotic stress responses (Bartels & Sunkar, 2005). They were also differentially expressed in other herbicide resistant weed species e.g. in multiple herbicide resistant American sloughgrass (*Beckmannia syzigachne* Steud.; Wang, 2022).

## 5.5 Conclusions

Evolving resistance in black-grass to pre-emergence herbicides e.g. flufenacet is a challenge for modern agriculture in Western Europe. It is well known that resistance to the VLCFA-inhibitor flufenacet is metabolism-based due to enhanced GST activity. Using the recently published black-grass genome (Cai et al., 2023) in combination with transcriptomic data derived from flufenacet susceptible and resistant populations, this study sheds further light on the phenomenon of metabolic herbicide resistance. A genome-wide GST analysis revealed 115 GST genes belonging to 11 different classes found in black-grass genome, a large number for a diploid species and a favorable condition for adaptation to regular herbicide treatments in modern agriculture. In particular, the large number of tau and phi class GSTs is likely to be the result of a combination of duplication mechanisms, such as WGD and tandem gene duplication, which are certainly influenced by TEs. Further duplication may be promoted by continuous herbicide selection pressure. Finally, this study points out the need of a standard protocol for genome-wide GSTome analyses to compare orthologous genes between species in term of sequence and biochemical activities.

The most abundant classes tau and phi, but also some other differentially expressed genes often occurred in clusters of genes with high sequence similarity. Nevertheless, promoter sequence and protein sequence as well as expression patterns rarely correlated. One exception was the potential involvement of E2F/DP factors in three clustered GSTUs, which were previously shown to be upregulated in flufenacet resistant black-grass and validated as flufenacet-detoxifying GSTs *in vitro*. Two potential E2F/DP binding sites were identified in all GSTs of the cluster on opposite strands. A sequencing of the promoter regions of susceptible and resistant plants of GSTU13670 revealed that the two motifs were conserved, but adjacent sequences differed between susceptible and resistant plants. In an EMSA with the most upstream motif (motif1), a clear shift with a strong band deriving from the susceptible population Herbiseed was observed, indicating repressor activity at the E2F/DP binding motif. However, further analyses are necessary to identify the protein(s) in the complex. Additionally, co-expression patterns in relation to the complex promoter sequences could be further elucidated using machine-learning based approaches and targeted comparisons of the upstream regions of the clustered GST genes (Chr3_CL2) between susceptible (Herbiseed) and resistant (Kehdingen2) populations could further improve the understanding of how promoter sequences affect herbicide resistance.

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# 6. General Discussion

Black-grass (*Alopecurus myosuroides* Huds.) is a notorious weed species in cereals in Western Europe, which is mainly controlled by the use of herbicides in combination with good agronomic practices, known as Integrated Weed Management (IWM). During the last decades, resistance to post-emergent herbicides, especially the leaf-applied acetyl-CoA-carboxylase- (ACCase; HRAC Group 1) and acetolactate synthase-inhibitors (ALS; HRAC Group 2) – has evolved (Peterson et al., 2018). This has led to the increased use of pre-emergent herbicides such as flufenacet, diflufenican, prosulfocarb and, more recently, cinmethylin, as solo applications or in mixtures. These herbicides can be used in order to slow down black-grass spread. The most widely used active ingredient (a.i.) for grass weed control in Europe, and particularly in Germany, is the very-long-chain fatty acid (VLCFA) synthesis inhibitor flufenacet (HRAC Group 15), which is typically used in pre-emergence (Menne et al., 2012). It was introduced to the German market in 1998 (BVL, 2022) and is classified in the category of herbicides that are less prone to evolve resistance (S. R. Moss et al., 2019).

However, reduced sensitivity of various weed species to VLCFA synthesis-inhibitors, such as the dicotyledons tall waterhemp (*Amaranthus tuberculatus* (Moq.) JD Sauer; Strom et al., 2019) and Palmer amaranth (*Amaranthus palmeri* S. Watson; Brabham et al., 2019) and mostly to monocotyledons, has been already described. The list of monocotyledonous weeds includes the following species: common wild oat (*Avena fatua* L.) (Mangin et al., 2017), common barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv.; Juliano et al., 2010), Italian ryegrass (*Lolium multiflorum* (Lam.); Dücker, Zöllner, Lümmen, et al., 2019; Rauch et al., 2010), and rigid ryegrass (*Lolium rigidum* Gaud.; Busi et al., 2018; Busi & Powles, 2013). Black-grass is also affected by evolving resistance against several VLCFA-inhibitors, including the α-oxyacetamide flufenacet (Dücker, Zöllner, Parcharidou, et al., 2019).

The reduced efficacy of flufenacet against black-grass belongs to the non-target site resistance spectrum (NTSR), caused by enhanced metabolism of the a.i. by glutathione transferases (GSTs) (Dücker et al., 2020; Dücker, Zöllner, Parcharidou, et al., 2019). That holds true as well for other grass weeds such as ryegrass (Dücker, Zöllner, Lümmen, et al., 2019) and several crops (Bayer AG, 2017; Bieseler et al., 1997; Gould & Lemke, 2002). Target site resistance (TSR) to flufenacet is very unlikely to occur; the main reason is that its putative binding site the very-long-chain 3-oxoacyl-CoA synthases (synonym: keto-acyl-CoA-synthases - KCSs) have a highly conserved cysteine residue in their active centres which is crucial for the catalytic function (Böger et al., 2000; Krähmer et al., 2018). Besides, several enzymes of this family were shown to have redundant activities (Tanetani et al., 2013; Todd et al., 1999). Thus, a single mutation is unlikely to have a significant effect on the resistance level.

In order to detect constitutively differentially expressed genes (DEGs) between flufenacet susceptible (Herbiseed or H, Appel or A) and resistant (Kehdingen1 or K1, Kehdingen2 or K2)

black-grass populations, a transcriptomic study was conducted (Dücker et al., 2020) using the black-grass reference transcriptome published by Gardin et al. (2015), as well the recently assembled genomes (Cai et al., 2023; Kersten et al., 2023). Based on similar studies, overexpressed candidate genes involved in herbicide resistance, as well as in the evolving resistance to flufenacet, have been described, but were not biochemically validated.

In this study, DEGs were obtained by aligning RNA-Seq data (Dücker et al., 2020) from four untreated black-grass populations (H, A, K1, K2) against the black-grass genome (Cai et al., 2023). Of the approximately 45,000 annotated protein-coding genes found in the black-grass genome, 134 genes with logFC values ≥ 1.5 were found to be significantly differentially expressed in both flufenacet resistant (K1 and K2) compared to both susceptible (H and A) populations. The majority of these genes showed upregulation. However, additional genes were found that were differentially expressed only in each of the resistant populations.

Exploring the DEG list, genes coding for enzymes involved in metabolic resistance such as cytochrome P450s (CYPs), NADPH-dependent FMN reductases (FMNs), UDP-glucuronosyl/UDP-glucosyl transferases (UGTs) and glutathione transferases (GSTs) were found among them, with the majority to be upregulated. Focusing on the abundance of DEGs belonging to the FMN, GST, UGT and CYP classes, it is noteworthy that approximately 10%, 3.5%, 2.7%, 1% of the common DEGs were revealed, but K2 showed additionally 3.3%, 9.5%, 6.7%, 4% of DEGs respectively. CYPs and their necessary partner proteins FMN reductases are taking part in xenobiotics detoxification, and they are associated with herbicide resistance (Werck-Reichhart et al., 2000). The same holds true for the UGT proteins (Huang et al., 2021). Among the common DEGs, four GST genes were found, three of which were correspondingly listed as DEGs in a previous study (Dücker et al., 2020) using the same RNA-Seq data and aligned to the black-grass reference transcriptome (Gardin et al., 2015). The sequences of the most upregulated GST genes (ALOMY3G13667 or GSTU13667, ALOMY3G13668 or GSTU13668, ALOMY3G13670 or GSTU13670, ALOMY5G35766 or GSTF35766) derived from the above two analyses were obtained by RACE PCR using genetic material from the most resistant population (K2), and cloned into plasmids expressed in bacteria (*Escherichia coli*). In addition, the well-described AmGSTF1 (corresponds to ALOMY3G11300 or GSTF11300), which was frequently upregulated in multiple herbicide resistant black-grass (Cummins et al., 2013) and found only statistically significantly upregulated in K1, was chosen to be expressed as recombinant protein in *E. coli* for further *in vitro* biochemical experiments. The respective cytosolic recombinant GST proteins were tested on flufenacet, and as well various other a.i., such as acetochlor, *S*-metolachlor, pyroxasulfone, prosulfocarb, cinmethylin, diflufenican, pendimethalin, fenoxaprop-ethyl, fenoxaprop-acid, clodinafop-propargyl, clodinafop-acid and mesosulfuron-methyl.

The recombinant proteins GSTU13667, GSTU13668, GSTU13670 share more than 80% of sequence similarity and all the three possessed the active site serine (Ser15), the invariant proline (Pro57) and the quasi-invariant aspartic acid (Asp162). Moreover, they have conserved the residues involved in binding glutathione (G-site) such as lysine (Lys42 and Lys55), valine (Val56), serine (Ser69), but not the residue at position 68, which is critical for GSH binding. In GSTU13667 and GSTU13670 this position is filled by glutamic acid (Glu68) as expected by a tau class GST (Sylvestre-Gonon et al., 2019) in contrast to GSTU13668 where it is occupied by aspartic acid (Asp68). However, all three proteins could detoxify flufenacet *in vitro*. The recombinant enzymes GSTU13667 and GSTU13670 metabolised flufenacet to a flufenacet-glutathione (GSH) conjugate, whereas GSTU13668 produced flufenacet-alcohol. The flufenacet-GSH conjugate is the metabolite produced also *in vivo* studies in black-grass (Dücker, Zöllner, Parcharidou, et al., 2019), ryegrass (Dücker, Zöllner, Lümmen, et al., 2019) and some crops (Bieseler et al., 1997). Nevertheless, flufenacet-alcohol has been described as an intermediate metabolite of the flufenacet-oxalate metabolite found in crops as wheat, maize, soybeans and sunflower (Bayer AG, 2017; Gould & Lemke, 2002). A mutated protein of GSTU13668, called GSTU13668m, with an amino acid substitution at position 68, which is crucial for GSH binding, as mentioned above, was constructed and its activity was also tested against flufenacet. GSTU13668m performed the same activity and produced the same metabolite as the wild type GSTU13668, indicating that more than one amino acid are crucial for the different metabolite formation. The previously described AmGSTF1 (GSTF11300), known to also to act as peroxidase (Cummins et al., 1999) and be involved in plant secondary metabolism (Cummins et al., 2013), showed transferase activity, albeit at a low rate (Parcharidou et al., 2023). The other phi class GST (GSTF35766) showed the highest activity on acetochlor, compared to the other tested recombinant proteins and was able to degrade all the amount of pyroxasulfone within 2 hours (≥ 1.67 nmol product $min^{-1}$ $mg^{-1}$ protein, 100% of the substrate), showing cross-resistance of GSTF35766 towards flufenacet and acetochlor. No cross-resistance was shown for cinmethylin, diflufenican and pendimethalin, which were not degraded by any of the recombinant GSTs tested in *in vitro*. Therefore, herbicides containing the above a.i. can be used as alternatives for the control of flufenacet resistant black-grass populations. Regarding the post-emergent herbicides, the ALS inhibitor mesosulfuron-methyl and the ACCase inhibitor clodinafop-propargyl and its active metabolite clodinafop-acid were not degraded either by any of the GSTs tested. However, the ACCase inhibitor fenoxaprop-ethyl was detoxified by all the GSTs and the degradation rates of its active metabolite fenoxaprop-acid were in all cases lower than for the prodrug. It is noteworthy that GSTU13668 had the lowest activity on fenoxaprop-ethyl and did not detoxify fenoxaprop-acid. The involvement of various GST proteins in flufenacet detoxification and the relatively low detoxification rates (0.47 to 1.5 nmol product $min^{-1}$ mg $protein^{-1}$) suggest an additive effect of

the different GST genes and a polygenic generalist resistance (Comont et al., 2020b; Hawkins et al., 2019). That is in accordance with the slow resistance evolution and the low resistance level described under field conditions (Hull & Moss, 2012). In other cases, one overexpressed gene was responsible to confer multi-herbicide resistance as described by Han et al. (2021).

However, the mechanism behind the upregulation of GST genes in resistant black-grass population has not been elucidated. This study represents the first genome-wide analysis of the GSTome of black-grass. Using the black-grass genome assembled by Cai et al. (2023), 115 GST-annotated genes were revealed. They belong to 11 different classes as follows: 65 tau (GSTU), 35 phi (GSTF), 3 zeta (GSTZ), 3 lambda (GSTL), 2 theta (GSTT), 2 dehydroascorbate reductase (DHAR), 1 tetrachlorohydroquinone dehalogenase (TCHQD), 1 hemerythrin (GSTH), 1 glutathionyl-hydroquinone reductase (GHR), 1 metaxin (MTX) and 1 microsomal prostaglandin E synthase type 2 (mPGES2). However, there are only a few classes of GSTs that have biochemical activity on herbicides, and these are mainly the GSTU and GSTF classes (Labrou et al., 2015). There are also a few other GST classes that were not found in the black-grass genome as expected, or whose expected domains were missing, such as the γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ). This highlights the need for an established nomenclature of GST proteins and criteria for classifying a GST protein as such.

Black-grass exhibits a remarkably high number of GSTs relative to its diploid genome, surpassing counts observed in other diploid plant species such as thale cress (*Arabidopsis thaliana* (L.) Heynh., 55; Sappl et al., 2009), barley (*Hordeum vulgare* L., 84; Rezaei et al., 2013), rice (*Oryza sativa* L., 79; Jain et al., 2010), pink shepherd's-purse (*Capsella rubella* Reut., 49; He et al., 2016) and tomato (*Solanum lycopersicum* L., 90; Islam et al., 2017).

GST genes, particularly the classes phi and tau, are typically organised in clusters, with only intergenic regions between them, and within a given GST cluster, genes from the same GST class are found. The phenomenon of GST clustering is well described in other plant species such as in rice (Soranzo et al., 2004), thale cress (Dixon et al., 2002), pink shepherd's-purse (He et al., 2016), but also in humans (Nebert & Vasiliou, 2004). These clusters are likely a result of repeated gene duplication events.

More specifically, whole genome duplication (WGD) of black-grass occurred millions of years ago, which was mainly followed by small scale local duplication events (Cai et al., 2023). WGD was also likely the force of soybean GST expansion (Liu et al., 2015), whereas in pink shepherd's-purse and oilseed rape, the force was the tandem gene duplication (Khan et al., 2018; Wei et al., 2019). Despite the duplication events black-grass is a diploid species, due to diploidization events (Li et al., 2021; Qiao et al., 2019; Wolfe, 2001), that contributed to its current diploid state. In general, it has been shown that GST genes in plants have mostly evolved by within-chromosome gene duplication and unequal crossing over (Frova, 2003). The

black-grass genome has also particularly been chiselled by transposable elements (TEs). TEs account for more than the 81.7% of the black-grass genome. Similar proportions of TEs have been found in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) with values more than the 80% (Mascher et al., 2017; Wicker et al., 2018). These can move around the genome, affecting gene transcription, causing mutations, and even altering regulatory networks.

The most abundant and expressed GST classes in black-grass are GSTU and GSTF, which are plant specific. The reason for this expansion, and that these GSTs have not been lost during the millions of years of evolution may be fitness advantages e.g. due to their major role in detoxification of xenobiotics and defence responses to abiotic stress (Benekos et al., 2010; Jha et al., 2011; Karavangeli et al., 2005; Loyall et al., 2000).

Despite the similarities between some GST proteins and their clustering events, it was shown in the present study that their 5' upstream regulatory regions (5'URR) and *cis*-regulatory elements (CREs) are dissimilar. Especially 5'URRs of duplicated genes varied, which is supported by other studies in plants, as for instance in soybean, where it was observed that the regulatory regions of duplicated genes were most prone to high mutational rates (H.-J. Liu et al., 2015). In oilseed rape (*Brassica napus* L.) duplicated GST genes were described to have divergent expression patterns (Wei et al., 2019). It is also known that some genes after duplication, gained or lost CREs located in their promoters, which might also be associated with subfunctionalization and neofunctionalization of the downstream genes (Arsovski et al., 2015; Force et al., 1999).

Despite the differences at the 5'URRs, three clustered GSTs belonging to class tau (GSTU13667-68-70; Chr3_CL2) shared some common elements. These elements were, however, absent or mutated in the other two non-differentially expressed clustered GSTUs of the same cluster (GSTU13665-66; Chr3_CL2). They were conserved putative motifs of the E2F/DP factors complex. The DNA motif was present twice, once in the forward strand (130 bp upstream of the TSS) and once in the reverse strand (67 bp downstream of the TSS). An electrophoretic mobility assay (EMSA) was conducted using dsDNA motifs of the promoter of GSTU13670 derived from the flufenacet susceptible (H) and resistant (K2) black-grass and a shift was observed when using the K2/1 and H1 biotinylated-dsDNA probes which was more intense in the susceptible (H1) motif. This suggests that the E2F/DP complex might repress the transcription of the GSTU13670 tested *in vivo*. It is already known that the E2F factor forms heterodimeric complexes with the DP affecting the transcription of downstream genes, either acting as activators or repressors (van den Heuvel & Dyson, 2008). Until now, it has never been described as an element on the promoter of a GST gene, but on the promoter of a UGT (Ramirez-Parra et al., 2004). Related studies on E2F/DP involvement in transcription of genes have been conducted in wheat and thale cress (Heckmann et al., 2011; Ramirez-Parra et al., 2007).

In this study, the activities of the highly upregulated GSTs found in flufenacet resistant black-grass populations were tested on flufenacet and several other pre- and post-emergent herbicides. The GST proteins were shown to detoxify flufenacet *in vitro* at moderate rates, indicating an additive effect and slow evolution of resistance. Different DEGs found between the two flufenacet resistant populations suggest divergent resistance evolution, implying different biotypes in the field, making their control more difficult. The fact that some proteins were not active on other substrates used in the field already suggests good herbicide alternatives and combinations for black-grass resistance management. The knowledge gained from the black-grass genome investigation shows the abundance of the GST genes and the duplication events they have undergone. Duplication of some genes, including GSTs, could be a plant power to overcome herbicide treatment and develop resistance to herbicides. Thus, controlling weeds by chemical means alone could trigger more duplication events in the genome and make resistance management more complex. In addition, it has been shown that protein similarity and chromosomal location of GST genes do not correspond to the same gene expression, which is most likely controlled by the promoter sequence itself. This means that resistance traits found in the DNA can be passed on to the next generation of individuals, resulting in even more resistant black-grass populations.

# 7. Summary

This study aimed to reveal and validate differentially expressed GST isoforms in flufenacet resistant black-grass populations and their gene regulation at transcriptional level. This was achieved by obtaining their coding sequence and testing them as recombinant proteins *in vitro* on flufenacet and other pre- and post-emergent herbicides important for black-grass control in Europe, leading into insights of resistance evolution and management. In addition, owing to a recently sequenced and assembled black-grass genome, the realignment of RNA-Seq data was feasible using a splice-aware aligner in order to identify more differentially GST and other genes related to herbicide resistance. Some of these genes were commonly found differentially expressed in both resistant compared to both susceptible populations, implying a convergent (parallel) resistance evolution, but also many unique genes to each of the resistant population, showing a non-parallel resistance evolution. In total, five GST genes, three tau and two phi class GSTs, were tested *in vitro* and all of them were able to metabolise the VLCFA synthesis-inhibitor flufenacet into flufenacet-GSH conjugate or in a unique case flufenacet-alcohol. All exhibited low detoxification rates and might have an additive effect *in planta* after flufenacet application, which can explain the slow resistance evolution of flufenacet, underlying a polygenic and generalist resistance. However, it cannot be ruled out that other GST encoding genes contribute to flufenacet detoxification. Moreover, all tested GSTs were able to detoxify the VLCFA-inhibitor acetochlor and the ACCase inhibitor fenoxaprop-ethyl and one GST detoxified the VLCFA-inhibitor pyroxasulfone. But the other herbicides of the same mode of action tested were not affected, highlighting the fact that metabolic resistance is complex and does not necessarily confer strong resistance to a wide spectrum of herbicides. Besides, it was demonstrated that the same enzyme can confer cross-resistance with other modes of action, while other active ingredients of the same mode of action or even same chemical class may not be affected. Moreover, the alternation between active ingredients has also been shown to play an important role in slowing down the development of resistance.

This study is the first genome-wide GST analysis of black-grass. It revealed 115 GST genes belonging to 11 different classes (GSTU, GSTF, GSTL, GSTZ, GSTT, DHAR, TCHQD, GHR, GSTH, MTX, mPGES2), which is a large number for a diploid species and a favourable condition for adaptation to repetitive herbicide treatments in modern agricultural systems. The most abundant and expressed classes were the tau and phi class GSTs which were typically found in clusters. The high number of GST genes and the clustering event is most likely a result of a WGD and tandem gene duplication, which is also influenced by TEs movement within the genome. However, the 5'URRs containing CREs found to vary strongly and to not follow any pattern according to location or gene expression. Moreover, the potential involvement of E2F/DP factors in three clustered GSTUs was described for the first time in a GST promoter. Overall differences in expression between GSTs were greater than between

resistant and susceptible individuals, and although genes in the same cluster often follow similar expression patterns, promoter sequences are likely to have a stronger effect on gene expression than gene location.

# 8. Outlook

This study provided biochemical validation of the activity of the highest differentially expressed GST isoforms of flufenacet resistant black-grass on flufenacet and other pre- and post-emergent herbicides in an *in vitro* based assay, demonstrating the detoxification pathways of flufenacet and cross-resistance patterns. This knowledge will lead to a better understanding of the evolution of flufenacet resistance and may contribute to a better and less resistance-favoured weed management system in the field. Finally, understanding the molecular mechanisms that induce flufenacet resistance may provide a basis for improving crop protection products and product mixtures.

Analysis of the binding complex derived from the electrophoretic mobility assay (EMSA) would be important to confirm that E2F/DP proteins are responsible for the shift or to propose a protein responsible for the shift.

Moreover, during the current study, samples from flufenacet susceptible and resistant black-grass populations were harvested. RNA and DNA from the same individuals were extracted and sent for RNA/microRNA and bisulfite sequencing. The upcoming results of those experiments can shed light on another perspective regarding GST expression, since microRNAs could also affect expression of GST genes at post-transcriptional level and as well, methylation of the GST promoter can be another significant factor affecting the expression of the GST genes at transcriptional level.

Eventually, it would be of interest to continue this research with bioinformatics-based approaches such as Weighted Correlation Network Analysis (WGCNA) (Langfelder & Horvath, 2008), in order to find co-expression patterns of genes coding for TFs and GSTs or for any other relevant proteins involved in herbicide resistance. In addition, the construction of gene regulatory networks (GRN) (Springer et al., 2019) would be an advantage in order to explain the interactions between TFs and their target genes. The above can help to map the signalling pathway of GST gene expression. A more targeted approach to reveal specific isoforms associated with flufenacet resistance will be a nascent RNA-Seq (Wissink et al., 2019). After application of flufenacet, samples could be harvested and examined to see which transcripts are immediately produced. This will reveal the direct produced transcripts and not the steady state, giving useful hints for which isoforms are triggered due to flufenacet pressure. This will provide knowledge on top of the current analysis related to constitutive GST upregulation. A parallel high-throughput chromosome conformation capture (Hi-C) technique (Belton et al., 2012) to find evidence for the distance between genes and CREs, will provide evidence for the involvement of enhancers and silencers in the expression of downstream GST genes.

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# 10. Annex

## 10.1 Supporting information of Paper §4

EmGFP

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTG
GACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCC
ACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCT
GGCCCACCCTCGTGACCACCTTGACCTACGGCGTGCAGTGCTTCGCCCGCTACCCCGA
CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAG
CGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCG
AGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACG
GCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAAGGTCTATATCACC
GCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGACCCGCCACAACATCGAGG
ACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCC
CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCC
CAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACT
CTCGGCATGGACGAGCTGTACAAGTAA

ALOMY3G13667

ATGGCCGCGGGAGATGACCTGAAGCTGCTCGGCGCATGGGCAAGTCCGTTTGTCACCA
GGGTGAAGCTTGCACTCAGCTTCAAGGGCCTGAGCTTTAAAGACGTCGAGGAGGACCT
CGGTAACAAGAGCGAGCTCCTTCTCAGCTCCAACCCGGTGCACAAGAAGGTGCCCGTG
CTCCTCCACAACGGGAAGCCCATCTGCGAGTCCGTGGTCATCGTGCAGTACATCGACG
AGGCATTCGCCGGCACCGGCCCCTCCTTGCTTTCCGATGATCCCCATGAACGTGCCGT
TGCGCGCTTCTGGGCCGCCTACATTGACGACAAGCTTGTCGCCCCGTGGATACAGTCG
TTCAGGGGCAAGACAGAAGGGGAGAAGTCCGAGGGGATTAAGCAGACGTTTGCTGCAG
CGGAGACCTTGGAGGGTGCCCTCAGGGAGTGCTCCAAGGGGCAGGGCTACTTCGGCG
GCGAGAGCGTTGGTCTCGTGGACGTTTCGCTGGGGAGCCTCCTCTCCTGGCTGAAAGC
GACGGAGGTGATGTCCGGGGCCAACATCTTTGACCCTGTTAAGACTCCGCTCCTAGCG
GCATGGGTGGAGCGCTTCACTGAGCTTGATGCCGCCAAGGCGGCCTTGCCGGATGTC
GACAGGGTGGTCGAGTTCGCCAAGAAGAGGCAGGCACAGGCTGCCGCGGCCGCTGCC
GCTTTGGAGAACAAGTAA

ALOMY3G13668

ATGGACGCAGGAGATGACCTGAAGCTGCTCGGCGTATGGGCAAGTGCGTTTGTCATCA
GGGTGAAACTTGCACTGAGCTTCAAGGGGCTGAGCTTCGAGAACGTCGAGGAGGACCT
CGGCAACAAGAGCGAGCTCCTCCTCAGCTCCAACCCGGTGCACAAGAAGGTGCCCGTG

CTCCTCCACAACGGGAAGCCCATCTGTGACTCCATGGTCATCGTGCAGTACATCGACGA
GGCATTCTCCGGCACTGGCCCCTCCTTGCTTTCCTCCGACCCCCATGAGCGTGCCATT
GCTCGCTTCTGGGCCGCCTACATCGACGACAAGCTTATCGTCGCATGGAGGCAGGCGT
TCAGCAGCAAGACAGAGGAGGACAAGTCTGAGGGGACTAAGCAGATGTTTGCTGCACT
GGATACTTTGGAGGGAGCCTTGAGGGAGTGCTCCAAGGGGCATGGATACTTTGGAGGT
GAGAGCGTCGGACTCGTGGACGTATGGCTGGGGAGCCTGCTCTCCTGGCTGAAAGCG
AGCGCGGTGAACTCCGGGATCAAGATATTTGACCCCATTAAGACTCCCCTCCTGACGG
CATGGGTGGAGCGCTTCAGTGAGCTTGACGGCGCCAAGGCGGCCTTGCCGGACGTCG
ACAGGGTGATCGAGTTTGGAAAGATGAAGAAGTTATTATAG

ALOMY3G13668m

ATGGACGCAGGAGATGACCTGAAGCTGCTCGGCGTATGGGCAAGTGCGTTTGTCATCA
GGGTGAAACTTGCACTGAGCTTCAAGGGGCTGAGCTTCGAGAACGTCGAGGAGGACCT
CGGCAACAAGAGCGAGCTCCTCCTCAGCTCCAACCCGGTGCACAAGAAGGTGCCCGTG
CTCCTCCACAACGGGAAGCCCATCTGTGAGTCCATGGTCATCGTGCAGTACATCGACG
AGGCATTCTCCGGCACTGGCCCCTCCTTGCTTTCCTCCGACCCCCATGAGCGTGCCATT
GCTCGCTTCTGGGCCGCCTACATCGACGACAAGCTTATCGTCGCATGGAGGCAGGCGT
TCAGCAGCAAGACAGAGGAGGACAAGTCTGAGGGGACTAAGCAGATGTTTGCTGCACT
GGATACTTTGGAGGGAGCCTTGAGGGAGTGCTCCAAGGGGCATGGATACTTTGGAGGT
GAGAGCGTCGGACTCGTGGACGTATGGCTGGGGAGCCTGCTCTCCTGGCTGAAAGCG
AGCGCGGTGAACTCCGGGATCAAGATATTTGACCCCATTAAGACTCCCCTCCTGACGG
CATGGGTGGAGCGCTTCAGTGAGCTTGACGGCGCCAAGGCGGCCTTGCCGGACGTCG
ACAGGGTGATCGAGTTTGGAAAGATGAAGAAGTTATTATAG

ALOMY3G13670

ATGGCCGCAGGAGATGACCTGAAGCTGCTCGGCGTATGGGCAAGTCCGTTTGTCAGCA
GGGTGAAACTCGCACTGAGCTTCAAGGGCCTGAGCTTCGAGAACGTGGAGGAGGACCT
CGGCAACAAGAGCGAGCTCCTCCTCAGCTCCAACCCGGTGCACAAGAAGGTGCCCGTG
CTCCTCCACAATGGGAAGCCCATATGTGAGTCCGTGGTCATCGTGCAGTACATCGACGA
GGCATTCGCCGGCACTGGCCCCTCGTTGTTTTCCTCCGACCCCTATGACCGTGCCATT
GCCCGCTTCTGGGCCGCCTACGTCGACGACAAGCTTCTCGTCGCGTGGAGGCAGGCG
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TGGATACTTTGGAGGGAGCCTTGAGGGAGTGCTCCAAGGGGAAGGGATACTTTGGAGG
CGAGAGCGTCGGATTCGTGGACGTTTCGCTGGGGAGCCTGCTCTCCTGGCTGAAAGCG
AGCGCTGTGCACTCTGGGATCAAGATATTTGACCCCATTAAGACTCCCCTCTTGGCGGC
ATGGGTGGAGCGCTTCAGTGAGCTTGACGGCGCCAAGGCGACCTTACCGGATGTCGAC
AGGTTGGTCGAGTTTGCGAAGATGAGGAAGTTATTATAG

ALOMY5G35766

ATGGCGCCGGCTGTGAAGGTGTACGGGTGGGCGATGTCCCCGTACGTGGCGCGCGCG
CTGCTGTGCCTGGAGGAGGCCGGCGTCGAGTACGAGCTCGTGGCCATGAACCCCGAG
GCCGGCGATCACCTCCGCGCCGACTTCCTCGCCAAGAACCCCTTCGCTCAGGTCCCTG
TCCTCGAGGACGGCGACCTCACCCTCTTTGAGTCGCGCGCGATCGCGAGGCACGTGCT
GCGGAAGTACAAGCCGGAGCTGCTGGCGGGCGACGGCTCGCCGGAGGCGGCCGCGA
TGGTGGACGTCTGGATGGAGGTGGAGGCGCAGCAGCACCACGCCCCGACGGGCGCC
ATCATGATACAGTGCATCGTCGTCCCGCTCCGTGGCGGCGTGCGCGACCAGGGCGTC
GTCGACGAGAACGTCGCCAAGCTGAGGAAGGTGCTGGAGGTGTACGAGGCGCGGCTC
TCGGCGTCGAGGTACCTCGCCGGGGAATCGCTCACCCTCGCCGACCTCAGCCACTTCC
CCATGATGCGCTACTTCATGGACACCGAGTACGCGGCGCTGGTGGAGGAGCTCCCGCA
CGTGAAGGCGTGGTGGGAGGAGCTCAAGGCCAGGCCCGCTGCGAGGAAGGTCACGG
AGATCGGAGTTCACGCCGCCGAAGTTTGGGCTCGGAAAAAGGCTGAGCAGCAGTGA

ALOMY3G11300 (GenBank: AJ010453.1, *Alopecurus myosuroides* mRNA for glutathione transferase 2c)

ATGGCGCCGGTGAAGGTGTTCGGGCCGGCCATGTCGACGAACGTGGCGCGGGTGACT
CTCTGCCTGGAGGAGGTGGGCGCCGAGTACGAGGTGGTGAACATCGACTTCAACACCA
TGGAGCACAAGAGCCCCGAGCACCTCGCCAGAAACCCGTTCGGGCAAATCCCTGCTTT
CCAGGACGGGGATCTGCTTCTTTGGGAGTCCCGCGCGATCTCCAAATACGTGCTCCGA
AAATACAAGACGGACGAGGTCGACCTCCTGAGGGAGAGCAACCTGGAGGAGGCGGCG
ATGGTGGATGTGTGGACGGAGGTGGACGCCCACACCTACAACCCGGCGCTGTCCCCC
ATCGTGTACCAGTGCCTTTTCAACCCGATGATGCGTGGCCTCCCCACCGATGAGAAGGT
CGTCGCCGAGAGCCTGGAGAAGCTGAAGAAGGTGCTGGAGGTGTACGAGGCTCGCCT
CTCAAAGCACAGCTACCTGGCCGGGGACTTCGTCAGCTTCGCGGACCTCAACCACTTC
CCATACACCTTCTACTTCATGGCGACGCCCCATGCCGCGCTCTTCGACTCGTACCCGCA
CGTCAAGGCCTGGTGGGACCGCCTCATGGCCAGGCCTGCCGTCAAGAAGATCGCCGC
CACCATGGTTCCGCCCAAGGCGTGA

Figure S1**.** Nucleotide sequences: Full length cDNA of the genes EmGFP, ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m, ALOMY3G13670, ALOMY5G35766, ALOMY3G11300.

EmGFP

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLV
TTLTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVN

RIELKGIDFKEDGNILGHKLEYNYNSHKVYITADKQKNGIKVNFKTRHNIEDGSVQLADHYQQ
NTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

ALOMY3G13667

MAAGDDLKLLGAWASPFVTRVKLALSFKGLSFKDVEEDLGNKSELLLSSNPVHKKVPVLLH
NGKPICESVVIVQYIDEAFAGTGPSLLSDDPHERAVARFWAAYIDDKLVAPWIQSFRGKTEG
EKSEGIKQTFAAAETLEGALRECSKGQGYFGGESVGLVDVSLGSLLSWLKATEVMSGANIF
DPVKTPLLAAWVERFTELDAAKAALPDVDRVVEFAKKRQAQAAAAAAAALENK

ALOMY3G13668

MDAGDDLKLLGVWASAFVIRVKLALSFKGLSFENVEEDLGNKSELLLSSNPVHKKVPVLLHN
GKPICDSMVIVQYIDEAFSGTGPSLLSSDPHERAIARFWAAYIDDKLIVAWRQAFSSKTEEDK
SEGTKQMFAALDTLEGALRECSKGHGYFGGESVGLVDVWLGSLLSWLKASAVNSGIKIFDPI
KTPLLTAWVERFSELDGAKAALPDVDRVIEFGKMKKLL

ALOMY3G13668m

MDAGDDLKLLGVWASAFVIRVKLALSFKGLSFENVEEDLGNKSELLLSSNPVHKKVPVLLHN
GKPICESMVIVQYIDEAFSGTGPSLLSSDPHERAIARFWAAYIDDKLIVAWRQAFSSKTEEDK
SEGTKQMFAALDTLEGALRECSKGHGYFGGESVGLVDVWLGSLLSWLKASAVNSGIKIFDPI
KTPLLTAWVERFSELDGAKAALPDVDRVIEFGKMKKLL

ALOMY3G13670

MAAGDDLKLLGVWASPFVSRVKLALSFKGLSFENVEEDLGNKSELLLSSNPVHKKVPVLLH
NGKPICESVVIVQYIDEAFAGTGPSLFSSDPYDRAIARFWAAYVDDKLLVAWRQAFRGKTEE
EKSEGTKQMFAALDTLEGALRECSKGKGYFGGESVGFVDVSLGSLLSWLKASAVHSGIKIF
DPIKTPLLAAWVERFSELDGAKATLPDVDRLVEFAKMRKLL

ALOMY5G35766

MAPAVKVYGWAMSPYVARALLCLEEAGVEYELVAMNPEAGDHLRADFLAKNPFAQVPVLE
DGDLTLFESRAIARHVLRKYKPELLAGDGSPEAAAMVDVWMEVEAQQHHAPTGAIMIQCIVV
PLRGGVRDQGVVDENVAKLRKVLEVYEARLSASRYLAGESLTLADLSHFPMMRYFMDTEY
AALVEELPHVKAWWEELKARPAARKVTEIGVHAAEVWARKKAEQQ

ALOMY3G11300 (https://www.uniprot.org/uniprot/Q9ZS17)

MAPVKVFGPAMSTNVARVTLCLEEVGAEYEVVNIDFNTMEHKSPEHLARNPFGQIPAFQDG
DLLLWESRAISKYVLRKYKTDEVDLLRESNLEEAAMVDVWTEVDAHTYNPALSPIVYQCLFN
PMMRGLPTDEKVVAESLEKLKKVLEVYEARLSKHSYLAGDFVSFADLNHFPYTFYFMATPH
AALFDSYPHVKAWWDRLMARPAVKKIAATMVPPKA

Figure S2. Protein sequences: Sequences of the recombinant proteins EmGFP, ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m, ALOMY3G13670, ALOMY5G35766, ALOMY3G11300.

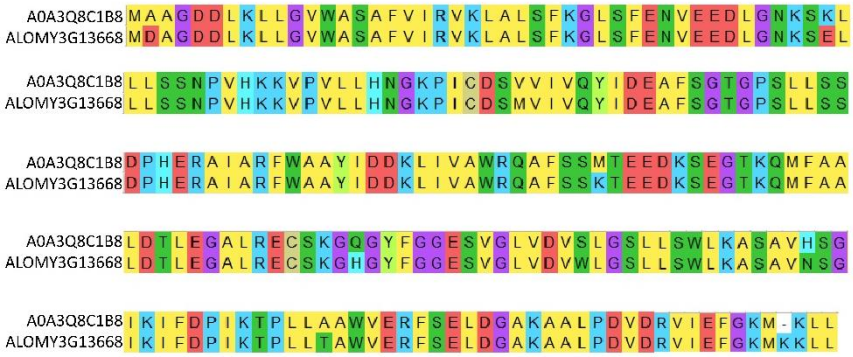


Figure S3. Alignment of ALOMY3G13668 and GST2 (*Alopecurus myosuroides* Huds. GSTU2, A0A3Q8C1B8, https://alphafold.ebi.ac.uk/entry/A0A3Q8C1B8). The alignment was produced by using MEGA11 (11.0.13 version).

Table S1. CDNB activity of recombinant EmGFP and GST proteins based on the Beer-Lambert law [activity = ($\Delta A_{340}$ x $min^{-1}$ / ε x l) x ($V_{reaction}$ / $V_{sample}$)].

| Protein | Activity (nmol x $min^{-1}$ x $mL^{-1}$) |
|---|---|
| EmGFP | 24 |
| ALOMY3G13667 | 398 |
| ALOMY3G13668 | 438 |
| ALOMY3G13668m | 476 |
| ALOMY3G13670 | 402 |
| ALOMY5G35766 | 174 |
| ALOMY3G11300 | 360 |

Table S2. Herbicides used for the study (listed alphabetically).

| Herbicide | Supplier company |
|---|---|
| acetochlor (100 mg) | Supelco, Merck, Darmstadt, Germany |
| cinmethylin (50 mg) | Supelco, Merck, Darmstadt, Germany |
| clodinafop-acid | product of [phenyl ring-U-$^{14}$C] clodinafop-propargyl† |
| [phenyl ring-U-$^{14}$C] clodinafop-propargyl | Institute of Isotopes Co., Ltd., Budapest, Hungary |
| [difluorophenyl-UL-$^{14}$C] diflufenican | Bayer CropScience AG, Isotope Chemistry, Wuppertal, Germany |

| | |
|---|---|
| fenoxaprop-acid | product of [phenoxy-UL-$^{14}$C] fenoxaprop-P-ethyl† |
| [phenoxy-UL-$^{14}$C] fenoxaprop-P-ethyl | Bayer CropScience AG, Isotope Chemistry, Wuppertal, Germany |
| [phenyl-UL-$^{14}$C] flufenacet | Bayer CropScience AG, Isotope Chemistry, Wuppertal, Germany |
| [pyrimidyl-2-$^{14}$C] mesosulfuron-methyl | Bayer CropScience AG, Isotope Chemistry, Wuppertal, Germany |
| metolachlor, *S*-[phenyl ring-$^{14}$C(U)] | American Radiolabeled Chemicals, Inc., St. Louis, MO 63146, USA via Biotrend, Köln, Germany |
| pendimethalin (100 mg) | Supelco, Merck, Darmstadt, Germany |
| prosulfocarb (250 mg) | Supelco, Merck, Darmstadt, Germany |
| pyroxasulfone (1 g) | Combi-Blocks, Inc., San Diego, CA, USA |

†Esterase from porcine liver (7.91 units; Sigma-Aldrich, Darmstadt, Germany) was added to a final concentration of 50 µM of $^{14}$C-clodinafop-propargyl and $^{14}$C-fenoxaprop-P-ethyl respectively and incubated for 30 min (room temperature) in the reaction buffer.

Table S3**.** Gradient of Liquid Chromatography (XLC Jasco) for (a) flufenacet, (b) fenoxaprop-ethyl, fenoxaprop-acid, S-metolachlor, acetochlor, pyroxasulfone, prosulfocarb, cinmethylin, pendimethalin, (c) mesosulfuron-methyl and (d) clodinafop-propargyl, clodinafop-acid

| (a) | Time (min) | A [water, 0.05 formic acid (%)] | B [acetonitrile, 0.05 formic acid (%)] |
|---|---|---|---|
| | 1.0 | 90 | 10 |
| | 1.5 | 50 | 50 |
| | 7.5 | 20 | 80 |
| | 8.10 | 0 | 100 |
| | 9.5 | 0 | 100 |
| | 10.0 | 90 | 10 |
| | 12.0 | 90 | 10 |

| (b) | Time (min) | A [water, 0.05 formic acid (%)] | B [acetonitrile, 0.05 formic acid (%)] |
|---|---|---|---|
| | 1.0 | 95 | 5 |
| | 5.0 | 95 | 5 |
| | 6.5 | 25 | 75 |
| | 13.0 | 0 | 100 |
| | 14.5 | 0 | 100 |
| | 15.0 | 95 | 5 |
| | 17.0 | 95 | 5 |

| (c) | Time (min) | A [water, 0.05 formic acid (%)] | B [acetonitrile, 0.05 formic acid (%)] |
|---|---|---|---|
| | 1.0 | 80 | 20 |
| | 8.0 | 0 | 100 |
| | 9.5 | 0 | 100 |
| | 10.0 | 80 | 20 |
| | 12.0 | 80 | 20 |

| (d) | Time (min) | A [water, 0.05 formic acid (%)] | B [acetonitrile, 0.05 formic acid (%)] |
|---|---|---|---|
| | 1.0 | 80 | 20 |
| | 1.5 | 25 | 75 |
| | 18.0 | 0 | 100 |
| | 19.5 | 0 | 100 |
| | 20.0 | 80 | 20 |
| | 22.0 | 80 | 20 |

Table S4. Gradient of Liquid Chromatography - Mass Chromatography for (a) flufenacet and (b) fenoxaprop-ethyl and fenoxaprop-acid

| (a) | Time (min) | A [water, 0.2 formic acid (%)] | B [acetonitrile, 0.2 formic acid (%)] |
|---|---|---|---|
| | 0.0 | 90 | 10 |
| | 1.0 | 90 | 10 |
| | 1.5 | 50 | 50 |
| | 7.5 | 20 | 80 |
| | 8.0 | 10 | 90 |
| | 10.0 | 10 | 90 |
| | 10.5 | 90 | 10 |
| | 15.0 | 90 | 10 |

| (b) | Time (min) | A [water, 0.2 formic acid (%)] | B [acetonitrile, 0.2 formic acid (%)] |
|---|---|---|---|
| | 0.0 | 95 | 5 |
| | 5.0 | 95 | 5 |
| | 6.5 | 25 | 75 |
| | 13.0 | 5 | 95 |
| | 14.5 | 5 | 95 |
| | 15.0 | 95 | 5 |
| | 20.0 | 95 | 5 |

Table S5. Sequence identity of tau (U) class ALOMY3G13667, ALOMY3G13668, ALOMY3G13668m and ALOMY3G13670 proteins using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)

| Proteins | Sequence identity (%) | | | |
|---|---|---|---|---|
| | ALOMY3G13667 | ALOMY3G13670 | ALOMY3G13668 | ALOMY3G13668m |
| ALOMY3G13667 | 100.00 | 84.00 | 81.33 | 81.78 |
| ALOMY3G13670 | 84.00 | 100.00 | 89.33 | 89.78 |
| ALOMY3G13668 | 81.33 | 89.33 | 100.00 | 99.56 |
| ALOMY3G13668m | 81.78 | 89.78 | 99.56 | 100.00 |

## 10.2 Supporting information of Paper §5

Table S1. Oligonucleotides used for annealing in order to create dsDNA for the sake of the electrophoretic mobility shift assay (EMSA). The scale synthesis of them was 50 nmol and their purification was performed under HPLC. The oligonucleotides were synthesised by the Thermo Fisher Scientific in the UK.

| Number | Oligonucleotides | Sequence (5´-3´) | dsDNA |
|---|---|---|---|
| 1 | biotinylated_ALOMYR1-FWD | TTGATCATTTCCTGCCAACCGA | K2 motif1 |
| 2 | biotinylated_ALOMYR1-REV | TCGGTTGGCAGGAAATGATCAA | |
| 3 | biotinylated_ALOMYS1-FWD | TTGATCATTTCCTGCCTCCCAA | H motif1 |
| 4 | biotinylated_ALOMYS1-REV | TTGGGAGGCAGGAAATGATCAA | |
| 5 | biotinylated_ALOMYR2-FWD | GCATCCAGCAGGAAACCATGGC | K2 motif2 |
| 6 | biotinylated_ALOMYR2-REV | GCCATGGTTTCCTGCTGGATGC | |
| 7 | biotinylated_ALOMYS2-FWD | GATGTAAGCAGGAAATCATGGC | H motif2 |
| 8 | biotinylated_ALOMYS2-REV | GCCATGATTTCCTGCTTACATC | |
| 9 | unbiotinylated _ALOMYR1-FWD | TTGATCATTTCCTGCCAACCGA | K2 motif1 |
| 10 | unbiotinylated _ALOMYR1-REV | TCGGTTGGCAGGAAATGATCAA | |
| 11 | unbiotinylated _ALOMYS1-FWD | TTGATCATTTCCTGCCTCCCAA | H motif1 |
| 12 | unbiotinylated _ALOMYS1-REV | TTGGGAGGCAGGAAATGATCAA | |
| 13 | unbiotinylated _ALOMYR2-FWD | GCATCCAGCAGGAAACCATGGC | K2 motif2 |
| 14 | unbiotinylated _ALOMYR2-REV | GCCATGGTTTCCTGCTGGATGC | |
| 15 | unbiotinylated _ALOMYS2-FWD | GATGTAAGCAGGAAATCATGGC | H motif2 |
| 16 | unbiotinylated _ALOMYS2-REV | GCCATGATTTCCTGCTTACATC | |
| 17 | biotinylated_ALOMY45FWD | GCGACTTGCTCAATACTCATTC | scrambled |
| 18 | biotinylated_ALOMY45REV | GAATGAGTATTGAGCAAGTCGC | K2 motif1, H motif1, H motif2 |
| 19 | biotinylated_ALOMY59FWD | CAGCCTAGTCGACAGAGAGCAC | scrambled |

| | | | |
|---|---|---|---|
| 20 | biotinylated_ALOMY59REV | GTGCTCTCTGTCGACTAGGCTG | K2 motif2 |

Table S2. Genes annotated as putative glutathione transferases (GSTs) assigned to families resembling the glutathione transferase structure, such as two repeated N-terminal thioredoxin domain (GST2N), aminoacyl-tRNA synthetase (aaRS) and aminoacyl-tRNA synthetase cofactor I (Arc1p).

| Gene | GST class |
|---|---|
| ALOMY1G05444 | GST2N |
| ALOMY2G22464 | aaRS |
| ALOMY3G13453 | GST2N |
| ALOMY5G32826 | aaRS |
| ALOMY6G44955 | Arc1p |
| ALOMY7G38121 | Arc1p |

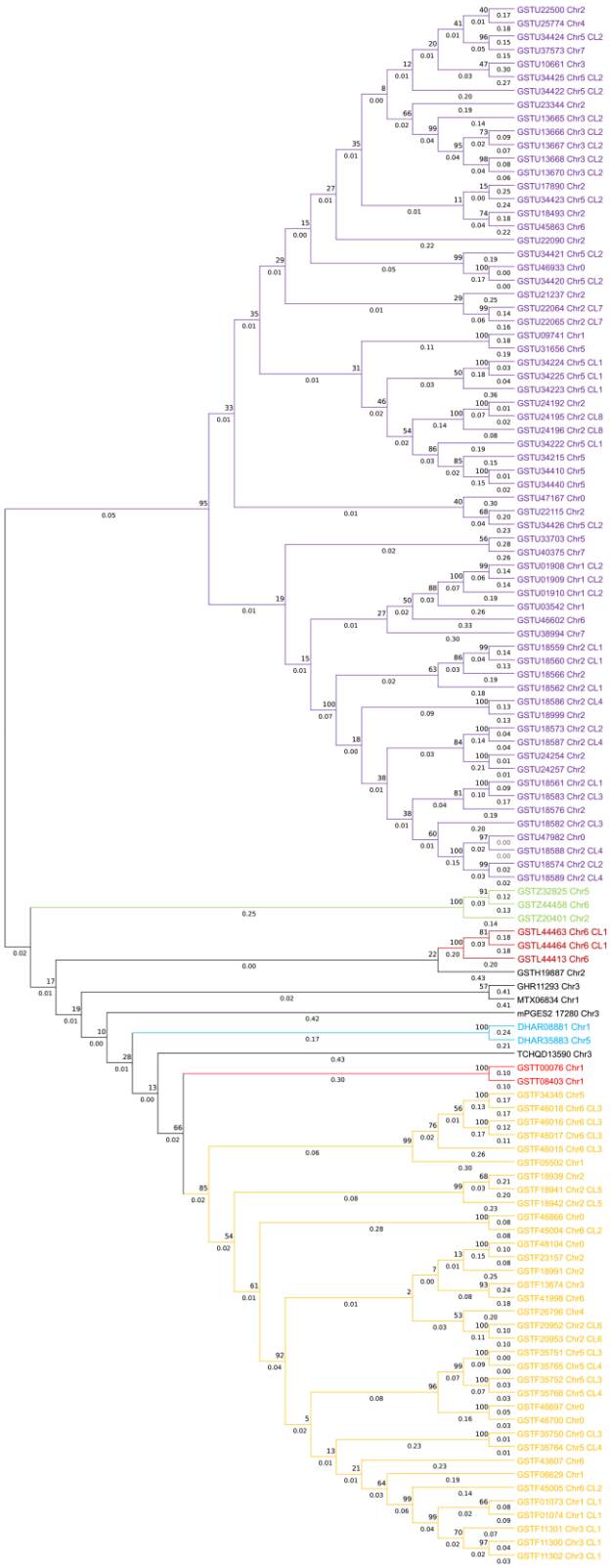


Figure S1. Vertical phylogenetic tree of glutathione transferase (GST) proteins found in the black-grass genome (Cai et al., 2023). The analysis was preformed using the neighbor-joining (NJ) method with 1000 bootstrap replicates and the p-distance model in the MEGA11 software (11.0.13 version; Tamura et al., 2021) The numbers on the nodes indicate the bootstrap values and the numbers on the branches the residues corresponding to protein dissimilarity.

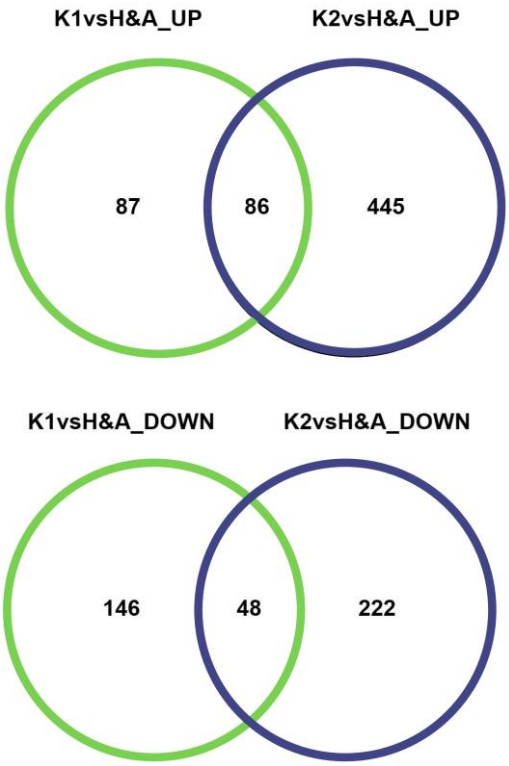


Figure S2. Venn diagrams of the commonly significantly upregulated (UP) and downregulated (DOWN) genes in K1 and K2 compared to H and A created using Venny 2.1.0.(Oliveros, 2007)

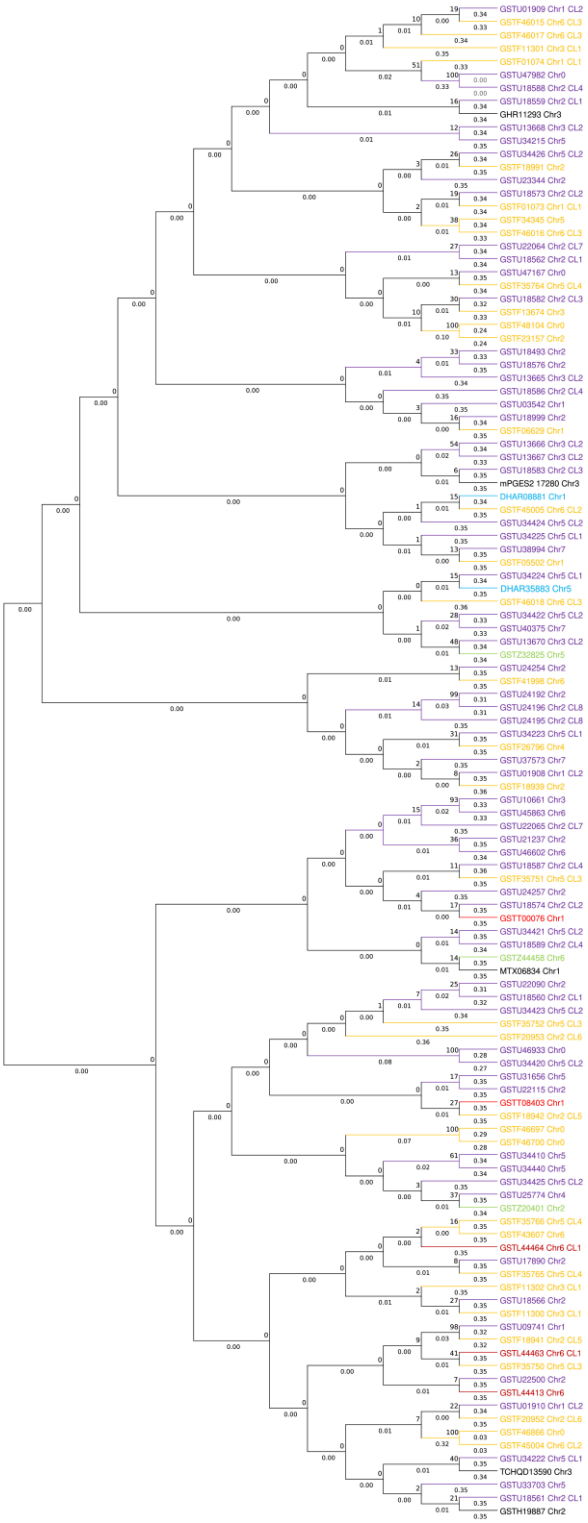


Figure S3. Vertical phylogenetic tree of the 2kb 5´ upstream regulatory region of glutathione transferase (GST) genes found in the black-grass genome (Cai et al., 2023). The analysis was preformed using the neighbor-joining (NJ) method with 1000 bootstrap replicates and the p-distance model in the MEGA11 software (11.0.13 version; Tamura et al., 2021). The numbers on the nodes indicate the bootstrap values and the numbers on the branches the percentage corresponding to nucleotide dissimilarity.

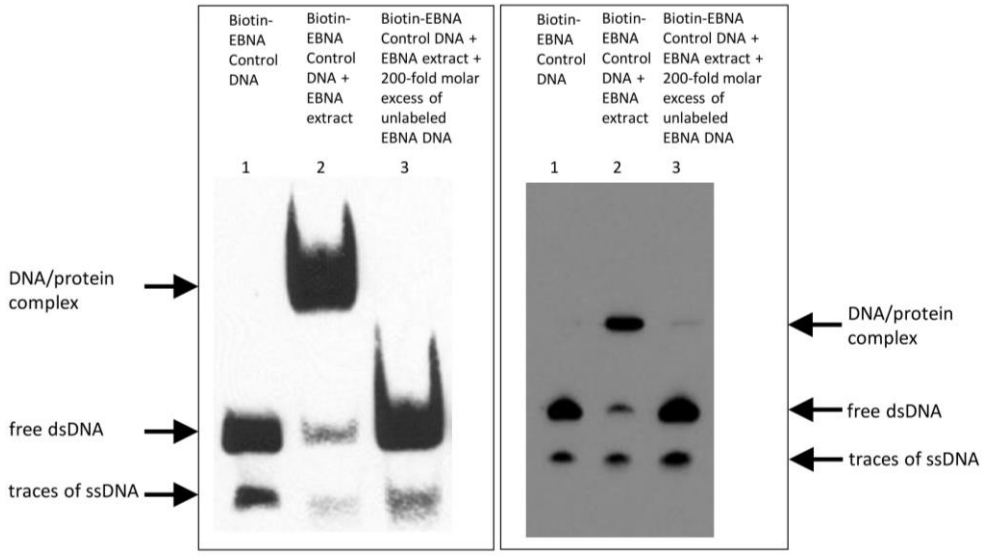


Figure S4. Control nylon H+ membranes of electrophoretic mobility assay (EMSA) visualised by chemiluminescence. Reactions of the control using the LightShift® Chemiluminescent EMSA kit data, as shown in the user manual (left side) and as they were found in the laboratory (right side).


Figure S5. *In silico* analysis of transcription factor binding sites on the 2kb 5´ upstream regulatory region of glutathione transferase (GST) genes found in the black-grass genome (Cai et al., 2023), preformed using the „Binding Site Prediction" tool of PlantRegMap (Jin et al., 2017; Tian et al., 2020).

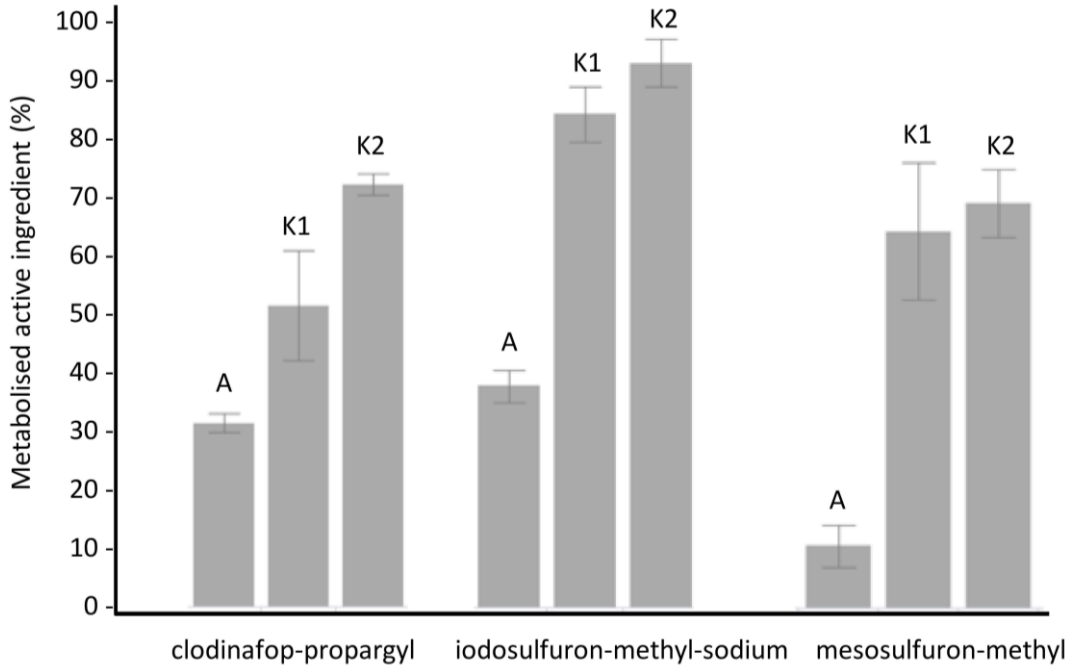


Figure S6. *In vivo* metabolism of active ingredients (clodinafop-propargyl, iodosulfuron-methyl-sodium, mesosulfuron-methyl) used in post-emergent herbicides products, by the sensitive population A, and the flufenacet resistant populations K1 and K2.

## 11. Acknowledgements

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## 12. *Curriculum vitae*

### PERSONAL DATA

| | |
|---|---|
| Name: | Evlampia Parcharidou |
| Email: | evlampia.parcharidou@gmail.com |
| Born: | 05.07.1993, in Thessaloniki |

### PROFESSIONAL EXPERIENCE

04/2023 – today

**Research Assistant / Lab Manager**
Institute of Molecular Biology, Mainz

07/2019 – 02/2024

**PhD Candidate at Georg-August University Göttingen / Bayer AG, CropScience Division**
Title: “The role of glutathione transferases in herbicide detoxification – a genome-wide study on flufenacet resistant black-grass”

### PUBLICATIONS

2024

**Genome-wide study of glutathione transferases and their regulation in flufenacet susceptible and resistant black-grass (*Alopecurus myosuroides* Huds.)**
Parcharidou et al.

2023

**Recombinant glutathione transferases from flufenacet-resistant black-grass (*Alopecurus myosuroides* Huds.) form different flufenacet metabolites and differ in their interaction with pre- and post-emergence herbicides**
Parcharidou et al.

2020

**Flufenacet activity is affected by GST inhibitors in blackgrass (*Alopecurus myosuroides*) populations with reduced flufenacet sensitivity and higher expression levels of GSTs**
Dücker et al.

2019

**Enhanced metabolism causes reduced flufenacet sensitivity in black-grass (*Alopecurus myosuroides* Huds.) field populations**
Dücker et al.

Mainz, 25.03.2024

# 13. Declarations

Declaration by the doctoral candidate

**at the Georg-August-Universität Göttingen**

Parcharidou, Evlampia
Mainz, Germany

I intend to produce a dissertation on the topic of
„The role of glutathione transferases in herbicide detoxification - a genome-wide study on flufenacet resistant black-grass“ at Georg-August-Universität Göttingen.

In this, I shall be supervised by Prof. Andreas von Tiedemann.
I submit the following declaration:
1. The opportunity for the existing doctoral project was not made commercially available to me. Especially, I have not engaged any organisation that seeks thesis advisers against a fee for the preparation of dissertations or performs my obligations with respect to examination components entirely or partly.
2. I have until now and shall in future accept the assistance of third parties only in a scope that is scientifically justifiable and compliant with the legal statutes of the examinations. I shall specifically complete all parts of the dissertation myself; for contributions of shared authorship, the parts created by me are/will be marked separately; I have neither, nor will I, accept unauthorised outside assistance either free of charge or subject to a fee.
3. The regulations to ensure good scientific practice at the University of Göttingen are observed by me.
4. A corresponding doctorate has not been applied for at any other university in Germany or abroad; the submitted dissertation or parts of it have not been/will not be used for another doctoral project. Otherwise, I have provided corresponding information on the topic, period, university and supervisor. Furthermore, I am aware of the fact that untruthfulness with respect to the above declaration repeals the admission to complete the doctoral studies and/or subsequently entitle termination of the doctoral process or withdrawal of the title attained.

Mainz, 25.03.2024