Investigation of glutathione transferases at the gene regulation level in flufenacet resistant black-grass populations (*Alopecurus myosuroides* Huds.)

Untersuchung von Glutathiontransferasen auf Genregulationsebene in Flufenacet-resistenten Acker-Fuchsschwanzpopulationen (Alopecurus myosuroides Huds.)

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Abstract

The herbicide flufenacet, applied in pre-emergence, acts as an inhibitor of the synthesis of very long-chain fatty acids (HRAC group 15) and is an important component in the control of grasses in winter cereals, especially when resistance to other herbicide groups already exists. However, decreases in sensitivity due to increased flufenacet metabolism by glutathione transferase activity have already been described in some black-grass (*Alopecurus myosuroides* Huds.) populations. So far, little is known about the mechanisms of gene regulation in metabolically resistant weeds. Therefore, we aligned RNA-seq data from two sensitive black-grass populations and two black-grass populations with reduced sensitivity to flufenacet against the recently sequenced black-grass genome. In a differential gene expression analysis, an upregulation of genes involved in metabolic detoxification pathways, such as cytochrome P450 monooxygenases (CYPs), glycosyltransferases (GTs), glutathione transferases (GSTs) and ATP-binding cassette (ABC) transporters was observed. It was found that 7% of the GST genes in the two populations with reduced flufenacet sensitivity were significantly upregulated even without any herbicide application (constitutively) when compared to the two sensitive populations. Three of these genes are located next to each other on the third chromosome and represent a cluster. For each of the upregulated GST genes 3,5 kb of the upstream promoter region were investigated in silico for potential transcription factor binding sites (TFBSs). Some of them share common upstream motifs, such as an *ocs* element, previously described as enhancer element for GSTs. In addition, other motifs could act as potential binding sites for transcription factors (TFs) that were found upregulated. These belonged to various classes including WRKYs, basic helix-loop-helices (bHLH), basic leucine zippers (bZIP) or MADS-boxes. A better understanding of the regulation of resistance-associated genes can contribute to improve diagnosis of herbicide resistance and help in predicting the evolution of cross-resistance, as well as contribute to the search for new active ingredients.

Keywords: metabolic resistance, glutathione transferase, transcription factor binding site, flufenacet, black-grass

Zusammenfassung

Das Vorauflaufherbizid Flufenacet wirkt als Hemmer der Synthese sehr langkettiger Fettsäuren (HRAC-Gruppe 15) und ist ein wichtiger Baustein in der Bekämpfung von Ungräsern in Wintergetreide, v.a. wenn bereits Resistenzen gegen andere (Nachauflauf-) Herbizidgruppen vorliegen. Jedoch wurden bereits bei einigen Acker-Fuchsschwanzpopulationen (*Alopecurus myosuroides* Huds.) Sensitivitätsunterschiede aufgrund von erhöhten Flufenacetabbauraten durch Glutationtransferaseaktivität beschrieben. Bisher sind die Mechanismen der Genregulation bei metabolisch resistenten Unkrautern kaum bekannt. In unserer Studie wurden daher RNA-Seq-Daten von zwei sensitiven Acker-Fuchsschwanzpopulationen und zwei

Acker-Fuchsschwanzpopulationen mit verminderter Sensitivität gegenüber Flufenacet gegen das kürzlich sequenzierte Acker-Fuchsschwanzgenom aligniert. In einer differenziellen Genexpressionsanalyse wurde eine Hochregulation von Genen beobachtet, die an metabolischen Detoxifizierungsprozessen beteiligt sind, wie Cytochrom-P450-Enzyme (CYPs), Glycosyltransferasen (GTs), Glutathiontransferasen (GSTs) und ATP-bindende Kassette (ABC)-Transportern. Es wurde festgestellt, dass 7 % der GST-Gene in den beiden Populationen mit verminderter Flufenacet-Sensitivität im Vergleich zu den beiden Sensitiven konstitutiv (ohne vorherige Herbizid-Applikation) signifikant hochreguliert waren. Drei dieser Gene sind auf dem dritten Chromosom nebeneinander lokalisiert und bilden ein Gen-Cluster. Jeweils 3,5 kb der vor dem Transkriptionsstartpunkt gelegenen Promoterregionen der hochregulierten GST-Gene wurden in silico auf potenzielle Transkriptionsfaktor-Bindungsstellen (TFBSs) untersucht. Einige von ihnen teilen gemeinsame vorgelagerte Sequenzmotive wie das *ocs*-Element, das u.a. als pflanzliches Enhancerelement für GSTs beschrieben wurde. Darüber hinaus wurden weitere Motive gefunden, die als potenzielle Bindungsstellen von Transkriptionsfaktoren (TFs) fungieren könnten, z. B. WRKY, basische Helix-Loop-Helix (bHLH), basische Leuzin-Zipper (bZIP) oder MADS-Boxen. Ein besseres Verständnis der Regulation resistenzassoziierter Gene kann die Diagnostik von Herbizidresistenz vereinfachen und dazu beitragen, die Evolution von Kreuzresistenzen besser vorherzusagen, sowie zur Verbesserung der Wirkstoffforschung führen.

Stichwörter: metabolische Resistenz, Glutathiontransferase, Transkriptionsfaktor-Bindungsstelle, Flufenacet, Acker-Fuchsschwanz

Introduction

During the last decades black-grass (*Alopecurus myosuroides* Huds.) has become a problematic weed species in cereals in Western Europe (PETERSON et al., 2018) and populations already resistant against post-emergent herbicides inhibiting acetyl-CoA-carboxylase and acetolactate synthase become increasingly widespread (HRAC Groups 1 and 2). As a consequence, agronomic practices have been adapted to this situation and black-grass control gradually shifted to autumn treatments. Hence, pre-emergence herbicides are playing an increasingly important role in the control of this weed to slow down resistance evolution (BAILLY et al., 2012; MOSS, 2017). For example, flufenacet, an α-oxyacetamide herbicide inhibiting the synthesis of very long-chain fatty acids (VLCFAs; HRAC Group 15), plays a key role for the control of grasses in winter cereals in Europe (MENNE et al., 2012). Analytical studies in black-grass showed that the detoxification of flufenacet is controlled by glutathione transferases (GSTs) (DÜCKER et al., 2019b). Yet, little is known so far about the mechanisms of gene regulation in metabolically resistant weeds. Nevertheless, recent studies of sensitive black-grass populations and populations with a decrease in flufenacet sensitivity revealed upregulation of several enzymes involved in the detoxification of xenobiotics as well as several transcription factors (DÜCKER et al., 2020). Therefore, in that study, an investigation at the gene regulation level using the newly sequenced and assembled black-grass genome was undertaken to look for transcription factors that potentially can affect GST expression. Transcription factors (TFs) are proteins (trans-acting factors), which regulate the expression of genes by binding to specific sequence motifs (*cis* regulatory elements or CREs) of the DNA strand, while regions of DNA coding for them called *trans* regulatory elements (BIŁAS et al., 2016). Most TFs bind on the major groove of the DNA double strand, which allows more room to assess the sides of the bases and offers more distinct patterns of hydrogen bond donors and acceptors on the bases (PABO & SAUER, 1992). CREs are usually in the 1 kbp upstream region of the transcription start site (TSS), however, they can also be found up to 1 Mbp upstream (Fig. 1). So far, several plant GST promoters are known to possess an *ocs* (octapine synthase) element, which serves as binding site for basic leucine zipper transcription factors (bZIP; (CHEN et al., 1996; MARRS, 1996)).

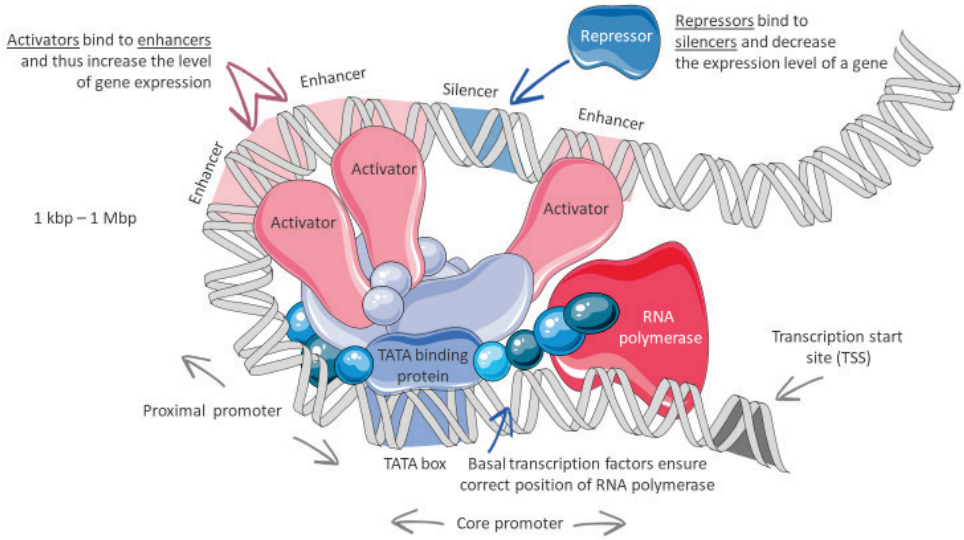

Figure 1 Regulation of transcription by transcription factors in eukaryotes. This image was created using smart.servier.com, based on information modified from SPRINGER et al., 2019.

Abbildung 1 *Regulation der Transkription durch Transkriptionsfaktoren bei Eukaryoten. Die Abbildung wurde nach SPRINGER et al., 2019 mit smart.servier.com erstellt.*

The work presented here is preliminary and aiming to understand better 1) which genes are differentially expressed between sensitive and flufenacet-resistant black-grass populations and 2) how differentially expressed GST genes potentially involved in flufenacet detoxification in black-grass can be regulated by various TFs.

Material and methods

Differentially expressed genes (DEGs)

RNA-seq reads derived from sensitive black-grass populations as well as populations with a statistically significant shift in flufenacet sensitivity (DÜCKER et al., 2020) were aligned against the newly sequenced and assembled black-grass genome (Lichun Cai, Clemson University, personal communication, 2021) using a splice aware aligner (STAR aligner; version 2.6.1d). A differential gene expression analysis was completed with edgeR (version 3.32.1; ROBINSON et al., 2010) in R Studio using thresholds of logFC ≥ 2 and FDR ≤ 0.01 (version 1.2.1335). Additionally, some GSTs defined as candidate genes described by DÜCKER et al. (2020) were investigated.

Candidate transcription factors

Several software programs were used for the investigation of TFs and CREs. PromoAlggen was used for the analysis of binding factors and DNA binding sites using the option “Plants” (MultiSearch Promoter Sites). Further criteria used were a maximum matrix dissimilarity rate of 5%, RE ≤1 and the prerequisite that all motives are common in all the 10 upstream GSTs regions. Additionally, barley (*Hordeum vulgare* L.) was chosen as plant species matrix for a search in PlantRegMap. Cut-off thresholds were q ≤ 0.05 and p ≤ 1e-4. In addition, manual investigation of CREs took place. For this purpose, known CREs described in the literature, such as *ocs* elements (MARRS, 1996) were investigated.

Results

Differentially expressed genes (DEGs)

In total, 3.041 differentially expressed genes were detected in black-grass populations with reduced flufenacet sensitivity. A set of genes involved in detoxification pathways of xenobiotics was detected, such as cytochrome P450s (CYPs), glutathione transferases (GSTs), glucosyltransferases (GTs) and ATP binding cassettes (ABC). In addition, various transcription factor families (FARRELL in BASSETT, 2007, p.9-10) belonging to the families of leucine zippers (ZIP), basic helix-loop-helices (bHLH), MADS-box factors, zinc fingers, WRKYs and as well genes belonging in AP2/ERF superfamily were found to be differentially expressed. A closer look at the most highly upregulated GSTs (logFC ≥ 2) detected in the black-grass populations with reduced sensitivity to flufenacet revealed that they comprise 7% of the total genes annotated as GSTs in the genome (Tab. 1). Three of them were located next to each other on chromosome 3 and two of them were located in direct proximity on chromosome 5 (with logFC = 1,78).

Candidate transcription factors

Investigation of the 3,5 kbp region upstream from the transcription start site (TSS) was done to find transcription factors potentially involved in the expression of GSTs, except for ALOMY2_3, where its upstream region is interrupted by another gene and for this reason only the 2,76 kbp upstream was investigated. The outcome of our analyses revealed various potential binding factors and regulatory elements that may be involved. In most of the GST promoters, TFs belonging to the families of zinc fingers, MADS-box and Dof factors, as well belonging to the AP2/ERF family were discovered. In addition, other elements were detected by manual investigation (not automatically done by an algorithm). These include *ocs* elements, specifically in the promoters of ALOMY2_2, ALOMY3_4, ALOMY3_5, ALOMY3_6, ALOMY6_10 as well as a GCC box in ALOMY2_3, ALOMY3_4, ALOMY5_9.

Table 1 Highly upregulated (logFC ≥ 2, FDR ≤ 0,01) glutathione transferases found in black-grass (*Alopecurus myosuroides* Huds.) populations with reduced flufenacet sensitivity

Tabelle 1 *Stark hochregulierte (logFC ≥ 2, FDR ≤ 0,01) Glutathiontransferasen, die in den Acker-Fuchsschwanzpopulationen (*Alopecurus myosuroides *Huds.) mit geringer Flufenacet-Sensitivität detektiert wurden*

Number	Chromosome	Name of GSTs	GST name**
1	2	ALOMY2_1	-
2	2	ALOMY2_2	-
3	2	ALOMY2_3	-
4	3	**ALOMY3_4**	GST1
5	3	**ALOMY3_5**	GST2
6	3	**ALOMY3_6**	-
7	5	ALOMY5_7	-
8	5	*ALOMY5_8*	-
9*	5	*ALOMY5_9*	GST4, GST5
10	6	ALOMY6_10	GST6

*logFC = 1.78 smaller than 2; **GST name based on DÜCKER et al., 2020; bold: Genes located next to each other on the chromosome 3; italics: Genes located next to each other on the chromosome 5

Discussion

Many genes belonging to the families of CYPs, GSTs, GTs and ABC transporters were found to be upregulated in the populations with reduced sensitivity to flufenacet. All these gene families code for enzymes potentially involved in the detoxification pathways of xenobiotic compounds (e.g. herbicides) in

plants (COLEMAN et al., 1997). It is noteworthy that 7% of the GST genes annotated in black-grass genome were found upregulated in the populations with decreased flufenacet sensitivity. All or some of these GST genes could play an important role in the detoxification of flufenacet in these populations, since GSTs catalyze the first step of flufenacet detoxification in black-grass (DÜCKER et al., 2019b) and in other grasses as maize or ryegrass (*Lolium* spp.) (BIESELER et al., 1997; DÜCKER et al., 2019a). The functional activity of these genes require confirmation. Manual investigation found ocs elements upstream of the TSS in ALOMY2_2, ALOMY3_4, ALOMY3_5, ALOMY3_6 and ALOMY6_10. These are 20 bp consensus sequences containing a tandem core sequence of ACGT. They have been already described in promoters of phytopathogens and in plant GST promoters (MARRS, 1996). Their role in the plant GST promoters is stress-inducible. Interestingly, both software programs used implied the presence of Dof (DNA-binding One Zinc Finger) binding motifs. OBP1 (or AtDof1) has been found to be responsible for a specific interaction with a bZIP protein (OBF) in *Arabidopsis thaliana* (L.) Heynh. This interaction results in stimulation of bZIP binding to an ocs element of the AtGST6 promoter (combinatorial control) (CHEN et al.,1996). In this work, the induction of AtGST6 expression was triggered by salicylic acid and hydrogen peroxide (H2O2), which reveals once more the central role of GST proteins in plant defense. Therefore, the populations with reduced sensitivity to flufenacet could be exhibiting enhanced oxidative stress levels (CAVERZAN et al., 2019; KAUR, 2019) since resistance to various modes of action has already evolved. GSTs might be triggered more easily in resistant rather than in sensitive populations. In addition, GCC boxes were found in ALOMY2_3, ALOMY3_4 and ALOMY5_9, which is recognized by the ERFs (ethylene responsive factors). The latter also appeared as binding factors in the results of the in silico analyses and were also upregulated in our DEG analysis. GCC boxes are typically found in pathogenesis related genes (OHME-TAKAGI & SHINSHI, 1995; SATO et al., 1996; ZHOU et al., 1997) and elevated ethylene levels are responsible for GST expression (LIEBERHERR et al., 2003; SMITH et al., 2003). Considering that the expression of GSTs can be regulated by phytohormones, a functional role of the GCC box in plant GST promoters cannot be excluded. Moreover, there are cases where synergistic effects of ERF (AtEBP) and bZIP (OBF4; ocs element binding factor) have been described in *Arabidopsis thaliana* (L.) Heynh (BÜTTNER & SINGH, 1997), but the functional importance remains unknown.

Outlook

This work will contribute to improve the diagnosis of metabolic herbicide resistance, will help to develop better predictions of the evolution of cross-resistance, as well as contribute to the search for new active ingredients. Knowing the TFs which regulate the transcription of genes coding for enzymes involved in herbicide detoxification pathways e.g. GSTs, will contribute fundamentally to a greater understanding of metabolic weed resistance. Therefore, TFs could serve as potential markers for sequencing-based diagnostics. Despite the high number of trans-acting factors detected in silico, this does not confirm their function in vivo. Further experiments studying protein-DNA interactions to verify the functions of TFs and CREs are necessary to clarify this.

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