Possible involvement of glutathione S-transferases in imazamox detoxification in an imidazolinone-resistant sunflower hybrid

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Fast response of IMI-R and IMI-S sunflower hybrids to the herbicide imazamoxf

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1. Introduction

Sunflower (*Helianthus annuus*) is an important agricultural oilseed crop worldwide. According to the Food and Agricultural organization (FAO), sunflower is the first oilseed crop in Europe with annually approximately 16 million ha of farmland (http://faostat.fao.org/). Weeds are a major problem in sunflower production. Broadleaf weeds cause considerable yield losses that are estimated at about 20-70 % (Sala *et al.*, 2012).

The Clearfield® technology in sunflower has been developed to allow the use of imidazolinone herbicides as a post-emergence control a wide range of weeds, including broad leaf and parasitic weeds (Pfenning *et al.*, 2008). Imidazolinone herbicides were discovered and developed in the 1980s and 1990s (Shaner and Singh, 1997). They control weeds by inhibiting the acetohydroxyacid synthase (AHAS) enzyme, also known as acetolactate synthase (ALS), which is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids valine, leucine and isoleucine in plants (Duggleby and Pang, 2000). The Clearfield® production system consists of a combination of imidazolinone herbicides with imidazolinone resistant sunflower hybrids, which allows the control of a wide range of weeds, including those for which no other herbicide control in sunflower cultivation can be applied (Pfenning *et al.*, 2008). While the conventional sunflower cultivars are imidazolinone sensitive, Clearfield® sunflower hybrids can survive an otherwise lethal application of these herbicides (Pfenning *et al.*, 2008). AHAS-inhibiting herbicides are widely used in agriculture given their high weed control efficacy, high crop-weed selectivity, low use rates, low levels of mammalian toxicity and their favorable environmental profile (Shaner and Singh, 1997).Imazamox is a selective herbicide of the imidazolinone group used against annual and perennial grasses and broadleaf weeds applied in a wide spectrum of crops (Hess *et al.*, 2010).

The tolerance of Clearfield® sunflower hybrids to the imidazolinone herbicides is due to site of action resistance of the enzyme AHAS. However, little is known on how the tolerant genotypes detoxify the herbicide molecules that entered the cell. Shaner (2003) proposed an important role of glycosyltransferases (GTs) in imidazolinones herbicides detoxification through the conjugation with glucose. Increased accumulation of glutathione S-transferases (GSTs) related proteins have been detected in a proteomic research with *Medicago truncatula* treated with AHAS inhibitors (Holmes, 2006). More recently Garcia-Garijo *et al.* (2014) also reported increased activities of GSTs in imazamox-treated young legume seedlings. GSTs comprise a large enzyme family, catalyzing the conjugation of glutathione (GSH) with a number of pesticides and herbicides in plants (Schröder and Wolf, 1996).

The very early responses of the plants to abiotic stress are rather non-specific (Steinberg, 2012) and 24 h are enough to induce plant responses to stress factors such as herbicide application and to activate the defense mechanisms. In order to compare the non-specific responses of IMI-R and IMI-S sunflower hybrids to imazamox application we conducted several analyzes concerning the cellular anti-stress performance. The aims of our study were (1) to detect the herbicide-induced fast responses in IMI-R and IMI-sensitive (IMI-S) sunflower plants. For this purpose we subjected IMI-R and IMI-S sunflower plants to application of imazamox and investigated, lipid peroxidation, activities of some oxidative stress responsive enzymes, GST, GSH content and its redox state, and expression of genes related to the GSH metabolism.

2. Materials and Methods

2.1. Plant material and treatment - Seeds of the sunflower Clearfield® hybrid Mildimi (IMI-R) carrying the haplotype 5 of the *AHAS1* gene (*Imisun* trait) and IMI-sensitive conventional hybrid Albena (IMI-S), carrying haplotype 1 were used. The plants were grown as a hydroponic culture (4 plants per pot), with ½ modified Hoagland nutrient solution in controlled conditions. The imazamox was applied on 2-3 leaf pair stage, by spraying it on the leaves in a rate of 132 mg per plant, which is equivalent to 40 g ai ha^{-1}.

2.2. Determination of glutathione content - The reduced (GSH) and oxidized (GSSG) glutathione were determined according to the method described by Queval and Noctor (2007).

2.3. Determination of lipid peroxidation was estimated according to Dhindsa *et al.* (1981).

2.4. Determination of enzyme activities:

- *Glutathione peroxidase* (GPX, EC 1.11.1.9) according to Dixon *et al.* (1998b).
- *Glutathione reductase* (GR, EC 1.8.1.7) according to Zhang and Kirkham (1996).
- *Glutathione-S-transferase* (GST, EC 2.5.1.18) according to Habig *et al.* (1974).
- *Syringaldazine peroxidase* (SPOD, EC 1.11.1.7) according to Cuypers *et al.* (2002).

The protein extraction from leaves was performed according to Schröder and Götzberger (1997). The protein concentrations were measured according to the method described by Bradford (1976).

2.5. Gene expression analysis - RNA was extracted from frozen samples using the mirVana miRNA Isolation kit, according to the manufacturer's instructions (Life Technologies). First strand cDNA synthesis was performed with 1 µg total RNA using the High Capacity reverse transcription kit and a combination of oligo(dT)-primers and random hexamers according to the manufacturer`s instructions (Life Technologies). Quantitative PCR was performed with the 7500 Fast real-time PCR cycler (Applied Biosystems) and SYBR green Master Mix (Life Technologies). Primer sequences of reference genes were designed according to Fernandez *et al.* (2011). The amplification efficiencies of all primer sets were investigated by measuring a 2-fold serial dilution method and were approved when they were greater than 1.8. After geNorm analysis (Vandesompele *et al.*, 2002), two reference genes (ACT and PEP), were selected for normalization.

2.6. Polymerase chain reaction for AHAS haplotype confirmation by gene sequencing - gDNA was isolated from the IMI-R and IMI-S plants by the Plant DNeasy mini kit (QIAGEN), according to the manufacturer's instructions. Primers amplifying the *AHAS* gene region containing the relevant mutation were AHAS-F 5'-CACGATCATCAAACCGCACC-3' and AHAS-R 5'-GACCGTTACCGGTGCAGCCT-3'. The remainder of the PCR product was sent for DNA sequencing (Macrogen). The IMI-R was confirmed to have a haplotype 5 and the IMI-S a haplotype 1 genome at the *AHAS* locus.

2.7. Statistical analysis - Statistical analysis was performed using ANOVA (for $P< 0.05$). Two way ANOVA was used to compare the responses of both hybrids. Based on ANOVA results, a Duncan test for mean comparison was performed, for a 95% confidence level, to test for significant differences among treatments. In the figures, different letters (a, b, c) express significant differences.

3. Results

Imazamox-treated sunflower plants from IMI-R (Mildimi) and IMI-S (Albena) hybrids did not show any visual symptoms of injury one day after the treatment (DAT). Nevertheless, the performed biochemical and molecular analyses indicated significant differences in their physiological status. To highlight the fast responses of both hybrids to imazamox we studied the changes in the plant cell defence system and the glutathione-dependent metabolism (Figure 1).

The total glutathione content in the leaves of imazamox-treated plants from both cultivars was significantly increased (Figure 1A). In both hybrids, the higher glutathione content is due to an activation of the expression of the *GSH2* gene (Figure 1C). The observed increases of glutathione seemed to be differently utilized in both hybrids. In the IMI-S hybrid it might be involved in the significantly increased GR activity leading to slight change in GSH/GSSG ratio (Figure 1F), while in IMI-R hybrid its increase could be connected with the enhanced participation of GSTs enzymes in xenobiotic detoxification. The data presented significant activation of GSTs, measured with the two used substrates CDNB and fluorodifen (Figure 1 D and F).

The antioxidative defence system in IMI-S plants was significantly activated through increased activities of GR, GPX and SPOD (Figure 1F, H and G). In contrast, in IMI-R plants, the imazamox application did not induce such activation and antioxidative enzymes GPX and GR were not significantly activated. Only SPOD showed slight increase in IMI-R plants. The level of lipid peroxidation (Figure 1J), caused by imazamox 24 h after the treatment was increased with 35 % and 24 % in IMI-S and IMI-R hybrids, respectively.

4. Discussion

One of the earliest responses on many abiotic stresses involve ROS formation, which modify enzyme activity and gene regulation (Cramer et al., 2011). Overproduction of ROS is leading to oxidative stress in cells and can destroy cell organelles, cause membrane damage and inhibit gene expression (Sharma *et al.*, 2012). ROS formation has been reported in several stress conditions, including herbicide treatment (Stajner *et al.*, 2003). The determination of lipid peroxidation level, gene expression and activities of enzymes involved in anti-oxidative defense are good indicators of oxidative stress (Cuypers *et al.*, 2002).

Our results show dissimilarities in the fast stress responses and defense reactions of both sunflower hybrids markedly differing in their level of tolerance to the herbicide imazamox. In both tested sunflower hybrids the total amount of the metabolite glutathione was increased (Figure 1A) as a result of imazamox application from the first day after application, which was confirmed also at the molecular level by upregulation of the *GSH2* gene (Figure 1C). Meanwhile, the IMI-S plants were reacting through mainly activating the anti-oxidative cell defense network through increasing the activities of enzymes such as GR, GPX and SPOD (Figure 1F, 1H, 1G). In contrast in IMI-R hybrid only the SPOD had slightly increased activity and the activity of the rest measured antioxidative enzymes (GPX, GR) were not significantly affected by imazamox application. Our results also showed significantly increased levels of TBA reactive compounds (indicator of lipid peroxidation and a marker of oxidative stress) in leaves of imazamox-treated IMI-R sunflower plants (Figure 1J) which agrees with earlier reports (Zabalza *et al.*, 2005; Qian *et al.*, 2011). This indicates that the imidazolinone herbicides cause slight lipid degradation, which could be due to oxidative stress as a result of a disturbed redox balance. Indeed, until now, it

has not been reported that AHAS inhibitors cause significant oxidative stress and moreover, ROS production is not the main factor involved in growth inhibition. Slight or even no changes in the activities of anti-oxidative enzymes were described in legume root nodules in response to the herbicide imazamox (Garcia-Garijo *et al.*, 2014). Qian et al. have reported similar results in imazethapyr-treated *A. thaliana*, concluding that imazethapyr could stimulate ROS production, but that the anti-oxidative defense system is rather suppressed than activated, which causes an imbalance in the anti-oxidant system (Qian *et al.*, 2011).

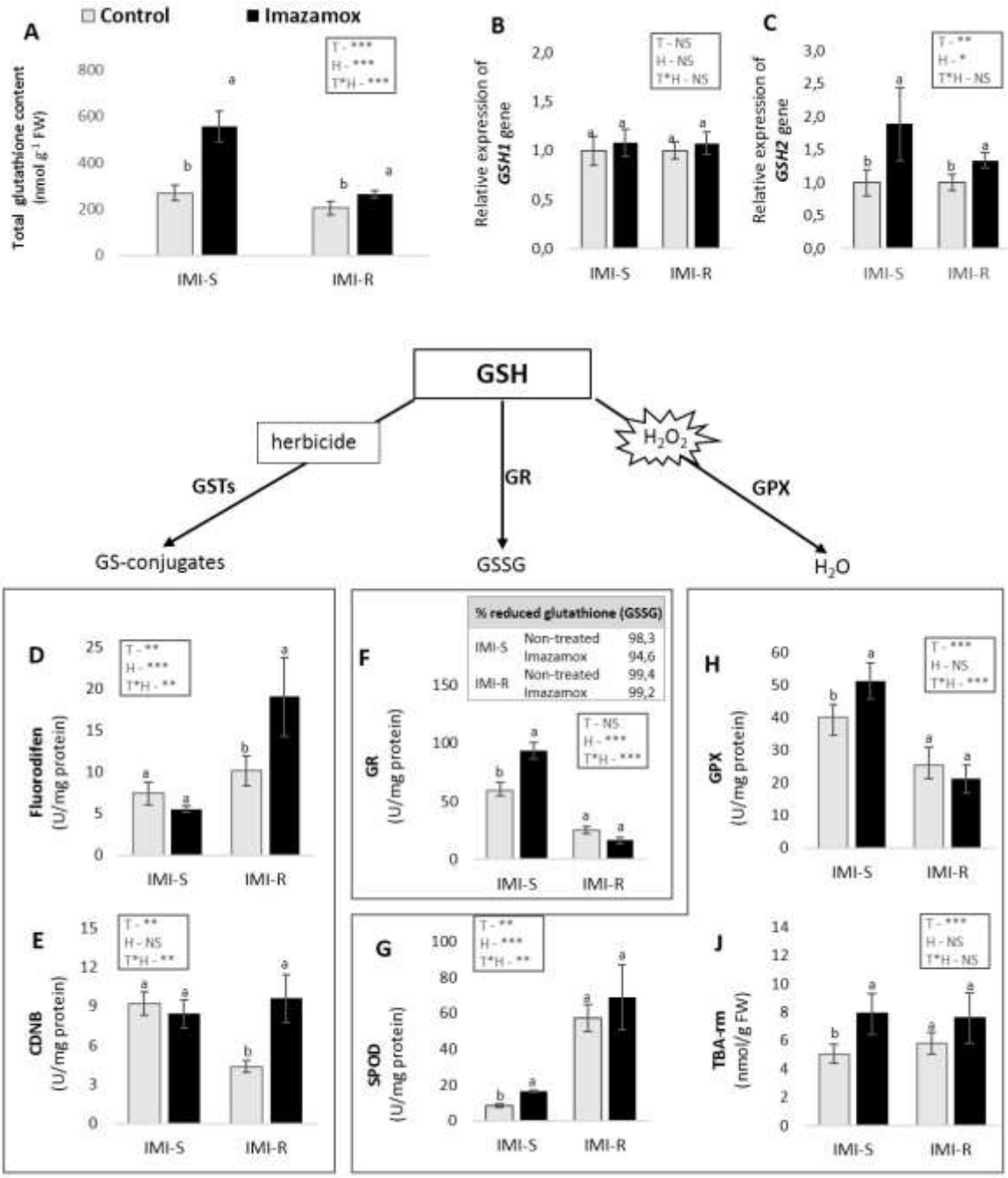

Figure 1: *An overview of the relative enzyme activities and metabolites content that play a role in either the detoxification of herbicides or the antioxidant defence mechanism in leaves of IMI-S and IMI-R sunflower hybrids treated with the herbicide imazamox:* ***A*** *– total glutathione (GSH) content;* ***B*** *– relative gene expression of GSH1 gene, encoding the enzyme gamma-glutamylcysteine syntethase (EC 6.3.2.2.);* ***C*** *– relative gene expression of GSH2 gene, encoding the enzyme glutathione synthetase (EC 6.3.2.3.);* ***D*** *– glutathione-S-transferase (GSTs) activity (substrate fluorodifen);* ***E*** *– glutathione-S-transferase (GSTs) activity (substrate chlorodinitrobenzene);* ***F*** *– glutathione reductase (GR) activity and percentages of reduced glutathione;* ***G*** *– syringaldazine peroxidase (SPOD) activity;* ***H*** *– glutathione peroxidase (GPX) activity;* ***J*** *– TBA-reactive compounds (TBArm). Error bars indicates standard deviation (SD). The values represent the mean of three biological replicates. Different letters (a, b) express significant differences between treatments (P < 0.05). The small text boxes within each graph show the significance levels of two way analysis of variance (ANOVA): * P = 0.05, ** P = 0.01, *** P = 0.001, NS – not significance).*

The tripeptide glutathione (GSH) has a multiple function in plant metabolism and stress defense. Its biological role is relate to reversible redox reactions, resulting in the coexistence of a reduced state (GSH) and an oxidized state (GSSG). The cellular GSH pool is mostly reduced under optimal conditions. (Jozefczak et al., 2012). GSH may also conjugate with numerous endogenous and xenobiotic compounds, through reactions catalized by glutathione S-transferases (GSTs) (Schröder et al., 2002,). Therefore the glutathione mediated detoxification system is valuable stress marker in ecophysiological studies (Tausz et al., 2004).

GSTs are a large enzyme family playing a central role in the cellular defense against foreign compounds. Imidazolinine herbicides are usually detoxified through glucose conjugation (Shaner, 2003), but based on proteomic research of *Medicago truncatula* plants treated with AHAS inhibitors GSTs were also reported to be involved in AHAS inhibiting herbicide detoxification (Holmes *et al.,* 2006). However, there are no reports proving that glutathione conjugation is part of the imidazolinones detoxification pathway. In our study the imidazolinone-tolerant sunflower hybrid showed definitely increased levels of GSTs with CDNB, but also with fluorodifen (Figure 1 E and D), which is an indication for activation of its detoxification machinery. These results suggest that in addition to the *AHAS1* gene mutation, conferring the resistance to imidazolinone herbicides in sunflower plants, the so-called non-target mechanism is also involved in the imazamox tolerance, through strengthened herbicide metabolization and detoxification. Analysis of transcript profiles in IMI-S (wild type) and IMI-R genotypes of *Arabidopsis thaliana* demonstrated that in wild-type plants, the genes which responded the first to imazapyr application were detoxification-related genes such as cytochrome P450s, glycosyl transferases, GSTs, and ABC-transporters (Manabe *et al.,* 2007). Recently, Garcia-Garijo et al. also reported increased GST activity in nodules of bean plants treated with imazamox (Garcia-Garijo *et al.,* 2014). The expression of GSH synthesizing gene *GSH2* induced by imazamox (Figure 1C), unambiguously illustrates the increased need of GSH in imazamox-treated plants. At the same time the GSH/GSSG ratio remained unchanged (Figure 1F), which corresponds with the fact that almost no changes in GR activity were observed in IMI-R sunflower plants (Figure 1F). All these results indicate that, in the tolerant hybrid, GSH is mainly involved in the initial herbicide detoxification pathway and not in ROS combatting mechanisms.

In conclusion, the comparison of both sunflower hybrids differing in their tolerance to the herbicide imazamox showed that the IMI-R and IMI-S sunflower have dissimilarities in the mechanisms involved in the rapid response to imazamox. The IMI-S hybrid Albena is activating the cell anti-oxidative defense system, while in the IMI-R hybrid Mildimi detoxification mechanisms, including enzymes such as GSTs are mainly triggered. After detailed analysis of the responses of the GSH-dependent detoxification system we may conclude that GSH participate in the detoxification pathway of herbicide imazamox in IMI-R sunflower plants and

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