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Contribution of Glutathione Transferases in the Selective and Light-Dependent Effect of Flumioxazin on Winter Wheat (*Triticum aestivum* L.) and Its Typical Weed Common Poppy (*Papaver rhoeas* L.)

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Abstract: Glutathione transferases (GSTs) are enzymes that catalyse modifications and conjugations of a range of organic and often cytotoxic compounds. GST enzymes with many functions—such as their conjugation activity against herbicides and their metabolites—can be induced and show light and circadian determination. The enzyme family, which is widespread in its function, also shows great diversity in its structure, which has been linked to its enzyme kinetic characteristics and physiological role at many points. In this study, we aimed to find out the role of different glutathione transferases in the herbicide responses to flumioxazin, as well as to determine how the antioxidant and detoxification response to herbicide treatment changes in the presence and absence of light. One of the herbicide treatments was carried out during the light period in the morning (9:00 a.m.), and the other before the end of the dark period (4:00 a.m.). The decrease in the maximal quantum efficiency of PS II and the reduction in the chlorophyll concentration supported the effect of the herbicide on *Papaver rhoeas*. In the guaiacol peroxidase POD and GST activity, there were large differences between the cultivated plants and the weed; both enzyme activities were much higher in the case of wheat. According to the activity of the antioxidant defence enzymes and GST gene expression data, the application of the photosynthesis inhibitor herbicide, flumioxazin, in the dark could allow the wheat antioxidant defence to switch on before the herbicide effect could appear in the light period. Phi and tau group GSTs were transcriptionally upregulated by the treatments in wheat plants (especially *TaGSTU1B*), while fewer changes were detectable in poppy weed (*PrGSTU4*). Based on our results, in the background of the greater and more successful response to flumioxazin may be—among other things—the higher degree of variability of the GSTU genes of wheat compared to poppies.

Keywords: flumioxazin; glutathione transferases; light; *Papaver rhoeas*; *Triticum aestivum*

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1. Introduction

The success of photo-inhibitory herbicide treatments can vary based on the presence and absence of light and on the circadian rhythm-controlled defence reaction of plants during the day or night, or under different light availability. It has been reported that phytotoxicity is less dominant when plants are exposed to low light levels compared to intense sunlight [1–3].

Among the several herbicides that influence photosynthesis, flumioxazin (WSSA Group 14) is a soil-applied pre- and post-emergence herbicide used to inhibit the development of dicotyledonous weeds, such as common poppy (*Papaver rhoeas*), redroot

pigweed (*Amaranthus retroflexus*), lambs quarter (*Chenopodium album*), and jimsonweed (*Datura stramonium*) [4–6]. According to the original mode of action of Group 14, flumioxazin inhibits the protoporphyrinogen oxidase. This enzyme is involved in the biosynthesis of chlorophylls and cytochromes [6–8]. There are many possible uses for the active ingredient: in certain industrial areas, it is also used as a non-selective herbicide, which is made possible by the fact that it can inhibit germination of a wide range of weeds, depending on soil moisture and other environmental factors [9,10]. The effectiveness as PRE treatment has been reported on many plant groups, including grasses [11] and crops, such as peanuts [12].

Flumioxazin is an herbicide that inhibits protoporphyrinogen IX oxidase; the enzyme catalyses the formation of protoporphyrin IX from protoporphyrinogen IX, which results in protoporphyrinogen IX accumulation. Oxygen accepts an electron from reduced protoporphyrinogen IX under light conditions, resulting in the formation of ROS, such as hydrogen peroxide. This herbicide treatment and ROS formation results in an oxidative burst, which causes damage to lipids, carbohydrates, and nucleic acids, and thus, may lead to cell death of plants [13,14]. In the detoxification of ROS and neutralisation of its harmful effect, the antioxidant system plays a role, thus protecting the plant from fatal damage [15,16]. However, the activity of antioxidant enzymes is significantly dependent on the presence or absence of light, and also on the circadian clock [17,18].

The antioxidant system of plants keeps reactive oxygen species (ROS) below the harmful level [15,16]. Among others, the superoxide dismutase (SOD) activity is at peak before the beginning of the dark period, and this activity continuously declines through the night [19].

In addition to the fluctuation of antioxidant enzymes during the day, the activity of certain detoxifying enzymes also follows a daily rhythm: the activity and expression levels of glutathione transferases (GSTs) reached their peak at the end of the light period, before both reduced under darkness in tobacco, tomato, and wheat [20–22].

The diverse group of plant GSTs (EC 2.5.1.18; GSTs) catalyse several different reactions, including the conjugation of glutathione (GSH) with electrophilic compounds to transform them to less toxic byproducts, which can be conveyed to the vacuole for subsequent metabolic processes [23,24]. Plant GSTs can be divided into at least fourteen distinct classes [22]. The tau and phi classes, which are the largest and the most diverse groups found in plants, play essential roles in mitigating environmental pollution caused by organic xenobiotics, such as herbicides [24–27].

The connections between tau class GST protein structures and their kinetic characteristics were analysed in soybean and sweet orange [28]. In soybean, an enzyme variant was reported to exhibit higher CDNB- and fluorodifen-conjugating activities [28,29]. Analysis of this enzyme variant revealed that the Trp114Cys point mutation was responsible for the altered properties, as the replacement of the bigger Trp residue by the smaller Cys induced conformational changes in the active site. Similar results were obtained in the case of sweet orange tau group GSTs, where changing a single mismatched residue (Glu117Lys) resulted in significant differences in the kinetic parameters [30].

The enzymatic conjugation of GSH with herbicides catalysed by the GSTs was reported to play an important role in the detoxification of selective herbicides in hexaploid bread wheat [31–33]. In the case of some active herbicide ingredients (chloroacetamide, dimethenamid, and sulphonylurea flupyrsulfuronmethyl), the decisive step of the process is the rapid conversion by GSH conjugation [33–37]. Phi and tau class GSTs are the most significant in terms of herbicide tolerance; certain members of both classes can be induced by safeners [37]. It was earlier reported that GSTs are frequently highly abundant in crops, which resulted in 20-fold more effective detoxification of selective herbicides in the crop than in the competing weeds [38].

The fate of the active ingredient flumioxazin in plant cells is not known in all details, so the role of GSTs in this process can only be inferred. Based on the detailed review of the transformation of herbicides that inhibit the enzyme protoporphyrinogen oxidase in plants, certain members of the active ingredient group are neutralised by GSTs [39,40]. In a

previous study, the degradation of flumioxazin by aquatic plants was investigated, and the role of phase II detoxification in pesticide degradation was emphasised, including in the form of GSH conjugation [41]. Furthermore, safener-induced GST activity increase was reported to have a protective role in cotton [42]. However, it is still not completely clear whether GSTs exert their protective effect through direct detoxification or whether they have a greater role in the elimination of secondary stress metabolites.

The involvement of GSTs in light-dependent cellular processes is multifaceted: some of these proteins turn out to be transducers of signalling pathways regulated by UV and red light [24,43–46]. Darkness or low light mostly reduced the conjugating activity of GSTs, while high light significantly increased GST activity and expression, and GSH contents [24].

The aim of our present study was to find out the role of different glutathione transferases in the herbicide responses to flumioxazin, as well as to determine whether the presence of light during spraying influences the intensity and course of the antioxidant and detoxification defence responses to the herbicide in the case of wheat crop and poppy weed.

2. Materials and Methods

2.1. Plant Materials and Treatments

During the experiment, common poppy (*Papaver rhoeas*) and two wheat *Triticum aestivum* L. cultivars were used. The cv. GK Ígéret is an improving quality (A2-A1) wheat line, while the cv. GK Arató is a forage quality (B1-A2) type. Both wheat lines were bred in the Cereal Research Non-Profit Ltd., Szeged, Hungary (https://www.gabonakutato.hu/en/ accessed on 2 August 2023).

Sixty healthy wheat grains and about one hundred healthy poppy weed seeds were sown in a 10 kg mixture of 9:1 soil and sand (Bioland Tőzegfeldolgozó Kft., Biatorbágy, Hungary) containing N (200–500 mg L^{-1}), P_2O_5 (200–500 mg L^{-1}); K_2O (300–600 mg L^{-1}), black peat (50%), white peat (50%), and $CaCO_3$ (2 kg m^{-3}), pH 5.5–7.5. The sampling was designed so that the phenophases corresponded to the early post-emergent treatment: wheat plants were 12–14 days old at the time of sampling (at least 3 leaves), and poppies had 3 leaf layers (suitable age for field spraying). Plant growth took place under the controlled conditions 12 h light/12 h dark and 23/20 °C with 50–60% humidity (6:00 a.m.–6:00 p.m. light period, 6:00 p.m.–6:00 a.m. dark period).

The leaves of wheat and common poppy were sprayed with Pledge 50 WP (flumioxazin) herbicide solution with a concentration of 3 mg/10 mL of formulation and 1.5 mg/10 mL active ingredient, which was diluted in sterile water. To be able to study the light-dependent effect of the herbicide, we treated the plants at two times: at dawn (4:00 a.m.) and morning (9:00 a.m.). After each treatment, samplings were performed in the second light cycle at 9:00 a.m. (Figure 1). The experiments were repeated three times, with independent plant generations. (Samples treated at 4:00 a.m. received an extra 3 h of light).

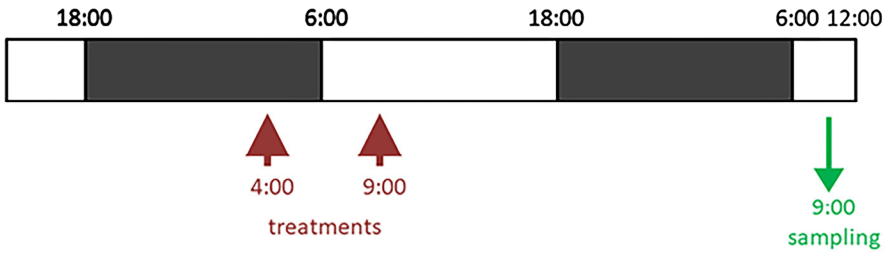

Figure 1. Time of treatments and sampling in two wheat (*Triticum aestivum* L.) cultivars and in common poppy (*Papaver rhoeas*) plants.

2.2. Determination of Antioxidant Activities

A total of 250 mg of leaf tissue of wheat and poppy weed were ground and mixed with 1.25 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% polyvinyl poly pyrrolidone (PVPP). After centrifugation (12,000× *g*, 4 °C, 20 min), the supernatant was used for the detection of superoxide dismutase (SOD; EC

1.15.1.1), catalase (CAT; EC 1.11.1.6), and guaiacol-dependent peroxidase (POD; EC 1.11.1.7) activities at 560, 240, and 470 nm, respectively, using a spectrophotometer (KONTRON, Milan, Italy).

The activity of SOD shows the ability of the enzyme to catalyse the reduction of NBT in the presence of riboflavin and light. One unit of SOD is equal to the enzyme amount used to inhibit fifty percent NBT reduction [47]. Catalase activity was determined by the decomposition of hydrogen peroxide and was followed spectrophotometrically, following the decrease in absorbance at 240 nm. One unit of CAT is equal to the amount of enzyme required to consume 1 μmol min^{-1} H_2O_2 [48].

The POD activity was determined following the increase in absorbance at 470 nm due to the oxidation of guaiacol. ε_{470} = 26.6 mM^{-1} cm^{-1}. The amount of enzyme catalysing the formation of 1 μmol min^{-1} 30 of oxidised guaiacol was defined as 1 U [48].

Glutathione transferase activity was determined spectrophotometrically using the 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich, St. Louis, MO, USA) artificial substrate. The reaction was started by the addition of CDNB; the increase in absorbance at 340 nm was determined. One U is equal to the amount of enzyme that produces 1 μmol of conjugated product in 1 min; ε_{340}=9.6 mM^{-1} cm^{-1} [49].

The protein contents of the leaf extracts were measured by the method of Bradford [50].

2.3. Measurement of Maximal Quantum Yield of PSII Photochemistry

To measure the maximal quantum yield of PSII in leaves of wheat and common poppy plants, a chlorophyll fluorometer (pulse amplitude modulation, PAM-2000; Heinz-Walz, Effeltrich, Germany) was used. The minimal fluorescence yield in a dark-adapted state (F_0) was determined after leaves were kept in dark for 15 min. After this, the maximal fluorescence in the dark-adapted state (F_m) was measured, which allowed the calculation of the maximal quantum efficiency of PSII photochemistry ($F_v/F_m = (F_m - F_0)/F_m$) [51].

2.4. Determination of Photosynthetic Pigment Concentration

A total of 25 mg of leaves of wheat and common poppy were ground using 750 μL of 100% (*v*/*v*) acetone (cold) and incubated for one day at 4 °C. After a centrifugation step (12,000× *g* for 20 min at 4 °C), the pellet was washed with 750 μL of cold acetone/Tris buffer (80:20 *v*/*v*, pH = 7.8) for one more day at 4 °C. After centrifugation (12,000× *g*, 20 min, 4 °C), the absorbance of the supernatants was measured by spectrophotometer in a glass cuvette at 470, 537, 647, and 663 nm (KONTRON, Milan, Italy). Concentrations of photosynthetic pigments were calculated according to Sims and Gamon [52].

2.5. Determination of H_2O_2 Concentration

The amount of H_2O_2 in leaves was measured spectrophotometrically, as described by Velikova et al. [53]. After the homogenisation of 0.2 g of leaf tissue with 1 mL of ice-cold 0.1% trichloroacetic acid, the samples were centrifuged (10.000× *g* for 20 min at 4 °C). The reaction mixture contained 0.25 mL of 10 mM phosphate buffer (pH 7.0), 0.5 mL of 1 M potassium iodide (KI), and 0.25 mL of the supernatant. The absorbance of the samples was measured after 10 min at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared from H_2O_2 stock solution, and using the extinction coefficient 0.28 μM^{-1} cm^{-1}.

2.6. RNA Extraction, cDNA Synthesis, and Expression Analysis by Quantitative Real-Time PCR

The changes in the transcript amounts of wheat and common poppy GST genes were determined by quantitative real-time PCR (RT-qPCR). RNA was isolated from 100 mg plant material, following the description of Chomczynski and Sacchi [54] and Csiszar et al. [55]. The RT-qPCR oligonucleotides (and the *Papaver rhoeas* GST-like sequences) are listed in Table S1. The changes in the transcript amounts of GST genes were monitored as published earlier [56]. The Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific™, Waltham, MA, USA) reaction mixture was used for the quantitative PCR (qRT-PCR; qTOWER Real-

Time qPCR System, Analytik Jena, Jena, Germany) reaction, following the manufacturer's instructions. We used the 18S and EF sequences as high and low internal controls, respectively. Data were calculated using the $2^{-(\Delta\Delta Ct)}$ method [57]. Data were normalised using the control ΔCt values of common poppy and the GK Ígéret cultivar on a log2 scale, and the results were shown on a heat map.

For our experiments, several common poppy and wheat sequences were chosen (Table S1). One partial poppy weed GST sequence is available in GenBank (MW306747.1). Other *Papaver rhoeas* GSTs were selected using the dataset of Subramaniyam et al. [58]. The used datasets contained the sequenced transcriptomes of *Papaver rhoeas*. BLAST Searching was performed using *Papaver somniferum* GSTs or GST-like sequences (XM_026567272, XM_026567096, AF118926, AF118925, AF118924, and XM_026527770, XM_026561865 led to searching results) in *Papaver rhoeas* (SRR7345724 and SRR7345723) transcriptome sequence libraries. Wheat sequences were chosen according to the enzyme properties, and previous data were obtained from Cummins et al. [32], Thom et al. [37], Gallé et al. [56], and Pelsőczi et al. [22].

According to Thom et al. [37], the TaGSTU1-1 protein shows outstanding conjugation activity against fluorodifen and metolachlor, and among the four tau group GSTs they examined, the CDNB conjugation activity was the second highest, and the activity against crotonaldehide (stress metabolite analogue) was the highest. TaGSTU3-3 shows conjugation activity towards Fenoxaprop and is characterised by higher GPOX activity. According to Cummins et al. [32], among the six phi group GSTs, TaGSTF1 protein showed the highest CDNB conjugating activity; TaGSTF3 exhibited the highest conjugating activity against benzylisothiocyanate and metolachor, and outstandingly high GPOX activity; while TaGSTF6 showed activity towards ethacrynic acid, which was a unique feature of the tested phi group GSTs [32]. Red light upregulates the expression of some genes of the mentioned protein, namely, *TaGSTU1B*, *TaGSTU3*, *TaGSTF3*, and *TaGSTF6* [22].

2.7. Alignment of the Secondary Structure

Amino acid sequences of TaGSTU1B, TaGSTU3, PrGST1, PrGST2, PrGST3, and PrGST4 were submitted to Proteus2 [59] in order to obtain the secondary structures. An alignment was created using Clustal Omega [60,61], and 2dSS was used for the visualisation (http://genome.lcqb.upmc.fr/2dss/index.html accessed on 14 June 2023) [62].

2.8. Statistical Analysis

Antioxidant enzymes activities and hydrogen peroxide and pigment concentrations were determined with at least three parallel samples. In the case of qPCR sampling, three to six individual plants were sampled per biological replicate per treatment, and we worked with three technical replicates. In the figures, mean values are presented with standard deviation bars. Sigma Plot 11.0 software (SPSS Science Software, Erkrath, Germany) was used for statistical analysis. Analysis of variance (ANOVA) was performed to determine the difference among different treatments by Duncan's test, and a significant difference was found if $p \leq 0.05$.

3. Results

First, the daytime- and nighttime-dependent effects of flumioxazin were tested on the maximal quantum yield of the PSII photochemistry (F_v/F_m) of monocotyledonous wheat (GK Arató and GK Ígéret) and dicotyledonous poppy weed plants based on the measurements of chlorophyll fluorescence (Figure 2).

The maximal quantum yield of the PSII photochemistry (Fv/Fm) of wheat (GK Arató and GK Ígéret) and poppy weed plants showed a larger impact of herbicide treatments on poppy: both the 4:00 and 9:00 application of flumioxazin reduced Fv/Fm only in poppy weed. It was in correspondence with the chlorophyll a+b concentration: treatments decreased it in poppy weed, while there were no changes in carotenoids concentrations (Figures 2 and 3).

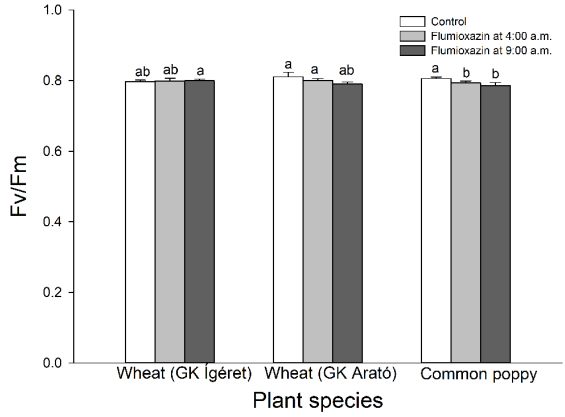

Figure 2. Changes in the maximal quantum yield of PSII photochemistry F_v/F_m in leaves of wheat (*Triticum aestivum* cv. GK Ígéret and cv. GK Arató) and poppy weed (*Papaver rhoeas*) treated with sterile water (white columns) and flumioxazin at dawn (4:00 a.m., grey columns) and in the light period (9:00 a.m., dark-grey columns). Sampling was performed in the second light cycle at 9:00 a.m. Means ± SE, $n = 3$. Means denoted by different letters indicate statistical difference at $p \leq 0.05$ as determined by Duncan's test.

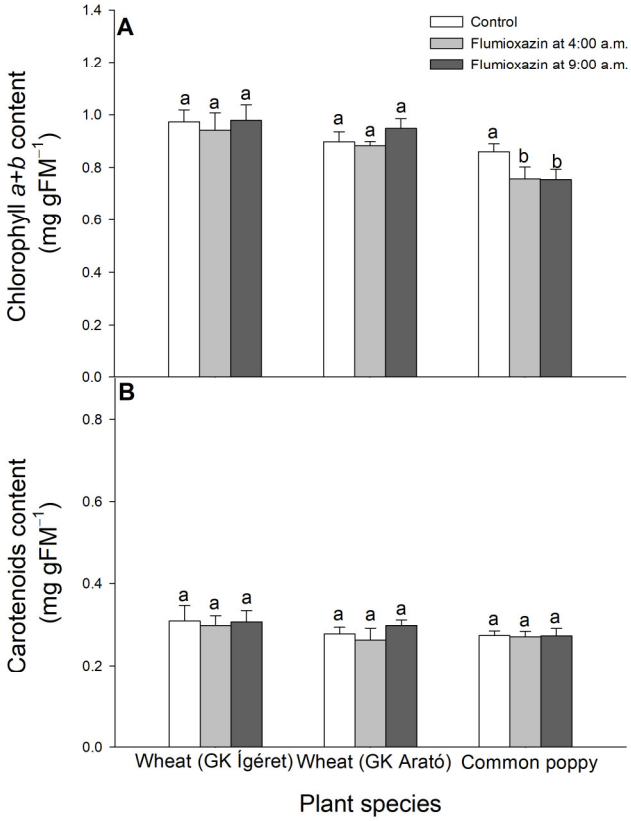

Figure 3. Differences in the chlorophyll *a* + *b* (**A**) and carotenoids concentration (**B**) in leaves of wheat (*Triticum aestivum* cv. GK Ígéret and cv. GK Arató) and poppy weed (*Papaver rhoeas*) with sterile water (white columns) and flumioxazin at different times: at dawn (4:00 a.m., grey columns) and in the light period (9:00 a.m., dark-grey columns). Sampling was performed in the second light cycle at 9:00 a.m. Means ± SE, $n = 3$. Means denoted by different letters indicate statistical difference at $p \leq 0.05$ as determined by Duncan's test.

The hydrogen peroxide amounts were increased in all plants, especially in common poppy leaves (ca. 3 times) (Figure 4). The constitutive level of H_2O_2 in wheat was three times higher than that of poppy.

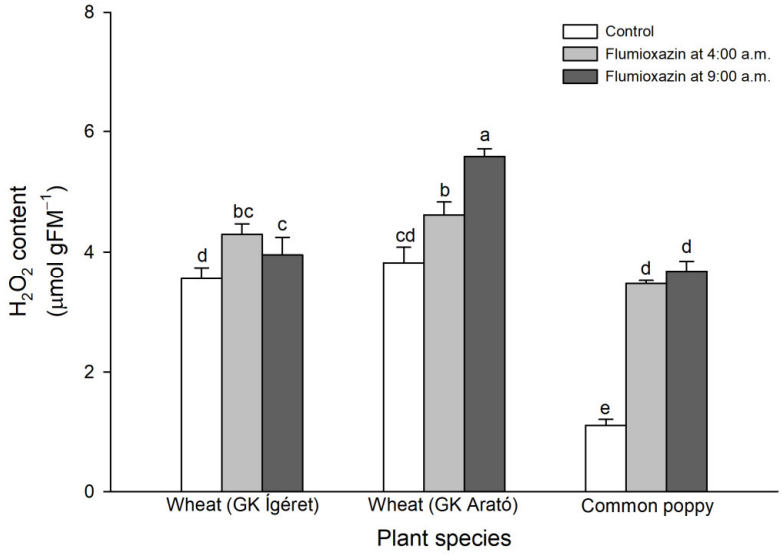

Figure 4. Differences in the H_2O_2 concentration in leaves of wheat (*Triticum aestivum* cv. GK Ígéret and cv. GK Arató) and poppy weed (*Papaver rhoeas*) treated with sterile water (white columns) and flumioxazin at different times: at dawn (4:00 a.m., grey columns) and in the light period (9:00 a.m., dark-grey columns). Sampling was performed in the second light cycle at 9:00 a.m. Means ± SE, $n = 3$. Means denoted by different letters indicate statistical difference at $p \leq 0.05$ as determined by Duncan's test.

There were large differences in POD activity between the untreated samples of wheat and poppy; the activities were significantly higher in wheat lines. SOD, CAT, and POD activity measurements further emphasised the differences between the responses to the herbicide of wheat and common poppy plants and showed even decreases in the enzymatic antioxidant activity of poppy weed. Spraying in the dark induced stronger measurable SOD and CAT, but not POD, activity in wheat cultivars (Figure 5).

GST activity in control conditions showed large differences between wheat and common poppy plants: the activity was higher and the treatments caused larger inductions in the two wheat lines (Figure 6).

In GK Ígéret, the treatments upregulated the gene expression of all the measured GSTs; higher values were detected when the herbicide was sprayed during the light period. The highest value was shown by the tau group *TaGSTU1B*. We were able to measure much smaller changes in GK Arató; in two cases (*TaGSTU1B* 4:00 a.m. and *TaGSTF6* 9:00 a.m.), the treatment resulted in a decrease in the relative expression of GSTs. In poppy weed, the expression of tau group GSTs was slightly enhanced by the treatment at nine o'clock, while spraying in the dark reduced it. The lowest measurable value was shown by the phi group *PrGSTF* in the case of the 4:00 treatment. Both treatments induced the expression of *PrGSTU4* (Figure 7).

Comparing the secondary structures of the detected tau group GSTs (according to the poppy sequences available in the SRR7345723 dataset and wheat sequences available in NCBI), surprising features were revealed. The amino acid and secondary structure composition of the substrate binding site (H-site) of the tau group GSTs TaGSTU3, PrGSTU3, and PrGSTU4 were more similar to each other than the two wheat tau group GSTs (TaGSTU1B and TaGSTU3) (Figure 8).

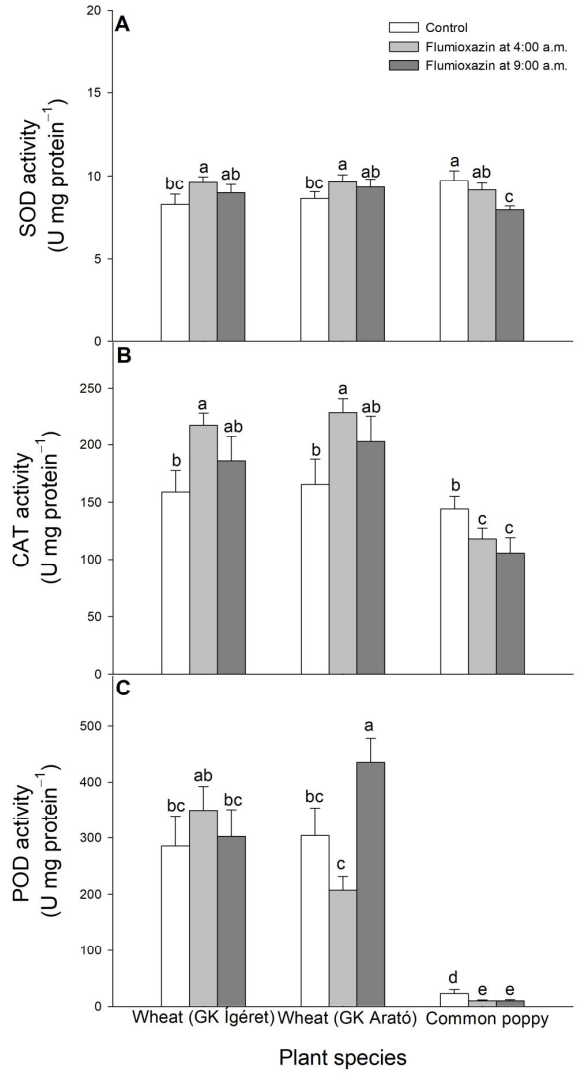

Figure 5. Differences in the activity of superoxide dismutase (SOD; (**A**)), catalase (CAT; (**B**)), and peroxidase (POD; (**C**)) in leaves of wheat (*Triticum aestivum* cv. GK Ígéret and cv. GK Arató) and poppy weed (*Papaver rhoeas*) treated with sterile water (white columns) and flumioxazin at different times: at dawn (4:00 a.m., grey columns) and in the light period (9:00 a.m., dark-grey columns). Sampling was performed in the second light cycle at 9:00 a.m. Means ± SE, $n = 5$. Means denoted by different letters indicate statistical difference at $p \leq 0.05$ as determined by Duncan's test.

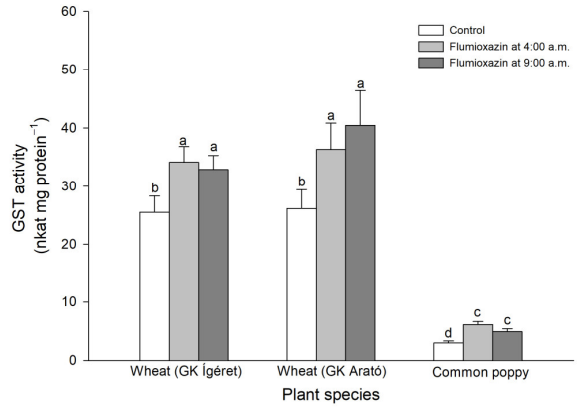

Figure 6. Differences in the activity of glutathione transferase (GST) in leaves of wheat (*Triticum aestivum* cv. GK Ígéret and cv. GK Arató) and poppy weed (*Papaver rhoeas*) treated with sterile water

(white columns) and flumioxazin at different times: at dawn (4:00 a.m., grey columns) and in the light period (9:00 a.m., dark-grey columns). Sampling was performed in the second light cycle at 9:00 a.m. Means ± SE, $n = 5$. Means denoted by different letters indicate statistical difference at $p \leq 0.05$ as determined by Duncan's test.

Highest: 4.434 Lowest: -1.138

	GK Ígéret			GK Arató				Poppy Weed		
	control	4:00 a.m.	9:00 a.m.	control	4:00 a.m.	9:00 a.m.		control	4:00 a.m.	9:00 a.m.
TaGSTU1B	0.000	2.226	4.434	0.000	−0.211	0.705	**PrGSTU1**	0.000	−0.774	0.295
TaGSTU3	0.000	2.736	2.446	0.000	0.904	0.908	**PrGSTU2**	0.000	−0.097	0.844
TaGSTF1	0.000	2.737	2.352	0.000	0.935	0.934	**PrGSTU3**	0.000	0.991	−0.011
TaGSTF3	0.000	2.354	3.115	0.000	0.249	0.067	**PrGSTU4**	0.000	2.663	1.315
TaGSTF6	0.000	2.497	2.380	0.000	0.405	−0.420	**PrGSTF1**	0.000	−1.138	−0.446

Figure 7. The relative expression level of 5-5 glutathione transferase (*GSTF, GSTU*) genes in leaves of wheat (*Triticum aestivum* cv. GK Ígéret and cv. GK Arató) and poppy weed (*Papaver rhoeas*). Relative transcript amounts of genes were determined by qPCR in leaf samples treated with sterile water and flumioxazin at different times: at dawn (4:00 a.m.) and in the light period (9:00 a.m.). Sampling was performed in the second light cycle at 9:00 a.m. Log2 of $2^{-(\Delta\Delta Ct)}$ data are presented as a heat map. Green colours show repression, while red colours show activation, as it is indicated on the upper colour scale bar.

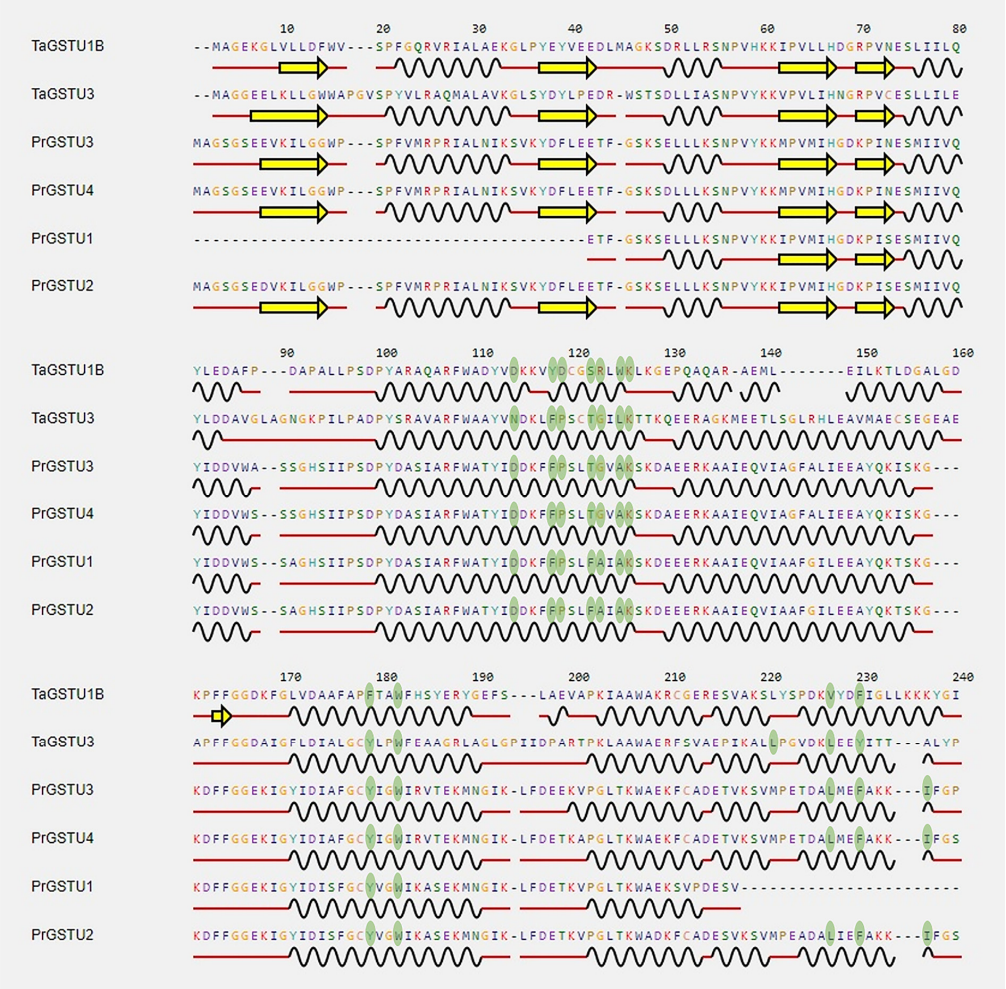

Figure 8. Alignment of the tau group wheat and poppy GST proteins and their secondary structure (2dSS, http://genome.lcqb.upmc.fr/2dss/index.html accessed on 14 June 2023). Green cycles denote the residues involved in the composition of the H-site (substrate binding site) of the enzymes.

4. Discussion

Weed control is of economic significance; thus, optimisation of herbicide utilisation has become one of the most important agricultural issues today [63]. In order to successfully control weeds in the future, it is necessary to develop new, innovative solutions. According to previous studies [64], the time of application can influence the effect of herbicides whose mode of action is related to the photosynthesis of plants. It is known that the phytotoxicity of some herbicides is higher in strong sunlight than in low light [2,3]. Due to this property, the optimal time of herbicide application—especially in the morning, when the activity of antioxidant enzymes is relatively low—can be one of the keys to the optimisation of herbicides [64].

The maximal quantum yield of the PSII photochemistry (F_v/F_m) of wheat (GK Arató and GK Ígéret) and poppy weed plants underlined the selective effect of herbicide treatments: the application of flumioxazin at both 4:00 a.m. and 9:00 a.m. reduced F_v/F_m only in poppy weed. At the same time, the values of Fv/Fm parameters revealed that the various flumioxazin treatments did not cause irreversible damage to the PSII reaction centres after 24 h [47], but the quantum yield decreased due to the herbicide-induced oxidative stress, which was in correspondence with the chlorophyll a+b concentration.

The antioxidant system of plants keeps ROS below harmful levels [15,16]. A possible first step of this process is the dismutation of superoxide radicals into molecular oxygen and H_2O_2, which is catalysed by the enzyme SOD [65]. H_2O_2 can be further utilised by CAT or by Class III peroxidases (PODs), which contribute to maintaining low levels of hydrogen peroxide [66–68]. It was reported that SOD activity is the highest at the end of the light period and decreases during the night in tomato plants [19]. In common poppy plants, the significant decreases in SOD, CAT, and POD activities indicate an herbicide caused the reduced antioxidant defence potential of the weed plant. The early application of the herbicide caused changes in wheat SOD and CAT enzyme activities; in this case, the 4:00 a.m. application of flumioxazin was a little more effective. The changes in the amount of hydrogen peroxide can be caused by large differences measured in the activity of the POD enzyme and reductions in its activity due to treatments (in the case of common poppy). The effect of the treatment in the concentration of hydrogen peroxide was also shown in the case of wheat, but assuming an effective antioxidant defence mechanism (e.g., high peroxidase activity) or effective harmful secondary metabolic product detoxification (e.g., by GST), there was no large difference in the photosynthetic pigment content and F_v/F_m value change. In addition, a correlation can be assumed between the higher POD activity measured in untreated wheat and the higher hydrogen peroxide level.

In addition to the generally estimated antioxidant enzymes, GST plays an important role in the plant detoxification system. This enzyme also exhibits circadian- and light-dependent activity, with maximum activity in the light period and in the early dark period [21]. In the two cultivars of wheat, GST activity was multiple times higher than that of the common poppy. Both herbicide treatments caused a significant GST activity increase (measured with CDNB substrate) in common poppy and wheat cultivars. Edwards et al. [69] reported that enhanced GST expression in wheat and its relatives is not the result of modern breeding, but is rather a characteristic of all the ancestral Triticum species used in its domestication.

The herbicide treatments caused several GST expression changes in wheat and common poppy plants: in wheat lines, the chosen all tau and phi group GST genes were induced, while in common poppy, higher inductions were only detectable in the case of one tau group gene (*PrGSTU4*). The highest elevation in the transcript amounts in wheat was connected to the tau group in wheat also (*TaGSTU1B*).

It was reported that the H-site in wheat TaGSTU1B contains Trp in the 117th position, while in the same positions, there is Leu in TaGSTU3 and Ala in all of the poppy GST proteins. In the case of some wheat GSTs (including TaGSTU1B and TaGSTU3), the enzyme activities (nkat mg^{-1} protein) were determined by Thom et al. [37]. Despite the fact that the Leu side chain in TaGSTU3 is smaller than that of the Trp117 of TaGSTU1B, the activity

of TaGSTU1B was 66 times higher than that of TaGSTU3. Among the previously described conserved structures of tau class GSTs [70], the residue Phe153 (position in TaGSTU4) [37] is part of the loop region between helices 5 and 6 present in all of the selected poppy and wheat tau class GSTs measured in this study (Phe148 in TaGSTU1B).

According to Thom et al. [37], TaGSTU3, among the wheat tau group sequences, has an unusual sequence including a unique three-amino-acid (residue) insertion before the Ser in the catalytic place. These, together with other differences in the amino acid sequence, led to the conclusion that this enzyme may not have a role in detoxification via glutathione conjugation [37]. The amino acid residues before the catalytic Ser differed in TaGSTU3 compared with the common poppy GSTs.

The second and third amino acids in the H-site are Tyrosine and Aspartic acid in TaGSTU1B, while in TaGSTU3 and the entire tau group GSTs in poppy, it is Phenylalanine and Proline. According to previous analysis of their secondary structure, these changes in the H-site (especially the Tyrosine—Phenylalanine), together with the changes in the 4th and 5th amino acids of the H-site, significantly influence the nature of the hydrophobic pocket of the H-site [37], which can lead to changed substrate (herbicide or stress metabolite) acceptance. Differences are presented in Figure 8.

The high degree of GST variability difference between wheat and poppy, which was also reflected in the secondary structure, can be observed even after the tau-group GST subgroup determination of poppy. Monticolo et al. [71] reported that the large and variable group of tau GSTs can be divided into subclasses in *Arabidopsis* and tomato. Their results highlighted that *Arabidopsis* tau call can be divided into four subclasses, and five subclasses were identified in tomato. Using homology searching of protein and cDNA sequences, we managed to classify the selected wheat and poppy GST sequences. TaGSTU1B and TaGSTU3 were found to be similar to tomato subclass 3 proteins and subclass 1, while all of the tau GST sequences were found to be similar to subclass 1. These data, confirmed by the differences in the secondary structures of GSTs, led to the conclusion that the GSTs of the tau group in wheat show greater diversity: they have at least one more subgroup (based on the poppy sequences available so far). Wheat TaGSTU1B, which represents the group missing in poppies, is light-regulated and drought- and senescence-induced, based on our previous data [22,56].

5. Conclusions

As a conclusion to the antioxidant defence and GST gene expression data, the application of the photosynthesis inhibitor herbicide flumioxazin in the dark could allow the wheat defence to switch on before the herbicide effect could appear in the light period. Based on our results, in the background of the greater and more successful flumioxazin response may be—among other things—the interspecies differences in GST and POD activity and the higher degree of variability of the tau GST genes of wheat compared to poppies: based on data so far, a tau GST subgroup is missing in poppy plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13082053/s1, Table S1. List of the used primers and the sequences of the selected *Papaver* genes.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to technical reasons.

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