Journal of Plant Sciences
2019; 7(6): 158-163
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20190706.15
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Responses of Detoxification Enzymes and Genes to Nicosulfuron Stress in Two Genotypes of Sweet Corn Differing in Nicosulfuron Tolerance

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To cite this article:
Guihua Lv, Guojin Guo, Jianjian Chen, Xiangnan Li, Zhenxing Wu. Responses of Detoxification Enzymes and Genes to Nicosulfuron Stress in Two Genotypes of Sweet Corn Differing in Nicosulfuron Tolerance. *Journal of Plant Sciences.* Vol. 7, No. 6, 2019, pp. 158-163.
doi: 10.11648/j.jps.20190706.15

Received: October 15, 2019; **Accepted**: November 12, 2019; **Published**: December 4, 2019

Abstract: Nicosulfuron, a sulfonylurea herbicide is registered for use on field, controns many annual and perennial grass weeds. The accumulation of nicosulfuron will increase toxicity and induce unfavorable oxidative stress in plants. Acetolactate synthase (ALS, EC 2.2.1.6) and glutathione transferases (GSTs, E. C.2.5.1.18), which participate in detoxification of xenobiotics and limit oxidative damages of cellular macromolecules, are important groups of cytoprotective enzymes. This study aims to investigate the toxic effect of nicosulfuron on the detoxification enzyme activities and gene expressions in maize seedling. Specifically, Nicosulfuron-tolerant sweet inbred line 'JP233' and nicosulfuron-sensitive sweet inbred line 'Z2H4' are subjected to 80mg kg^{-1} nicosulfuron treatment when the fourth leaves are fully developed, and the resulting effects are compared to those processed by water. After nicosulfuron treatment, it is found that ALS and GST enzymes activities of Z2H4 are significantly lower than those of JP233. Compared to Z2H4, nicosulfuron treatment increases the expression levels of *GST1*, *ALS1* and *ALS2* genes in JP233. These results suggest that the increased transcription level of these detoxifying enzymes might play a vital role in reducing the toxicity of nicosulfuron and the oxidative stress induced by nicosulfuron in maize seedlings. The research will improve our understanding of the function of maize detoxification enzymes and genes in herbicide metabolism.

Keywords: Nicosulfuron, Sweet Corn, Acetolactate Synthase, Glutathione Transferase, Gene Expression, Oxidative Stress, Toxicity

1. Introduction

Acetolactate synthase (ALS, EC 2.2.1.6) is the first enzyme to participate in the biosynthesis of branching chain amino acids (valine, leucine and isoleucine), and is the target enzyme of five groups herbicide metabolism, which corresponding to imidazolinones (IMI), sulfonylureas (SU), pyrimidinyl-thiobenzoates (PTB), triazolopyrimidines (TP) and sulfonyl-aminocarbonyltriazolinones (SCT) [1-3]. The SU and IMI herbicides are not competitive inhibitors of ALS because they do not directly bind to the active site of the substance. Instead, these herbicides bind within the substrate-access channel of the ALS enzyme in plants [4]. Duggleby et al. (2008) showed that SU herbicides are better ALS inhibitors than IMI herbicides [1]. Because the SU herbicides feature more hydrogen bonds in the chemical structure, which can block the pathway at a position much closer to the ALS active site in the depth of the channel, thereby inhibiting the activity of ALS. A large number of studies have demonstrated that mutations in ALS-related loci will damage the activity of ALS enzymes, causing plants lose their resistance to herbicides [5, 6]. In addition, repeated use of the same herbicide will also diminish plants' resistance to herbicides. Therefore, it is very necessary to understand the intrinsic mechanism of ALS enzyme in improving the resistance of maize to nicosulfuron from physiological, biochemical and molecular level.

The plant glutathione transferase, also referred as glutathione S-transferase (GST, EC 2.5.1.18), is a large and

diverse group of enzymes that catalyze the binding of electrophilic xenobiotic substrates to the tripeptide glutathione (GSH) [7]. GSTs play a vital role in herbicide detoxification, tyrosine metabolism, hormone homeostasis, hydroxyperoxide detoxification, regulation of apoptosis and responses to biotic and abiotic stresses in plants [8, 9]. Many studies have shown that the over-expression of specific GST isozymes in transgenic plants will enhance plant tolerance to herbicides and oxidative stress [2, 3, 10]. The importance of GST in herbicide metabolism has been clearly demonstrated in transgenic GST plants, particularly for tobacco. For instance, Karavageli et al. (2005) transferred Corn *GST1* to tobacco, realizing significantly improved tolerance to alachlor in transgenic tobacco [11].

In this study, the sweet corn inbred line JP233 which is tolerant to nicosulfuron, and the sweet corn inbred line Z2H4 that is sensitive to nicosulfuron were employed to: (1) investigate the ALS and GST enzyme activity of maize seedlings in the presence of nicosulfuron and (2) explore the gene expression levels of *ALS* and *GST* in maize seedlings in response to nicosulfuron treatment.

2. Materials and Methods

2.1. Experimental Design

The experiment was conducted at the Tangxi farm of the Dongyang Maize Research Institute of Zhejiang Province (29º16'N, 120º13'E), with a subtropical monsoon climate. The tested soil contained fundamental nutrients composed of 30.26 g kg^{-1} organic matter, 3.27 g kg^{-1} total nitrogen, 119.32 mg kg^{-1} alkaline hydrolyzed nitrogen, 20.29 mg kg^{-1} available phosphorus, and 108.28 mg kg^{-1} available potassium. The sweet corn inbred line JP233 (nicosulfuron-tolerant) and the sweet corn inbred line Z2H4 (nicosulfuron-susceptible) were developed by the Dongyang Maize Research Institute of Zhejiang Province. The inbred line JP233 grew normally after being sprayed with nicosulfuron. In contrast, the growth of the inbred line Z2H4 was inhibited by nicosulfuron, which ultimately caused the death of plants. A split-plot experimental design was performed with nicosulfuron treatment to define the main plots and inbred lines within subplots. The row length of one plot was 6 m, and the row width was 0.6 m. We set up eight rows per plot, with each plot measuring 36 m^2 in area.

2.2. Pesticide Treatments

In 2018, a herbicide concentration screening test was designed in the field. Maize seedlings were treated with nicosulfuron at the four-leaf stage using a laboratory pot-sprayer equipped with a nozzle. Nicosulfuron concentrations of 0 mg kg^{-1} (control), 20 mg kg^{-1}, 40mg kg^{-1}, 80 mg kg^{-1}, 120 mg kg^{-1}, 160 mg kg^{-1}, 200 mg kg^{-1}, 240 mg kg^{-1}, 280 mg kg^{-1} and 320 mg kg^{-1} were applied to each of the plot to screen for JP233 and Z2H4 under a wide range of herbicide concentrations. This initial screening test revealed that for a nicosulfuron concentration of 80 mg kg^{-1}, JP233 plants were able to attain normal growth via self-defense mechanism, while Z2H4 plants either wilted or died (Table 1). Then, an experiment was conducted in the field. Maize seedlings were treated with nicosulfuron at the four-leaf stage using a laboratory pot-sprayer, equipped with a nozzle. The nicosulfuron concentration was maintained at 80 mg kg^{-1} to investigate enzyme activities of maize seedlings. It was found that seedlings of Z2H4 either died or wilted after 15 DAT, data were collected every 2 days after 0 DAT.

Table 1. *Effect of nicosulfuron on survival rate of sweet maize seed of JP233 and Z2H4.*

Spraying concentration [mg kg–1]	JP233			Z2H4		
	CK	Number of seedlings after spraying	Survival rate [%]	CK	Number of seedlings after spraying	Survival rate [%]
0	449	449	100	446	446	100
20	447	441	98.65	446	6	1.34
40	443	429	96.84	444	0	0
80	441	431	97.73	445	0	0
120	444	395	89.96	444	-	
160	439	337	76.76	450	-	
200	443	188	42.43	447	-	
240	449	103	22.93	445	-	
280	441	41	9.29	445	-	
320	441	24	5.44	448	-	

2.3. Glutathione S-transferase (GST, EC 2.5.1.18) Activity

After nicosulfuron treatment, at 0 DAT, the seedling leaves were sampled, frozen in liquid nitrogen, and then stored at –80°C until subsequent analysis. Enzyme activities were measured spectrophotometrically and absorbances were recorded with a UNICO™ UV-2000 spectrophotometer (UV-2000, UNICO, USA). Enzymes were extracted by grinding 0.5 g of leaf samples in 5 ml of phosphate buffer (pH 7.5) containing 1 mM EDTA, 1% PVP, 1mM DTT, and 1 mM PMSF. The homogenate was centrifuged at 15,000 × *g* at 4°C for 30 min and the supernatant was collected to measure the enzyme activity.

GST activity levels were assayed according to a method initially described by Mimmo et al. (2015) [12]. Changes in the absorbance of the reaction solution described above at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$) caused by the formation of conjugate production were recorded for 5 min. The enzyme activity was calculated and expressed in nmol $min^{-1}\ g^{-1}$ (FW).

2.4. Acetolactate Synthase (ALS, EC 2.2.1.6)

ALS was extracted from nicosulfuron treatment maize leaves using methods of enzyme extraction from Fan et al. (2001) [13]. Enzymes were extracted by grinding 5 g of leaf samples in 10 ml of potassium phosphate buffer (pH 7.5) containing 1 mmol L^{-1} sodium pyruvate, 0.5 mmol L^{-1} TPP, 10 μmol L^{-1} FDA, and 0.5 mmol L^{-1} $MgCl_2 \cdot 6H_2O$. The homogenate was filtered with 8 layers of gauze and centrifuged at 24,400 × *g* at 4°C for 20 min, the supernatant was slowly added to $(NH_4)_2SO_4$ to 50% saturation. After the precipitation for 2 hours, enzyme pellet was centrifuged at 24,400 × *g* at 4°C for 30 min, the precipitate was dissolved in 6ml enzyme solution (0.1mol L^{-1} potassium phosphate buffer, 20 mmol L^{-1} sodium pyruvate, and 0.5 mmol L^{-1} $MgCl_2 \cdot 6H_2O$). The crude enzyme solution was obtained, and stored at -20°C. The above operations were all performed at 0–4°C.

A reaction mixture containing 0.9ml enzyme reaction liquid (0.1mol L^{-1} potassium phosphate buffer, 20 mmol L^{-1} sodium pyruvate, 0.5 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 0.5 mmol L^{-1} TPP, and 10 μmol L^{-1} FDA), 1ml of enzyme extract, and 0.1 mol L^{-1} potassium phosphate buffer (pH 7.0) was heated to 35°C for 1 h in the dark, then add 0.2mL of 3 mol L^{-1} H_2SO_4 to stop the reaction, decarboxylate for 15min in a 60°C water bath, add 1mL of 0.5% creatine and 1mL of 5% naphthol (dissolved in 2mol sodium hydroxide solution) to the mixture to determine the acetoin by color development at 60°C water bath for 15 min, cooling to room temperature. The red color complex was cooled to temperature by water and the absorbance was measured at 525 nm by using a UNICO^{TM} UV-2000 spectrophotometer (UV-2000, UNICO, USA). ALS specific activity was demonstrated as nmol acetoin mg^{-1} protein h^{-1}. Protein has been measured by the method of Coomassie blue according to Bradford (1976) [14].

2.5. RNA Isolation and Real-time RT-PCR

Maize seeds were soaked for 12h at 25°C, and germinated for 24h at 27°C until white. Seeds of uniform size were selected and planted in plastic pots with fertile soil. In order to avoid the impact of the environment on the growth, the seeded plastic pots were placed in an artificial climate chamber with a photoperiod of 24 h, an illumination intensity of 12,000lx, a culture temperature of 25°C during the day, 22°C at night, and a relative humidity of 70%. Watering every day during the culture period. Maize seedlings were treated with nicosulfuron at the four-leaf stage using a laboratory pot-sprayer, equipped with a nozzle. Water treatment as a control. According to Liu et al. (2015) [15], the best response time for corn to nicosulfuron herbicide is 24h. Therefore, after spraying herbicide treatment for 24h, samples were frozen in liquid nitrogen and stored in -80°C refrigerator for the determination of gene expression.

Total RNA of maize leaves (control and treatment of JP233 and Z2H4) was isolated via the RNA pure Plant Kit (Kangwei company, China). One microgram of total RNA was used to synthesize the cDNA using the HiScript II Q RT Super Mix reagent (Vazyme, China). Primers were designed using the Premier primer 3.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) according to gene sequences in the GenBank database. The PCR efficiency of all primers for gene expression analysis was examined (Table 2). Only primers with higher amplification efficiency (>90 %) were used for this experiment. Maize cDNA sequences of ALS and GST were searched against GeneBank, www.maizegdb.org and www.ncbi.nlm.nih.gov/ (Table 3). PCR amplification was performed with the HiScript II Q RT Super Mix reagent (Vazyme, China) on a real-time PCR detection system according to the manufacturer's instructions (CFX Connect Optics Module, Singapore). The $2^{-\Delta\Delta ct}$ method was used to calculate the relative transcript levels, and the *gapdh* gene was used as the house-keeping gene. Three biological repeats were performed for each analysis. The expression of each transcript was normalized against the amount of *gapdh* control transcript in each sample. Values are means ± standard error (SE) of three biological repeats.

Table 2. *House-keep ing gene and RT-PCR primer information.*

Sequence ID	Forward Primer	Reverse Primer
GRMZM2G116273	GGGGAACCACCGACCAGAAAG	GCGTAGGGCGTAGCAAACAGG
GRMZM2G143008	TTCTTCCTCGCCTCCTCTGGTC	ACAAAGCGTCGCAACTCCTCAC
GRMZM2G143357	TGCTAAAGGGTTCAACATTCC	ACAGTCCTGCCATCACCATCC

Table 3. *Information about candidate genes.*

Name	Locus name	Location	Chromosome	Transcript length (bp)	Translation length (aa)
GST1	GRMZM2G116273	178412108-178414563	8	929	214
ALS1	GRMZM2G143008	167860522-167862740	5	2233	638
ALS2	GRMZM2G143357	101325841-101328029	4	2189	638

2.6. Data Analysis

Microsoft Excel and SigmaPlot 12.5 were used for data processing and mapping, and each reported data point represents the mean ± standard error (SE) of three replicates combined in the three experimental repeats. SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) was used to conduct an analysis of variance (ANOVA), and mean values were compared via the least significant difference (LSD) test at a p-value < 0.05 threshold indicated significant difference.

3. Results

3.1. ALS Enzyme Activity

After nicosulfuron treatment, the ALS activity of JP233

(nicosulfuron-tolerant) increased at 1 DAT and no significant change was observed subsequentlly, while the ALS activity of Z2H4 (nicosulfuron-susceptible) significantly decreased at 7 DAT (Figture 1). Compared to JP233-CK, the ALS activity of JP233 was significantly increased at 1, 3, 5, and 7 DAT by 129.40%, 113.22%, 94.24%, and 73.81%, respectively. However, compared to Z2H4-CK, nicosulfuron treatment significantly decreased the ALS activity of Z2H4 at 7 DAT by 41.55% (Figure 1). These results indicate that JP233 might resist the negative effects of nicosulfuron through regulating the activity of ALS enzyme.

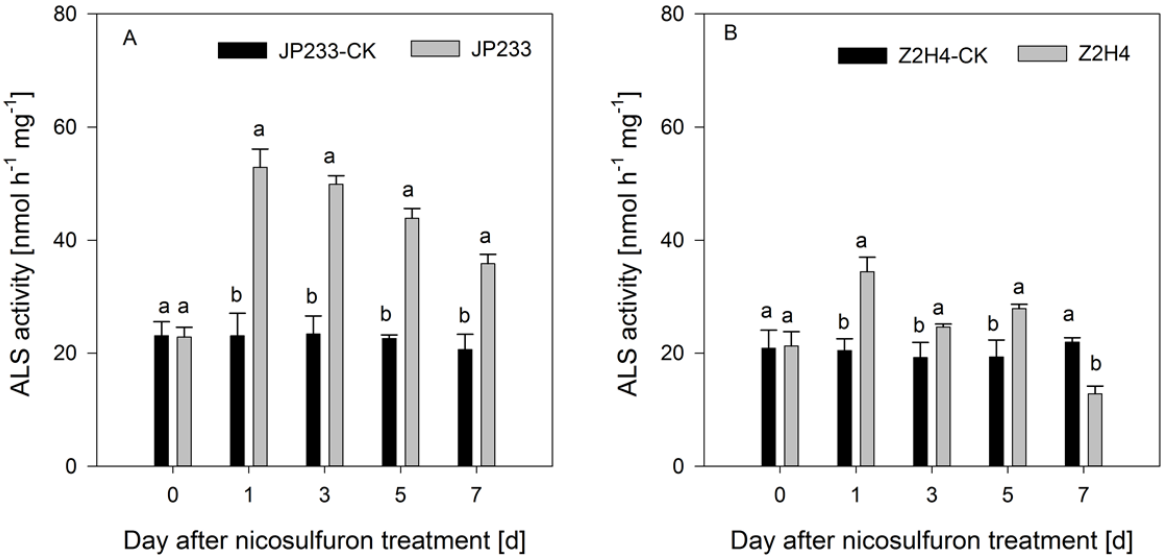


Figure 1. *Effects of nicosulfuron on acetolactate synthase (ALS) in leaves of JP233 and Z2H4. JP233-CK: water treatment in JP233; JP233: nicosulfuron 80 mg kg^{-1} treatment in JP233; Z2H4-CK: water treatment in Z2H4; Z2H4: nicosulfuron 80 mg kg^{-1} treatment in Z2H4. Vertical bars represent the SE (n = 3). Small letters indicate differences in values obtained on different days after nicosulfuron treatment (P<0.05) according to a least significant difference (LSD) test.*

3.2. GST Enzyme Activity

Nicosulfuron treatment induced significantly increasement of GST activity in JP233. Compared to JP233-CK, the GST activity of JP233 significantly increased at 1, 3, 5, and 7 DAT by 43.29%, 51.42%, 56.71%, and 52.23%, respectively. Instead, the GST activity of Z2H4 reached its maximum value at 3 DAT, and then steadily reduced. After 7 DAT, GST activity of Z2H4 was 29.05% lower than that of Z2H4-CK (Figure 2). The GST activity of JP233 was 35.45−48.29 nmol min^{-1} g^{-1} (FW) after 1 DAT, with an average of 44.49 nmol min^{-1} g^{-1} (FW). By contrast, the GST activity of Z2H4 was 15.13−39.29 nmol min^{-1} g^{-1} (FW), with an average of only 28.81 nmol min^{-1} g^{-1} (FW). The average GST activity of JP233 was 54.43% higher than that of Z2H4. These results imply that sweet corn inbred line JP233 might obtain a strong anti-detoxification ability by increasing the GST activity.

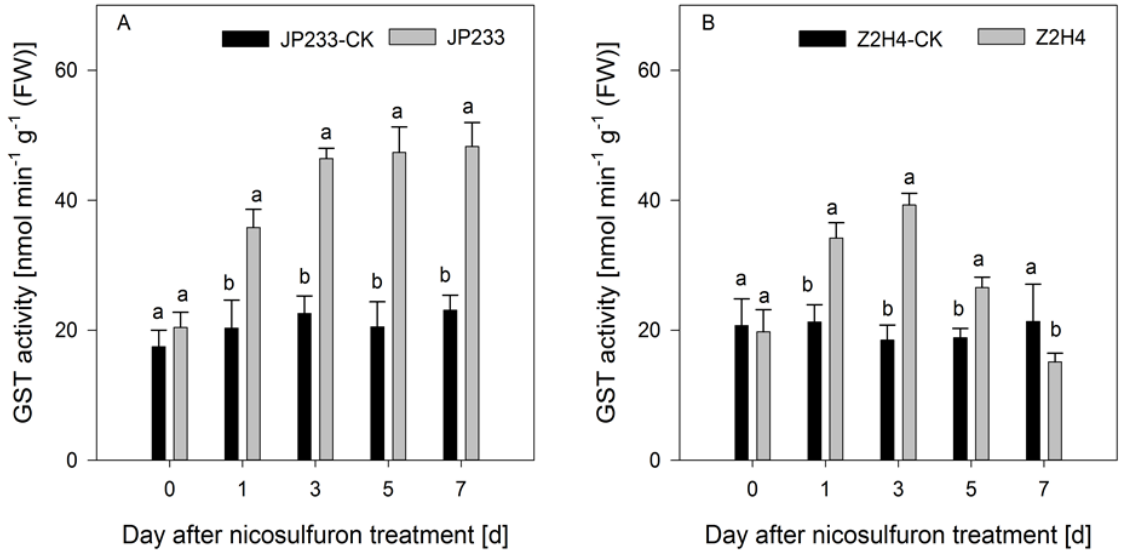
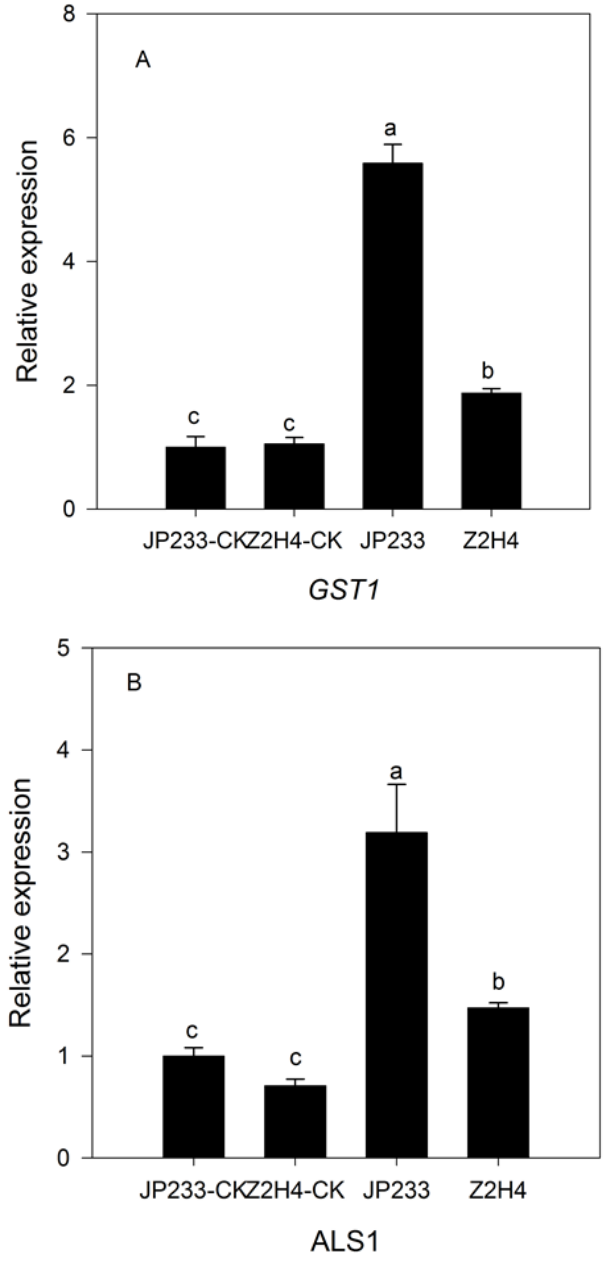


Figure 2. *Effects of nicosulfuron on glutathione transferases (GST) in leaves of JP233 and Z2H4. JP233-CK: water treatment in JP233; JP233: nicosulfuron 80 mg kg^{-1} treatment in JP233; Z2H4-CK: water treatment in Z2H4; Z2H4: nicosulfuron 80 mg kg^{-1} treatment in Z2H4. Vertical bars represent the SE (n = 3). Small letters indicate differences in values obtained on different days after nicosulfuron treatment (P<0.05) according to a least significant difference (LSD) test.*

3.3. Gene Expression Involved in Nicosulfuron Detoxification

The expression levels of detoxification enzymes in leaves of maize and their control group were measured 24 h after nicosulfuron treatment. The obtained results showed that, compared to the control, the *GST1* gene was significantly upregulated in both inbred lines, while *GST1* transcription level of JP233 was significantly higher than that of Z2H4. In addition, nicosulfuron also remarkably affected the expression level of *ALS* genes. After nicosulfuron treatment, *ALS1* and *ALS2* of JP233 were significantly higher than those of control at 24h treatment, showing enhances of 219.03% and 127.86%, respectively (Figure 3). While the expression levels of *ALSs* in Z2H4 had no significant variation, although slight increase was found in *ALS1*.



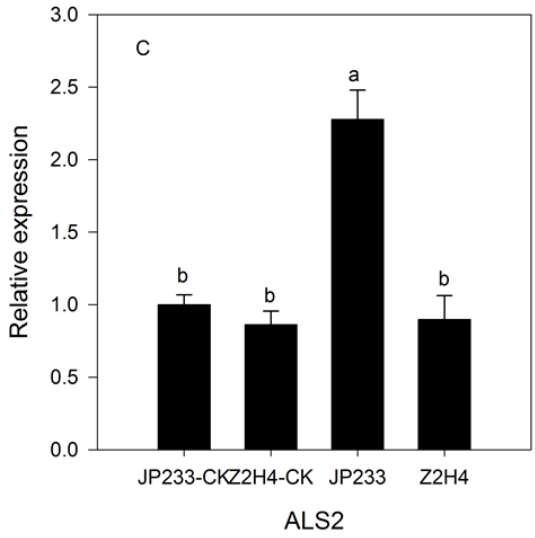


Figure 3. *Transcriptional levels of different antioxidant detoxification enzymes, expressed relative to the control, in leaves of maize seedlings exposed to nicosulfuron (A. GST1, B. ALS1, C. ALS2). JP233-CK: water treatment in JP233; JP233: nicosulfuron 80 mg kg^{-1} treatment in JP233; Z2H4-CK: water treatment in Z2H4; Z2H4: nicosulfuron 80 mg kg^{-1} treatment in Z2H4. Vertical bars represent the SE (n = 3). Small letters indicate differences in values obtained on different days after nicosulfuron treatment (P<0.05) according to a least significant difference (LSD) test.*

4. Discussion

ALS is a key enzyme in the metabolism of sulfonylurea herbicides. The tolerance of plants to sulfonylurea herbicides is closely related to the activity of ALS enzymes [16]. In this study, after nicosulfuron treatment, the ALS activity of JP233 increased significantly compared with that of the control. While in Z2H4, the activity of ALS enzymes increased initially and then decreased significantly (Figure 1). It suggested that nicosulfuron might effectively inhibit the ALS enzyme activity in sensitive plants. In addition, we also measured the expression of genes encoding detoxification enzyme after a nicosulfuron treatment time of 24 hours. The expression level of *ALS1* and *ALS2* genes of JP233 were significantly upregulated, while *ALSs* genes of Z2H4 did not change significantly compared to that of the control (Figure 3B and C). This indicated that the *ALS1* and *ALS2* genes might actively respond to the metabolism of the nicosulfuron herbicide in resistant plants. These genes were closely related to the detoxification of nicosulfuron in maize.

Extensive studies have revealed that plant resistance to herbicides is related to GST enzyme activity, indicating that GST plays an important role in the metabolic detoxification of herbicides in plants [3, 10]. Hatton et al. (1996) reported that the enhanced GST enzyme activity significantly increased the resistance of soybeans and corn to herbicides [17]. Sommer and Boger (1999) studies evidenced a similar increase in the activity of GST, which helps to enhance the resistance of plants to herbicides [18]. In this study, after spraying herbicide, the GST enzyme activity of resistant inbred line JP233 increased substantially, and the activity of GST enzyme remained at a high

level. While the GST activity of Z2H4 increased initially and then decreased rapidly. It was reasonably to conclude that Z2H4 had no sustained resistance to herbicide stress. After 24 h of spraying, the *GST1* gene was up-regulated in both inbred lines. This could account for the increase of GST enzyme activity in the two inbred lines in the early stage of spraying. However, *GST1* of JP233 was 216.02% higher than that of Z2H4. This might explain why JP233 could resist herbicides compared with Z2H4. At the same time, the continuous increase of stress time might attribute to the decline of GST enzyme activity in Z2H4. Sytykiewicz (2011) used juglone to treat maize seedlings and measured the expression of *GST1* gene at 4, 6 and 8 days after treatment. The expression of *GST1* gene increased considerably at 4 days after treatment [19]. The expression of *GST1* gene decreased significantly in the 6 and 8 days after treatment. It indicated that with the prolongation of the treatment of corn seedlings by juglone, the increasing oxidative stress in the seedlings disturbed the redox balance in the plants, which eventually led to a decrease in the expression of *GST1* gene and a significant reduce in GST activity.

5. Conclusion

In this study, the enzymes activities of ALS and GST in nicosulfuron-tolerant sweet inbred line 'JP233' are significantly higher than that in nicosulfuron-sensitive sweet inbred line 'Z2H4' under nicosulfuron treatment. Meanwhile, the detoxification related genes *GST1*, *ALS1* and *ALS2* in JP233 also show a higher transgene level compared to. Z2H4. These results suggest that the increased transcription level of these detoxifying enzymes and genes might play a vital role in reducing the toxicity of nicosulfuron and the oxidative stress induced by nicosulfuron in maize seedlings.

Acknowledgements

This work was supported by maize breeding support discipline of Zhejiang Academy of Agricultural Sciences (10102000318CC1601G/018), Hebei Natural Science of Foundation (304020163) and Major Agriculture Science Foundation of Upland Grain Crops Breeding of Zhejiang Province (grant No.2016C02050-9-1).

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