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RESEARCH ARTICLE

The regulation of *glutathione s-transferases* by gibberellic acid application in salt treated maize leaves

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Abstract Plant hormones and antioxidant system changes occur during plants' exposure to stress conditions. Although the interactions of some plant hormones (abscisic acid, salicylic acid, jasmonic acid, nitric oxide, and ethylene) with the glutathione *s*-transferase (GST) enzyme, which is one of the antioxidant enzymes, have already been reported, the influence of gibberellic acid (GA_3) on this enzyme under saline conditions has not yet been reported. Plant material for the experiments was obtained from M14G144 cultivar of maize (*Zea mays* L.) plants grown as a soil culture in growth chambers at 22 °C, 65–70% moisture, 16-h light/8-h dark conditions, and with full strength Hoagland solution for 8 days under controlled growth conditions. Then, the plants were exposed to salt stress (350 mM NaCl and 100, 300, and 500 ppm GA_3) simultaneously. In maize leaves, GA_3 treatment alleviated the physiological parameters under salt stress. Specifically, the treatments with 100 and 500 ppm of GA_3 were able to trigger GST enzyme and isoenzyme activities as well as hydrogen sulfide accumulation and anthocyanin content, although the lowest malondialdehyde, hydrogen peroxide, and superoxide radical content were under the treatment of 300 ppm of GA_3. Besides this, GST gene expression levels were found to be upregulated between 1.5 and fourfold higher in all the plants treated with GA_3 at different concentrations in proportion to salt stress. These results first indicated that the reason for the changes in GA_3-treated plants was the stimulating role of this hormone to maintain GST regulation in maize plants.

Keywords Anthocyanin · Phytohormone · Reactive oxygen species · Salinity · *Zea mays*

Introduction

Plant hormones are important chemicals that play efficient roles in plants under stressful conditions. Many researchers point out that these hormones (cytokinin (CTK), abscisic acid (ABA), salicylic acid (SA), gibberellic acid (GA_3), jasmonic acid (JA), auxin (IAA), and nitric oxide (NO)) could regulate response mechanisms by inducing osmolyte accumulation, the antioxidant defence system, increasing photosynthetic capacity, and regulating ion balance. One of these hormones, gibberellic acid, is a plant hormone with a tetracyclic diterpene structure that plays major roles in seed germination, stem elongation, the beginning of flowering, nutrient uptake, and fruit development (Li et al. 2010).

The DELLA (aspartic acid–glutamic acid–leucine–leucine–alanine) proteins have been known to act as molecular switches in GA_3 signaling. They are negative regulators of this signalling, and their degradation in the ubiquitin/proteosome pathway has been identified as the key event of the GA-stimulating process (Gomi and Matsuoka 2003). Lastly, it was determined that the soluble receptor GA-Insensitive Dwarf 1 (GID1), in rice and Arabidopsis, binds the bioactive GA hormone and generates the GID1-GA complex, which suppresses DELLA proteins (Ueguchi-Tanaka et al. 2005). The accumulation of DELLA transcriptional regulators represses GA signalling and limits plant growth.

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In recent years, gibberellic acid has been used as a "regulator" to increase stress tolerance in plants. Research with GAs showed that exogenous applications of GA_3, such as foliar spraying and seed priming, increased plant performance under stress (Sofy et al. 2016; Jiao et al. 2019). GA_3 increases sugar and proline content, H_2S production, macro elements, antioxidant enzyme activities, and regulates lipid biosynthesis in plants under stress conditions (Liu et al. 2018; Wang et al. 2019a, b; Kaya et al. 2020; Ben Rhouma et al. 2020; Seckin Dinler et al. 2021). GA_3 interacts with other hormones, particularly ABA and ethylene, via DELLA proteins for growth regulation, as demonstrated under stress conditions (Gantait et al. 2015). GA draws attention through its antagonistic crosstalk with ABA during the germination stage in plants, which was determined in Arabidopsis mutants (Seo et al. 2006; Kundu and Gantait 2017). Therefore, it is substantial to discuss the effects of GA_3 on ABA accumulation under stress conditions during the seedling stage as well as germination time (Yuan et al. 2011).

Glutathione *s*-transferase (GST; E.C. 2.5.1.18) constitute a multiple-functional enzyme family that includes plant growth, development, and stress response. Firstly, it was discovered in the 1960s with the use of drugs in the detoxification of the metabolism of animals (Wilce and Parker 1994). It was discovered in plants in the 1980s under herbicide (chloro-s-triazine) toxicity via conjugation reactions in maize (Timmerman et al. 1989). This family is mainly used in plants as a detoxification pathway to produce S-glutathionyl (R-SG) products by conjugating glutathione (γ-Glu-Cys-Gly) to various hydrophobic, electrophilic, and xenobiotic compounds (Marrs et al. 1996). However, soluble GSTs are classified as GSTU (tau), GSTF (phi), GSTT (theta), GSTZ (zeta), GSTL (lambda), DHAR (dehydroascorbate reductase), and TCHQD (tetrachlorohydroquinone dehalogenase). Beside this, GSTs were identified in many plants, such as *Zea mays, Glycine max,* and *Arabidopsis thaliana,* which have 101, 55, and 82 GSTs, respectively (McGonigle et al. 2000; Estevez and Hernandez 2020).

Additionally, different studies have proposed the relevance of GSTs in cell signalling, modification of ion channels, oxidation–reduction reactions, and post-translational (PT) glutathionylation of proteins (Dixon 2010). It is also known that this enzyme plays a role in signal transduction and is a transporter of anthocyanin, flavonol, salicylic acid, and auxin homeostasis (Chen and Singh 1999; Edwards and Dixon 2005; Gill and Tuteja 2010; Yu and Powles 2014; Liu et al. 2019). As Edwards et al. (2000) mentioned, the GST enzyme could be used as a marker for stress tolerance in plants.

The most widely used method for improving stress resistance in plants is the transgenic plant created with GSTs. For example, the overexpression of the *TaGSTU1B* and *TaGSTF6* genes increased the drought stress in *Triticum aestivum* plants (Nutricati et al. 2006). Furthermore, ectopic expression of *OsGSTU4* genes and tau class GSTs in rice plants resulted in salt stress tolerance in Arabidopsis plants (Sharma et al. 2014). Additionally, Arabidopsis plants showed increased tolerance to salt and drought stress via overexpressed *LeGSTU2* gene expression (Xu et al. 2015). Benekos et al. (2010), on the other hand, reported that overexpression of GmGST in tobacco plants increased tolerance to the herbicide alachlor, which inhibited plant growth. It has recently been reported that *Juglans regia JnGSTTau1* plays a beneficial role in osmotic tolerance (Yang et al. 2019). Lastly, Galle et al. (2021) reported that there is a positive correlation between GST transcription and GSH redox potential.

Since its first detection in maize plants, it has been proven that *glutathione S-transferases* are significant enzymes in plants' lives for stress adaptation as well as their interactions with plant hormones. Aside from its hormone ligand role (auxin, cytokinin), there have been some reports on the relationship between this enzyme and stress-induced plant growth hormones (ABA, ETH, JA, SA, and NO). In the past few years, Levine et al. (1994) and Polidoros and Scandalios (1999) showed that H_2O_2 could induce *GST* gene expression. Apart from this, Moons (2003) first reported that plant stress hormone (ABA) caused an accumulation of *OsGSTU3* under salinity in rice roots. Otherwise, Zettl et al. (1994) reported that GST enzyme activity is stimulated by auxin, salicylic acid, ethylene, methyl jasmonate, and abscisic acid, whereas it is bound by auxin and cytokinins. Moreover, in the Arabidopsis plant, *AtGSTF2* and *AtGSTF6* were encouraged by low amounts of salicylic acid and ethylene (Lieberherr et al. 2003). Also, no donor (SNP) treatment induced *GST* gene expressions in soybean leaves under salt stress (Dinler et al. 2014). In the same year, Csiszar et al. (2014) determined that the high SA concentrations induced GSTs that decreased salinity in tomato. In recent years, studies with genome-wide analysis of GSTs in soybean revealed that 18% of hormone-responsive elements (gibberellins, abscisic acid, ethlylene, methyl jasmonate, auxin, and salicylic acid) were present (Ahmad et al. 2020). Beside this, JA treatment maintained higher GST activities under cromium stress in *Brassica paracihensis* (Kamran et al. 2021). Similarly, Wang et al. (2019a, b) determined that the *TaGSTU62* could be upregulated by ABA and downregulated by GA_3 in *Triticum aestivum* under normal conditions within hours. Lastly, Ahmad et al. (2021) found that individual GA_3 applications caused an increase in GST enzyme activity under cadmium stress in *Cicer arietinum.*

From all these findings, it could be suggested that GST is quite an important enzyme to perceive external signals and interact with stress hormones for plant survival under extreme conditions. As shown in the figure, the findings of this study support this viewpoint by first demonstrating that

GA3 induces GST regulation and then presenting a detailed analysis of physiological and biochemical changes in salt-treated maize leaves.

Material and method

Plant growth

Seeds of maize (*Zea mays* L.) M14G44 cultivar, procurred from (MAY-Agro seed company Bursa), were used as the experimental material for the present study. The seeds were sown in plastic pots, contains 5 seeds and the mixture of soil. After germination, seedlings were grown in a growth room at 25 °C (16 h day/8 h night photoperiod), light intensity of 350 µmol m^{-2} s^{-1} and exposed with Hoagland solution for 3 days. The pots were divided into eight groups. Control (1), NaCl 350 mM (2), GA_3 100 ppm (3), GA_3 300 ppm (4), GA_3 500 ppm (5), NaCl + GA_3 100 ppm (6), NaCl + GA_3 300 ppm (7), NaCl + GA_3 500 ppm (8) and 8 days old plants were applied with Hoagland solution for 10 days. Then, the plants were harvested on the tenth day and the samples were stored at -80 °C. This experiment was done for three replicates. All GA_3 (100, 300, 500 ppm) concentrations were applied with Hoagland solution in soil medium in growth chambers.

Relative water content

The leaves were weighed to determine their fresh weight. The leaves were floated on de-ionized water for 5 h under low irradiance and then the turgid tissue was blotted to removed excess water and their turgid weights were measured. Then, dry weights were determined after the leaves were dried in an oven at 70 °C for 48 h. The relative water content (RWC) was analyzed according to Smart and Bingham (1974).

SPAD analysis

The soil plant analysis index, (SPAD, measurement for chlorophyll content estimation) was evaluated by reading ten individual points on six seedlings of each treatment, which were measured with SPAD (SPAD-502; Konica Minolta Sensing Inc, Japan) equipment. Data were taken every 1–1.5 cm along the full length of each maize leaf.

Proline content

The proline contents of leaves were identified according to Claussen (2002). The absorbance of the reaction mixture was determined at 546 nm.

Superoxide radical content

O_2^- content was measured according to (Ke and Sun 2004) with some modifications. The specific absorption was determined at 530 nm.

Malondialdehyde and hydrogen peroxide content

The level of lipid peroxidation in leaf samples was measured as the malondialdehyde content according to the method specified by Rao and Sresty (2000). The MDA concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to determine the MDA concentration. Hydrogen peroxide level was determined according to Velikova et al. (2000). The absorbance reading was noted at 390 nm.

Determination of GA_3 and ABA levels

GA_3 and ABA levels (µmol g FW^{-1}) were implemented based on the procedures of gas chromatography/mass spectrometry liquid chromatography/tandem mass spectrometry (LC/MS/MS) to identify (Nehela et al. 2016). The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method used a single-step buffered acetonitrile (MeCN) extraction and salting out liquid–liquid partitioning from the water in the sample with $MgSO_4$. Dispersive-solid-phase extraction cleanup is applied to remove organic acids, excess water, and other components with a combination of primary secondary amine sorbent and $MgSO_4$; then the extracts are determined by mass spectrometry (MS) techniques after a chromatographic analytical separation. 2007.01 outlines the protocol in a box format. 1.5 g homogenized sample was weighed into a 5 ml falcon tube and 1.5 ml extraction solution (Acetonitrile containing 1% Acetic acid) was added to it and vortexed or shaken for 15 min. When the shaking process is finished, 1 Quechers extraction salt (6 g $MgSO_4$ + 1.5 g $C_2H_3NaO_2$) was added and shaken vigorously. Then it was centrifuged at 4000 rpm for 5 min. 8 ml of acetonitrile phase (upper phase) is transferred to a 1.5 ml dSPE clean-up tube (400 mg PSA + 1200 mg $MgSO_4$) and vortexed for 1 min. The dSPE tube was centrifuged at 4500 rpm for 5 min. After centrifugation, the solution was filtered through a 0.45 µm syringe filter and injected into the LC–MS/MS. If the sample result was outside the measuring range, the sample was diluted to enter the measuring range.

GSH and GSSG content

Total glutathione was analysed according to the method described by Sahoo et al. (2017). The change in absorbance

Table 1 The effects of gibberellic acid treatment on shoot lenght, relative water content and SPAD values of leaves in maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Columns with different letters represent significantly different ($P < 0.05$) values

	Shoot lenght (cm)	RWC (%)	SPAD
C	43.33 ± 1.528^{a}	93.39 ± 3.317^{a}	37.333 ± 3.194^{b}
S	22.66 ± 3.05^{d}	71.042 ± 0.669^{d}	28.667 ± 2.454^{a}
GA_3 (100 ppm)	44 ± 3.000^{a}	94.95 ± 4.514^{a}	41.067 ± 2.074^{b}
GA_3 (300 ppm)	48.66 ± 4.04^{b}	91.65 ± 3.264^{a}	39.800 ± 0.954^{c}
GA_3 (500 ppm)	48.33 ± 2.517^{b}	93.47 ± 7.563^{a}	40.533 ± 0.709^{c}
GA_3 (100 ppm) + S	34 ± 4.000^{c}	77.25 ± 5.983^{d}	39.367 ± 2.701^{b}
GA_3 (300 ppm) + S	39 ± 1.732^{c}	85.07 ± 0.564^{b}	41.867 ± 0.709^{b}
GA_3 (500 ppm) + S	37 ± 1.00^{c}	80.57 ± 5.384^{c}	39.233 ± 2.146^{b}

at 412 nm was measured. For the GSSG assay, the rate of absorption change at the same absorbance was determined spectrophotometrically.

GR enzyme activity

The glutathione reductase (GR; EC 1.6.4.2) activity was determined in accordance with Foyer and Halliwell (1976). The reaction mix contained 25 mM sodium phosphate buffer (pH 7.8), 0.12 mM NADPH.$Na_{4,}$ 0.5 mM GSSG, and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was read at 340 nm. Activity was calculated using the extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}). One unit of GR was defined as 1 mmol ml^{-1} reduced GSSG min^{-1}. The specific enzyme activity for all enzymes was expressed as mg^{-1} protein.

GST enzyme activity

The glutathione *s*-transferases (GST; EC 2.5.1.18) activity was measured with Habig et al. (1974). The absorbance change in 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using as substrate at the presence of reduced glutathione (GSH). The 1.0 ml in 2% ethanol enzyme assay mix contained 1.0 mM GSH (0.05 ml), 0.5 mM CDNB (0.02 ml), 0.68 ml of distilled water and 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4; pH 6.5). The enzyme activity stated as nmol CDNB conjugate formed/min/mg proteins and in nmol CDNB conjugate/min/g wet tissue, using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

GST isoenzyme activity

The equal amounts of protein were run in 10% (w/v) native PAGE using the method of Laemmli (1970) and stained for GST activity according to the method of Ricci et al. (1984). After electrophoretic run, the gel was equilibrated in 0.1 M KP buffer (pH 6.5) for 10 min and transferred to reaction mixture containing 1 mM CDNB, 4.5 mM GSH and 1 mM NBT in 0.1 M potassium-phosphate buffer (pH 6.5) at 37°C for 10 min. The gel was incubated at room temperature in 0.1 M Tris/HCl (pH 9.6) containing 3 mM phenazine methosulphate. Gels stained for GST activities were photographed with Image Acquisition and Analysis Software (4.6.00.0; UVP BioImaging systems, UK). The values are the average of data from 3 independent gels In densitometric analyses of GST activities, activities of control plants were taken as 100% and % of control values for each treatment are demonstrated in (Table 1.)

Gene expression analysis

In silico expression analysis of GSTs in maize

Genome-wide analysis was performed using bioinformatics tools in maize and 33 GST genes were identified (Liu et al. 2013). These identified genes were sorted and renamed according to their chromosome locations (Table 2). RNA-seq data was used to select the genes with the highest expression under salt stress among 33 *GST* genes. The expression profiles of *ZmGST* genes were evaluated employing the RNA-seq data sets, consisting of the transcriptional response to salt stress (Chen et al. 2012). To check out the *GSTs* genes' responses against different conditions in various samples, NCBI Sequence Read Archive (SRA) was employed to download the raw RNA-seq data-sets of accession numbers named PRJNA670840 (salt). According to this RNA-seq data, the expression of *GST* genes was determined at 5 different times (0, 24, 48, 72 and 120 h). The downloaded files were processed with the help of Galaxy Europe Server (usegalaxy.eu) including virtual bioinformatics tools. The mapping of reads was set up via the HISAT2 tool. Transcript and differential expression analyses

Table 2 Phytosome accession numbers of genes name in maize

	Gen ID	Phytozome ıdentfier		Gen ID	Phytozome Identfier
1	*ZmHSP1*	ZmB84.01G023600	18	*ZmHSP18*	ZmB84.03G300800
2	*ZmHSP2*	ZmB84.01G023700	19	*ZmHSP19*	ZmB84.04G317500
3	*ZmHSP3*	ZmB84.01G023800	20	*ZmHSP20*	ZmB84.05G232800
4	*ZmHSP4*	ZmB84.01G023900	21	*ZmHSP21*	ZmB84.05G265600
5	*ZmHSP5*	ZmB84.01G174400	22	*ZmHSP22*	ZmB84.06G123000
6	*ZmHSP6*	ZmB84.01G174700	23	*ZmHSP23*	ZmB84.07G184600
7	*ZmHSP7*	ZmB84.01G180700	24	*ZmHSP24*	ZmB84.07G184700
8	*ZmHSP8*	ZmB84.01G180900	25	*ZmHSP25*	ZmB84.08G170700
9	*ZmHSP9*	ZmB84.01G181200	26	*ZmHSP26*	ZmB84.08G187800
10	*ZmHSP10*	ZmB84.01G378000	27	*ZmHSP27*	ZmB84.08G298800
11	*ZmHSP11*	ZmB84.01G474500	28	*ZmHSP28*	ZmB84.09G105400
12	*ZmHSP12*	ZmB84.01G519700	29	*ZmHSP29*	ZmB84.09G168800
13	*ZmHSP13*	ZmB84.02G142500	30	*ZmHSP30*	ZmB84.09G241300
14	*ZmHSP14*	ZmB84.03G158800	31	*ZmHSP31*	ZmB84.09G241400
15	*ZmHSP15*	ZmB84.03G188900	32	*ZmHSP32*	ZmB84.10G052500
16	*ZmHSP16*	ZmB84.03G267000	33	*ZmHSP33*	ZmB84.10G109700
17	*ZmHSP17*	ZmB84.03G300700			

was performed in Stringtie 1.3.3 r.A gene was adopted as differentially expressed (DEG) if the P-value < 0.01 and a log2 transformed fold change value > 1. Expression analysis was summarized by preparing a log2FC based Heatmap via TBTools (https://github.com/CJ-Chen/TBtools) (Fig. 5). As a result of this mapping, 5 genes whose expression increased 3.5 times more were selected among the most *GST* genes. The One-way ANOVA was utilized to define the statistical significance for changes in relative *GSTs* genes expression levels. The means and standard deviation of the replications were checked by the least significant difference (LSD) test at P value ≤ 0.01.

RNA isolation and gene expression analysis

RNA isolations from the salt and GA-treated leaves of *Zea mays* were performed by FavorPrep™ Plant Total RNA Mini Kit (Favorgen, Taiwan) and following the manufacturer's protocol. The quality, integrity, and quantity of the isolated total RNAs were estimated by NanoDrop ™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis (Biorad, USA). The iScript ™ cDNA Synthesis Kit (Biorad, USA) was used to synthesize the cDNA from the isolated total RNAs. Expression analysis of *ZmGST* genes in the stress and hormone-treated leaves and of M13G44 cultivar was carried out on the Agilent Mx3000P (Stratagene, USA) with

Table 3 Primer sequences of Actin and *ZmGST*s genes in maize (*Zea mays*)

Gene	Primer sequence	References
Maize Actin F	GACCGAGGCCCCTCTCA	Chen et al. 2012
Maize Actin R	CATTCGAAGGTTTCAAACATAATCTG	
Z.mays GB2oX F	CCACCATGACGAGCGAGTTA	
Z. mays GB2oX R	CTCCCTGGCTGTTCACATCA	
ZmGST31F	CCTGGCTGGTGATGAGTTCA	
ZmGST31R	AGCTTCCTTCCTCTCTCGGT	
ZmGST2F	CCCCGAGTTCATCAAGCTGA	
ZmGST2R	GCACAGGTACCGGCAGATAG	
ZmGST8 F	GACGACAAGTACAAGAACCAGGG	
ZmGST8 R	TAGGCGAGGCTGTAGACCAT	
ZmGST10 F	GGCCCAAGAAAAGCGACAGG	
ZmGST10 R	TCGATGTACTGGACGATGACTG	
ZmGST9 F	CGACAAGTACAAGAACCAGGGG	
ZmGST9 R	TAGGCGAGGCTGTAGACCAT	

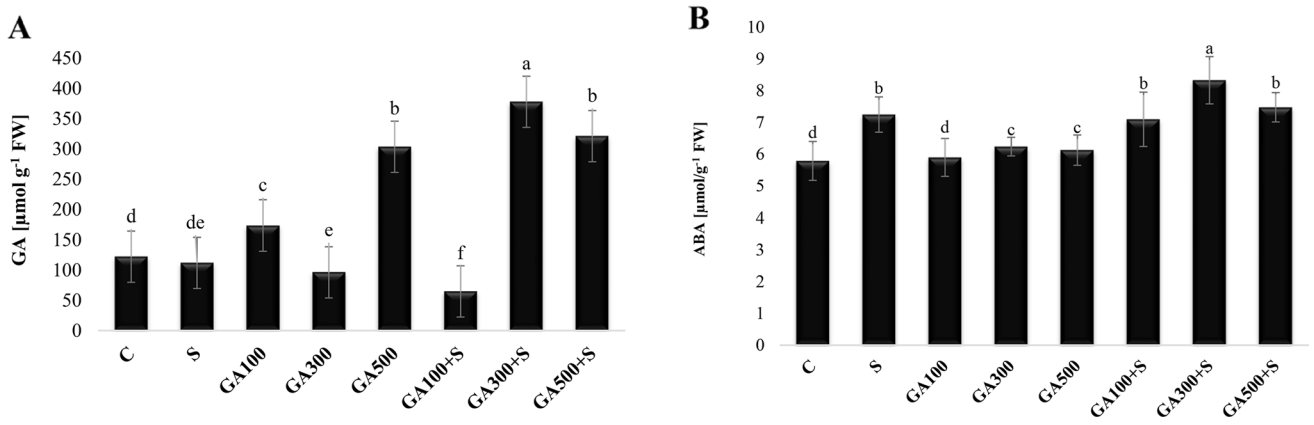


Fig. 1 The effects of gibberellic acid treatment on proline content (**A**), $O_2^{\bullet-}$ (**B**), MDA (**C**), H_2O_2 (**D**) of leaves in maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Columns with different letters represent significantly different (*P* value < 0.05) values. One-Way Analysis of Variance (ANOVA) was performed to compare the values of the parameters studied in each group. The samples were repeated three times during the experimental period

SsoAdvanced Universal SYBR Green Supermix (Biorad, USA). The conditions of the qRT-PCR experiments were as follows; denaturation at 98 ºC for 2 min; 40 cycles of 95 ºC for 15 s, 60 ºC for 20 s. The *Zea mays Actin* gene was used as an internal control (Chen et al. 2012). Relative expression of each *ZmGST* gene was measured in accordance with 2-ΔΔCt method. The primer sequences utilized in the qRT-PCR experiment was represented in (Table 3).

H_2S determination

The leaf H_2S content was determined by Nashef et al. (1977). Samples were powdered in liquid N_2 then 300 mg of frozen sample were extracted in a solution of 100 mM KH_2PO_4 (potassium-phosphate) buffer. The solution was exposed to centrifugation (15,000 g) at 4 °C for 15 min. A total volume of 2 ml of assay mixture containing 100 µl of supernatant, 188 µl of the extraction buffer and 5,5′-dithiobis was kept at 25 °C for 2 min for the quantification of H_2S. Absorbance values were determined at 412 nm on a UV–VIS spectrophotometer.

Anthocyanin content

The anthocyanin content was analyzed in accordance with Mancinelli et al. (1975). The leaf material was homogenized by adding 3 ml of 99% methanol, 1% HCl solution. Extraction and assay of anthocyanin samples were extracted with acidic methanol (1% HCl, w/v). The extracts, clarified by filtration or centrifuged at 10,000 rpm at 4 °C for 20 min. The resulting supernatant was used for determination of anthocyanins was analyzed at 530 nm (peak of absorption of anthocyanin) and 657 nm (peak of absorption of chlorophyll in acidic methanol; the absorption of chlorophyll in acidic methanol at 530 nm is 24–26% of that at 657 nm).

Statistical analysis

The samples were repeated three times during the experimental period. The experiment was carried out in a completely randomised design, and measurements were performed with six replicates (n = 6). Statistical variance analysis of the data was applied using ANOVA, and differences among treatments were checked using Tukey's honestly significant difference post-hoc analysis at 5% level. One-way Analysis of Variance (ANOVA) was performed to compare the values of the parameters studied in each group. The statistical significance level (a) was determined as 5% in the calculations, and the SPSS (IBM SPSS for Windows, ver.24) statistical package programme was used for analysis.

Results and discussion

Shoot length

In the present study, salt treatment decreased the shoot length by 47.70% compared to the control groups, while GA_3 (300 and 500 ppm) treatments increased the shoot length by 12.3 and 11.4%, respectively. However, all combinations of GA_3 and salt treatment increased this value (by 50, 72.1, and 63%, respectively) as compared to the plants grown under salinity conditions (Table 1). From these results, it can be said that GA_3 increases the shoot length by providing a stimulating effect on cell division under salinity and eliminating the damage caused by salt stress on cell elongation.

Conversely, Kaur et al. (2000) reported that GA_3 application increased the elongation in height by increasing the sucrose transport from the cotyledons to the stem under stress conditions in *Cicer arietinum* L. In our study, it can be suggested that sucrose transport and triggered cell division may have occurred with GA_3 application in maize shoots.

Relative water content

The results of this study demonstrated a marked reduction in relative water content (23.9%) under saline conditions compared to the control groups (Table 1). However, GA_3 individual treatments had no effect on the RWC of maize leaves as compared to the control groups. In our study, the GA_3 application alleviated the decrease in RWC values caused by salinity. The increases in RWC rates were 8.73 %, 19.7 %, and 13.4%, respectively. It is thought that the highest increase occurred due to the protective effect of GA_3 with the application of the second dose (300 ppm GA_3) under salinity (Table 1). In the present study, GA_3 application reduced the negative effects of salt stress in maize leaves. The results of the SPAD (chlorophyll content) value are given below due to the highest increase in RWC with the application of GA_3 + salt (300 ppm). When this data is compared to the results of proline contents, it can be stated that externally applied GA_3 in healing the damage caused by salt stress has a protective effect by increasing RWC and chlorophyll content without the need for a proline induction.

SPAD value

When the SPAD values of the control and salt treatments of the present study were compared, it was observed that there was a 23.2% decrease in maize leaves (Table 1). Only the application of GA_3 (100 ppm) increased concentrations by 9.9% compared to the control groups. In addition, GA_3 with salt stress treatment resulted in increased (37.33, 47.05, and 36.8%, respectively) chlorophyll content compared to salt stress alone. These findings can be interpreted as salinity-induced reduced chlorophyll content, which was tested using GA_3 while maintaining a constant leaf water status. Similarly, Ali et al. (2021) determined that GA_3 with salt stress treatment increased these values compared to just salt stress in sorghum plants.

Proline content

According to the results of these experiments, salinity led to a significant increase of 81.6% in the proline content of maize leaves (Fig. 1A). In the literature, although there are two views (stress tolerance marker or stress marker) that are related to amino acid accumulation in plants, numerous papers reported that increased endogenous proline content is related to higher saline tolerance in plants (Kishor et al. 2005). In accordance with these results, the report of Khan et al. (2019) determined that salt stress induced proline content in maize plants. Aside from that, GA_3 (100 ppm) and GA_3 (500 ppm) treatment alone increased this content, while the second dose of GA_3 (300 ppm) has a slight reduction when compared to the control groups, despite no change in RWC or SPAD value. This decrease can be explained by increasing the leaf area of maize leaves with the application of GA_3 (300 ppm). For this experiment, it could be suggested that the proline content per leaf was decreased in maize plants.

Otherwise, the smallest reduction of proline content was observed in the third dose of GA_3 (500 ppm) application under salinity according to salt treatment alone. Despite this result, the appropriate concentration of GA_3 for generating the minimal MDA content, which led to the least oxidative damage under salinity, was the second dose (300 ppm). This is explained by the increased length and leaf area of GA_3 (300 ppm)-treated leaves, as well as the increased sucrose concentration, improved growth, and increased ABA levels. Consequently, the results confirmed that GA_3 could be able to protect leaves by itself without needing proline accumulation.

Superoxide radical content and lipid peroxidation

When compared to control groups, the salt treatment increased this radical by 43.80% in maize leaves. All doses of GA_3 alone did not lead to any change, while the combination of GA_3 and salt stress reduced this content by 7.2, 24.60, and 13.45%, respectively (Fig. 1b). When examining these results, it was seen that the second dose of GA_3 was effective at alleviating the symptoms of this radical. In parallel with these findings, Wang et al. (2019a, b) reported that exogenous GA_3 application decreased radical content and lipid peroxidation in *Abelmoschus esculentus* L. In this work, the lipid peroxidation product (MDA) results are in accordance with the radical content (hydrogen peroxide and superoxide radical). Salinity induced by 65.45% of the MDA content, but individual GA_3 treatment did not cause any change compared to the control groups (Fig. 1c). In this study, salt stress increased reactive oxygen species by disturbing the physiological mechanisms in maize leaves. Also, all combinations of GA_3 and salt reduced MDA content as compared to salt stress alone, and the lowest MDA content was found using GA_3 (300 ppm) and salt (30.76%). In contrast to these findings, Tuna et al. (2008) determined that GA_3-sprayed maize plants provided biochemical improvement with reduced oxidative damage.

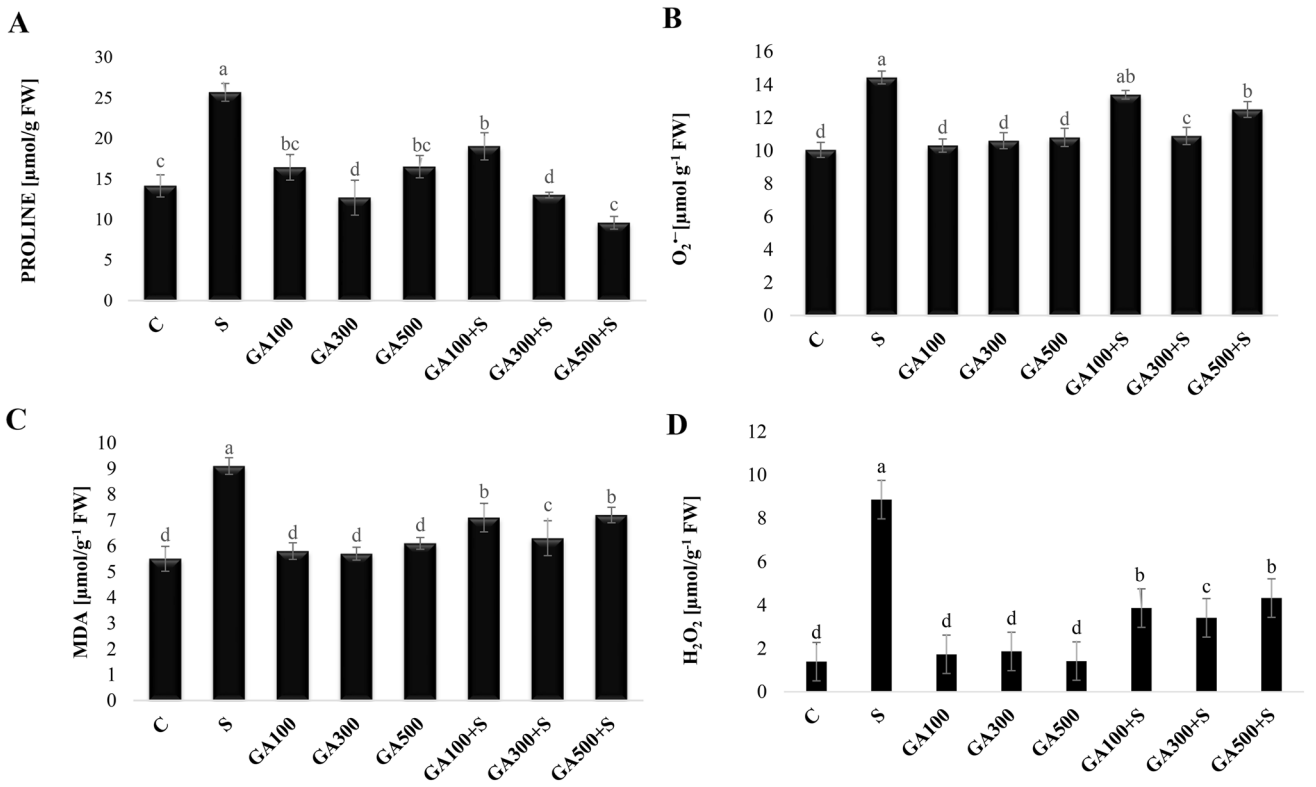


Fig. 2 The effects of gibberellic acid treatment on GA (**A**), ABA (**B**) hormone level of leaves in maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Columns with different letters represent significantly different (P value < 0.05) values. One-Way Analysis of Variance (ANOVA) was performed to compare the values of the parameters studied in each group. The samples were repeated three times during the experimental period

Hydrogen peroxide content

As stated earlier, in the current study, salt stress led to impaired metabolism in maize leaves by inducing reactive oxygen species as well as inhibition of chlorophyll, water content, and shoot length. Similar to the results of MDA, hydrogen peroxide content was increased by 6.38-fold with salinity, and this value was close to the control groups under GA_3 treatment alone (Fig. 1D). Like with the superoxide radical content, the combination of GA_3 and salt application restricted the hydrogen peroxide content, and the second dose of GA_3 was the most effective concentration. Similarly, Mukarram et al. (2021) found that exogenous GA_3 mitigated the effects of salt stress in *Trigonella foeum-graceum* L. plants via reducing reactive oxygen species. Here, we determined that GA_3-treated maize leaves had less oxidative accumulation compared to salt-treated ones.

Endogenous GA_3 level

In our results, salt treatment decreased endogenous GA_3 levels by 8.45% compared to the control group (Fig. 2A). In parallel with the results, Zhao et al. (2021) determined that drought stress reduced GA_3 levels in maize cultivars. Otherwise, exogenous GA_3 (100 ppm) and GA_3 (500 ppm) doses were induced by 1.4 and 2.4-fold, respectively, and reduced under GA_3 (300 ppm) treatment as compared to control. It is clear that GA_3 treatment has a positive impact on endogenous GA_3 accumulation, but the reaction to the second dose of GA_3 treatment (300 ppm) is very striking. This could be explained by the feedback inhibition of GA_3 biosynthesis in maize leaves. The combination of GA_3 (100 ppm) application and salt stress reduced this level, while GA (300 ppm) and salt stress induced a 3.37-fold (the highest) increase and GA_3 (500 ppm) and salt induced a 2.86-fold increase compared to the salt treatment alone. As alluded to above, GA_3 (300 ppm) is the best dose for the stress-treated maize leaves in this study. *Gibberellin 2-beta-dioxygenase* (*GA2ox*) was

also assessed in this experiment, and results showed that the expression of the *GA2ox* gene was reduced with GA_3 treatments, but it did not change while NaCl was applied.

Endogenous ABA level

ABA is stimulated to close stomata, and this stimulation induces a signal pathway in plants under stress conditions (Bharath et al. 2021). When examining the ABA results, it was determined that the values were increased by 25.21% in salt-treated leaves (Fig. 2B). However, GA_3 treatment alone (at 300 and 500 ppm) also increased ABA levels by 7.7% and 5.87%, respectively, compared to the control groups, although the differences are not noticeable. The combination of GA_3 and salt stress induced this level only at the second dose of GA_3 (300 ppm, 14.89%). This fact could be explained by the reports of Toh et al. (2008), which said that high levels of ABA inhibit GA_3 biosynthesis. Nevertheless, it has been known that GA_3 has an antagonistic effect on ABA in the germination and growth period, but ABA is induced by stress, which may regulate the negative feedback of GA_3 biosynthesis. The increased ABA is also consistent with the MDA, hydrogen peroxide, RWC, and shoot length values. In this study, when maize leaves were exposed to salt stress, GA_3 application was able to successfully induce ABA levels.

GSH/GSSG ratio, GST and GR enzyme activity

In the literature, this ratio (GSH/GSSG) is associated with the tolerance capacity of plants because of the effective glutathione pool created by the ascorbate–glutathione cycle. In our results, salinity reduced this ratio by 41.58% in maize leaves, while it was not changed with the GA_3 treatment alone compared to control (Fig. 3A). In our results, the salt treatment led to a 5.93-fold enhancement of the GST enzyme activity, while the GR enzyme activity was minimal. This can be explained by the increased ROS and MDA content. The GR enzyme was not able to induce the GSH pool and lead to oxidative damage, although GST consumed the GSH. In the current study, five different GST isoenzymes were expressed in maize leaves at day 10 under control conditions. Parallel with total GST enzyme activity, GST1, 3, and 5 were up regulated under salinity. Beside this, GA_3 (100 ppm) treatment alone induced GST1, 2, 3, and 5 compared to the control group. As shown in Fig. 4B, GST1 and GST2 were not changed in GA_3 (300 ppm), but a significant increase was observed in GST3 and GST5, while GST4 was suppressed. In addition, GST1, 2, 3, and 5 were upregulated with GA_3 (500 ppm). Otherwise, an increase was detected in GST3 and GST5 under GA_3 (100 ppm) and GA_3 (500 ppm) applications with salinity, whereas GST2 was not changed. These findings are also parallel with the report of Tascı et al. (2020), who determined that salt stress induced GST enzyme activity in *Glycine max* L. plants. Totally, GA_3 application alone did not affect GR enzyme activity, although GST enzyme activity was slightly induced (100 and 500 ppm) by 41.48% and 60.56%, respectively (Fig. 4A).

Nevertheless, the combination of GA_3 and salt stress maintained this ratio compared to the salt treatment alone, and the groups closest to the control were GA_3 (100 and 500 ppm). Also, GR and GST activities were higher at the first and third doses of the combination (Fig. 3c). Thus, it is clear that different doses of gibberellic acid could be able to stimulate GST enzyme activity differently. Although many reports have documented (Younesi and Moredi 2014; Uzal 2017; Ahmad 2010; Iftikhar et al. 2019; Mukarram et al. 2021; Shahzad et al. 2021) that exogenous GA_3 induced antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, catalase, peroxidase and glutathione reductase), limited studies (salinity; Tasci and Dinler 2020, cadmium stress; Ahmad et al. 2021) have been reported with the changes in glutathione *s*-transferase in *Zea mays* and *Cicer arietinum* plants.

Otherwise, it could be said that GSH is catalysed by the GST enzyme, and the GR enzyme is also effective in recycling the GSH with GA_3 treatment. However, the lowest MDA content had been observed under GA_3 (300 ppm) and salt treatment but not under GA_3 (100 ppm) or GA_3 (500 ppm); thus, this contrast could be coupled with an increased ABA level. As mentioned before, GA_3 protected the maize leaves from salinity by inducing growth (shoot length), maintaining water balance, increasing SPAD, RWC value, and ABA hormone levels, and also stimulating the antioxidant enzyme system.

Expression of *ZmGST* genes

In order to further study the mechanism by which GA_3 treatment could improve maize tolerance to salinity stress, the transcription levels of the *glutathione s-transferases* were studied. The expression levels of five *glutathione s-transferases (GSTs)* were assessed in maize plants subjected to different GA_3 treatments (0, 100, 300, and 500 ppm) under 350 mM saline conditions. The results displayed that the five *GST* genes revealed higher levels in GA_3-treated maize under salinity compared to control plants, indicating the important roles of GA_3 and *GST* under other abiotic stresses.

Gene expression patterns are thought to be important indicators that can be used to infer the functions of those genes (Kavas et al. 2016). To determine the potential functions of *ZmGST* genes, RNA-seq data from leaf tissues of plants subjected to salt stress at various time was analyzed. In accordance with Chen et al. (2012), the *ZmGSTs* with the highest active expression were selected for the RNA-seq data

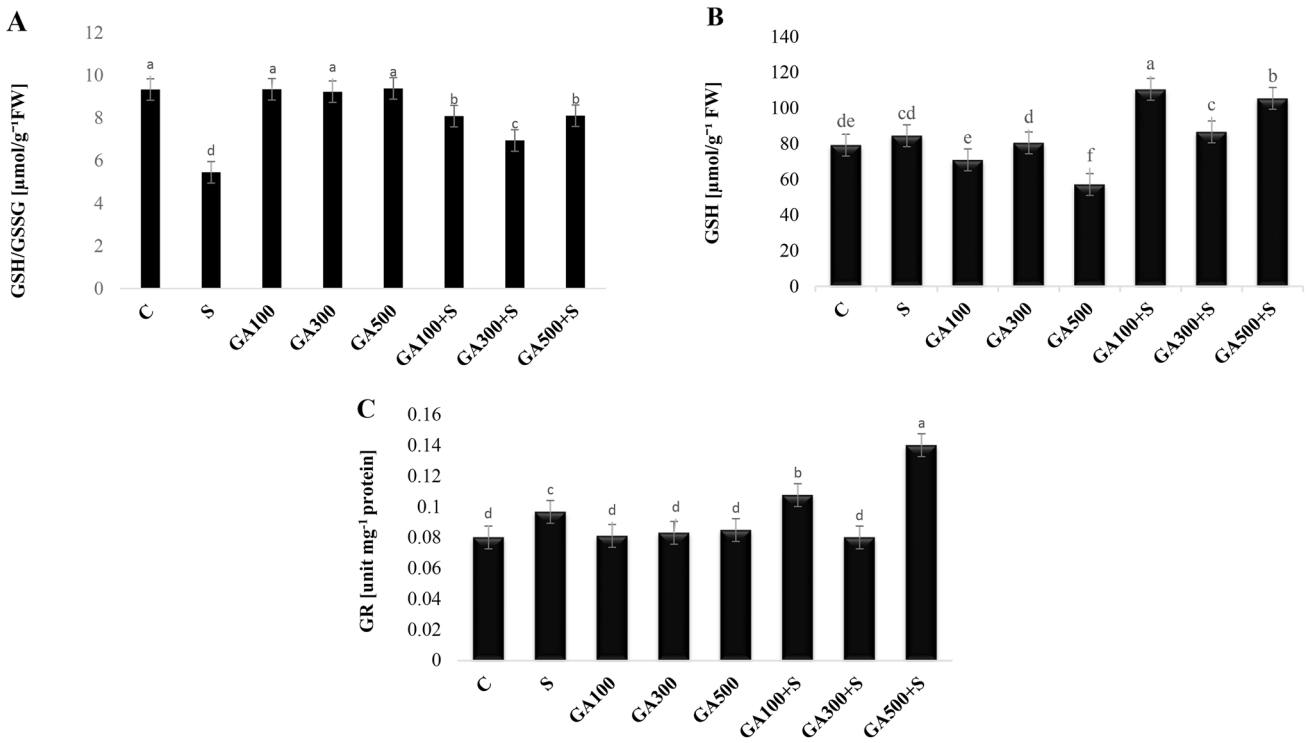


Fig. 3 The effects of gibberellic acid treatment on GSH/GSSG ratio (**A**), GSH content (**B**), GR enzyme activity (**C**) of leaves in maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Columns with different letters represent significantly different (P value < 0.05) values. One-Way Analysis of Variance (ANOVA) was performed to compare the values of the parameters studied in each group. The samples were repeated three times during the experimental period

between 0 and 120 h. In maize leaf tissues under salt stress conditions, *ZmGST2, 8, 9, 10,* and *31* were found to be 2.4, 2.7, 1.4, 2, and 1.8-fold upregulated, respectively, and these results were similar to RNA-seq data (Fig. 5).

This study investigated the interaction and correlation of *ZmGST* with GA_3 in order to better understand their potential roles in *Zea mays* L. *GST* gene expression levels were found to be higher in all plants treated with different concentrations of GA_3 compared to salt stress. *GST* gene expression levels were found to be upregulated between 1.5 and 4 times higher in maize plants treated with GA_3 at various concentrations compared to salt stress. According to our results, GA_3 is thought to be directly related to the expression of *GST* genes.

Anthocyanin content was found to be quite high in maize plants exposed to GA_3 application and salt stress. The increase in the expression levels of *GST* genes was found in parallel with the increase in this content. It was determined that the *Bronze2* (Bz2) gene, which was defined as the glutathione *S*-transferase gene in previous studies, was associated with anthocyanin (Bodeau et al. 1996). The *Bronze2* (Bz2) gene, which was previously identified by Bodeau et al. (1996) was renamed *ZmGST10* in this study, based on genome-wide analysis and chromosome location. *The ZmGST10* gene expression was found to be 2–fourfold *higher* in different concentrations of GA_3 treatment and GA_3 and salt treatment. According to our findings, the significant increase in *GST* gene expression level as a result of GA_3 application supports the high level of anthocyanin accumulation in plants. Especially under salt stress, according to MDA and H_2O_2 results, the best GA_3 concentration was determined to be 300 ppm, and the level of *ZmGSTs* genes was the highest (Fig. 6).

Hydrogen sulphide

In the results, salt stress increased H_2S content by 15.8% compared to the control groups, while GA_3 applications alone were different (5.8, 8.7, and 15.4%, respectively) from the values of the control treatment. However, concentrations of GA_3 (100 and 500 ppm) increased H_2S content (14.3 and 7.5%), while it was decreased in the GA_3 (300 ppm) application under salinity (5.7%), compared to the salt stress (Fig. 7A). In parallel with our results, salt application increased the H_2S content in tomato plants (daSilva et al. 2018). These results are also in agreement with the increase in GSH/GSSG, GST, and GR enzyme activities (between 100 and 500 ppm). Under chilling stress, similar results

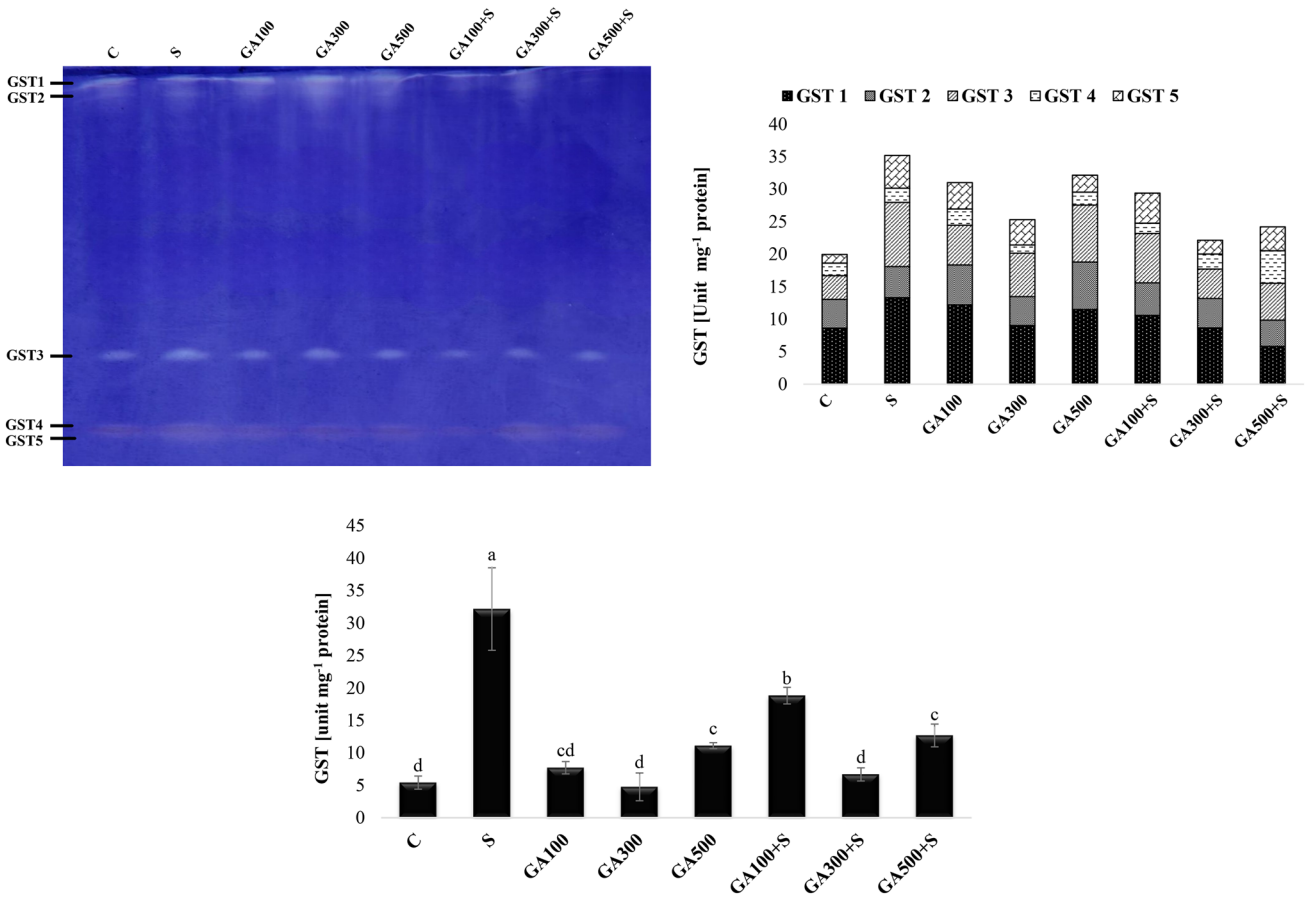


Fig. 4 The effects of gibberellic acid treatment on activity staining (**A**) and relative band intensity of GST isoenzymes (**B**) and total activity (**C**) in maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Columns with different letters represent significantly different (P value < 0.05) values. One-Way Analysis of Variance (ANOVA) was performed to compare the values of the parameters studied in each group. The samples were repeated three times during the experimental period

were reported in a recent study (Liu et al. 2020c) that H_2S increases GSH, the reduced/oxidized GSH (GSH/GSSG) ratio, and the expression of GSH-associated Tau-class *GST* genes. Based on these findings, it is possible to conclude that the GA_3 salinity treatment induced GST enzyme activities and genes in maize leaves by increasing H_2S content.

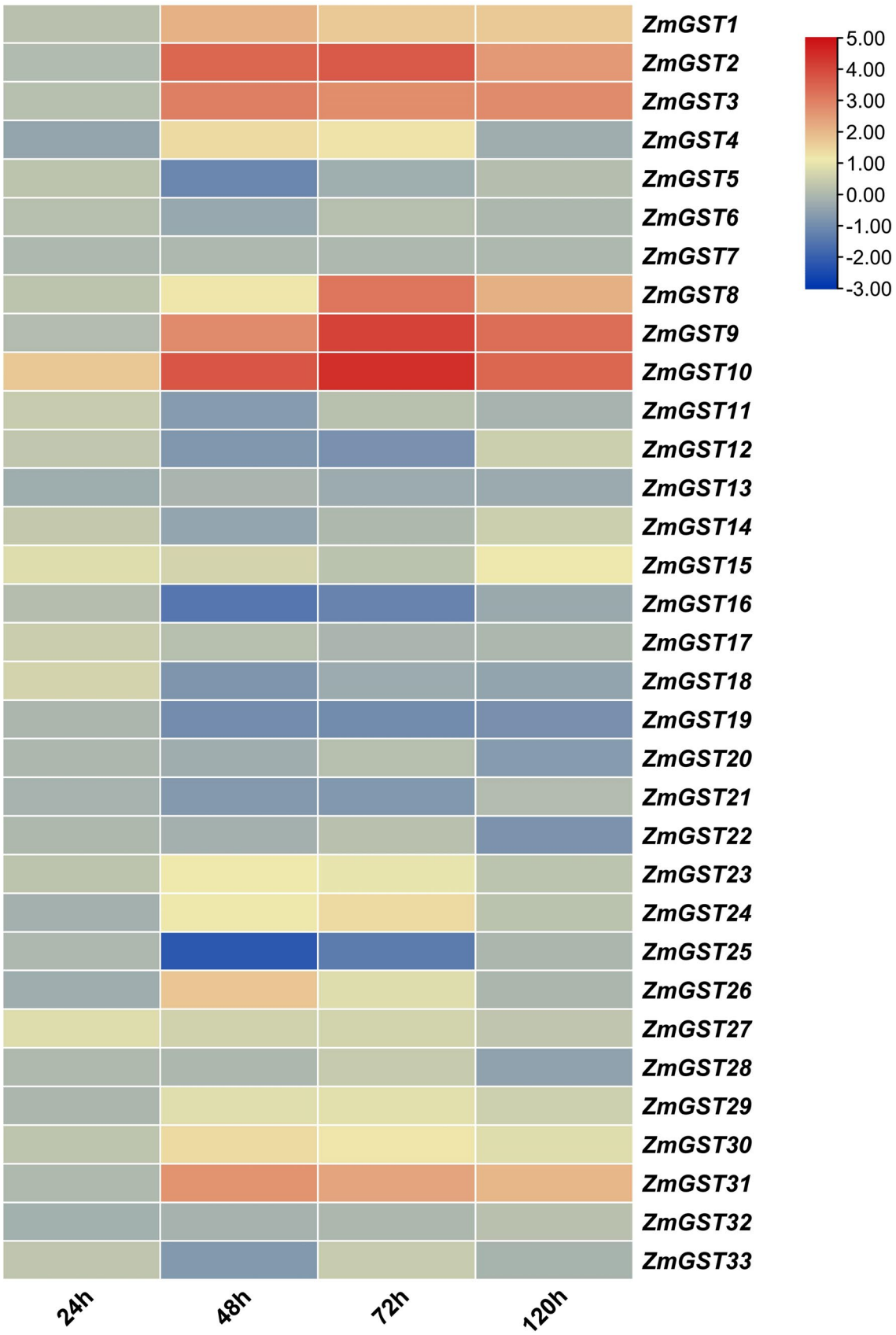
Anthocyanin content

The anthocyanin content was increased with NaCl in comparison to the control (by 20%), while it was decreased significantly (by 19.5 and 15.9%, respectively) by GA_3 (100 and 500 ppm), and the GA_3 (300 ppm) application did not change as compared to the control group. These results are parallel with the reports that anthocyanins were increased by salt stress in sorghum plants (Liang et al. 2018). Beside this, GA_3 (100 and 300 ppm) and salinity decreased significantly (14.01 and 38.6%, respectively), but GA_3 (500 ppm) did not change anthocyanin content as compared to NaCl alone (Fig. 7B).

Our results showed that GA_3 application (100 and 500 ppm) under salt stress increased anthocyanin content like GST enzyme activities under the same group in maize. It could be suggested that the increase in the amount of H_2S affected the GST enzyme activity positively and caused anthocyanin accumulation, but this increase was not greater than that in salt-treated maize leaves. In our study, when the results obtained were analyzed, it was noticed that there was a positive relationship between the groups in which GST activity increased (100 and 500 ppm) and the increase in anthocyanin content and H_2S.

Conclusions

GA_3 treatment improved maize plants' resistance to salinity by maintaining water balance, decreasing proline content,


ZmGST1
ZmGST2
ZmGST3
ZmGST4
ZmGST5
ZmGST6
ZmGST7
ZmGST8
ZmGST9
ZmGST10
ZmGST11
ZmGST12
ZmGST13
ZmGST14
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ZmGST16
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ZmGST25
ZmGST26
ZmGST27
ZmGST28
ZmGST29
ZmGST30
ZmGST31
ZmGST32
ZmGST33
24h
48h
72h
120h
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00

◂**Fig. 5** Heat map showing GSTs expression in RNA-Seq datasets. Red, positive log fold-change (log FC) defines higher expression in the first genotypes compared with the second; blue, negative log FC. The raw RNA-seq datasets were downloaded from NCBI Sequence Read Archive (SRA) under salt-treated leaf explants of control (0 h) and salt stress conditions (24, 48, 72 and 120 h) (PRJNA656794), The transcriptional analysis of downloaded files was done via Galaxy with *in silico* tools. The mapping of reads was done with the HISAT2 tool. Transcript assembly and differential expression analyses were accomplished with Stringtie 1.3.3, respectively. The genes having fold change value log2 > 1 and a *P* value < 0.01 were adopted as differentially expressed genes (DEGs). Heatmap based on the log2FC was prepared using TBTool.s

and increasing chlorophyll content, H_2S accumulation, shoot length, and ABA level. It is evident that in this study, GA_3 acted as an antioxidant for maize leaves. Besides this, the first and third doses (100 and 500 ppm) of this hormone could be able to trigger the GST enzyme and isoenzyme activity as well as H_2S accumulation and anthocyanin content in maize leaves under salinity, although the lowest MDA, hydrogen peroxide, and superoxide radical content were under the second dose (300 ppm) of GA_3. *GST* gene expression levels were found to be upregulated between 1.5

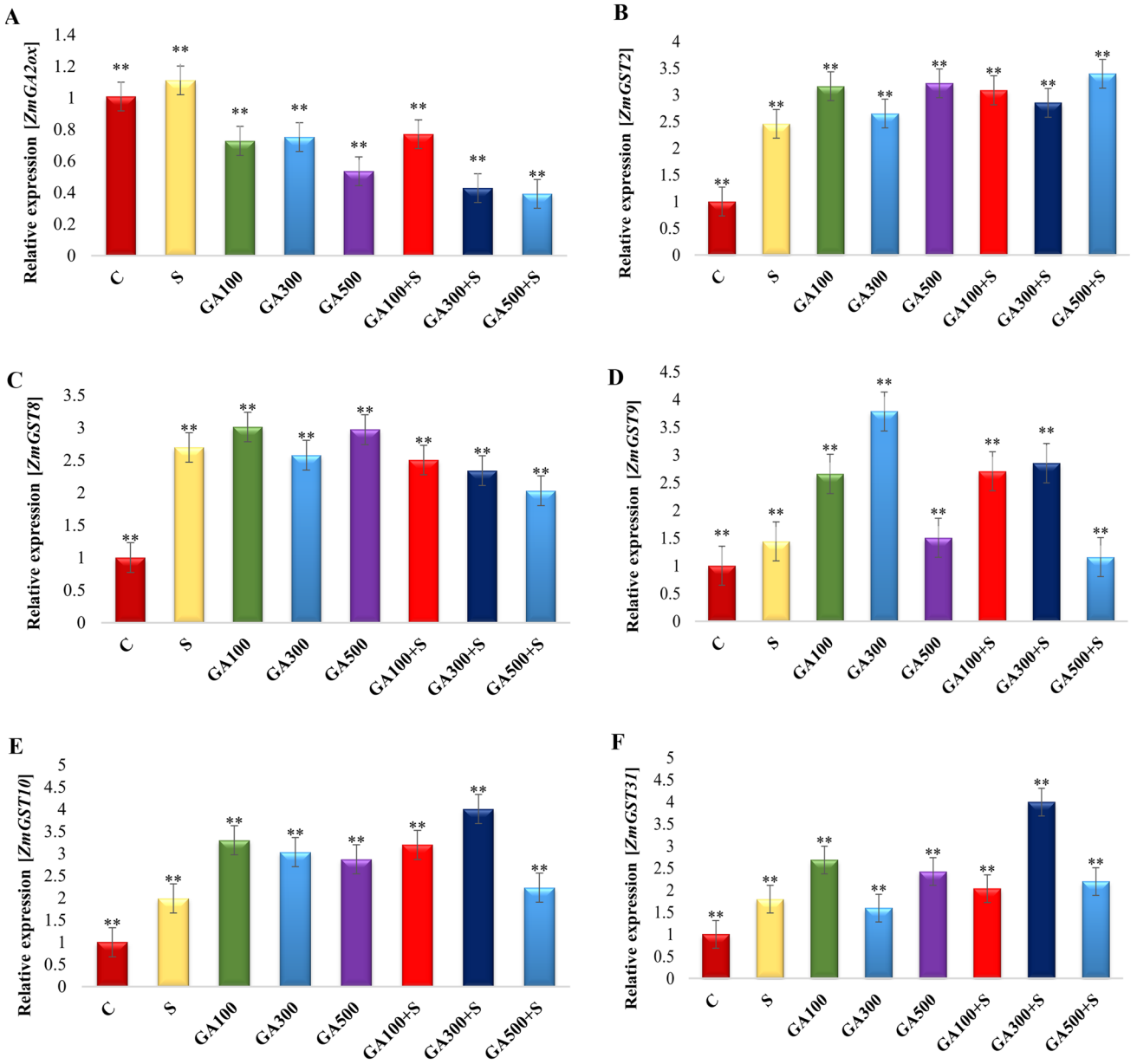


Fig. 6 The effects of gibberellic acid treatment on *GA2OX* (**A**), *GST2* (**B**), *GST8* (**C**), *GST9* (**D**), *GST10* (**E**), *GST31* (**F**) relative gene expressions in leaves of maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Data provided represents fold expression $2-\Delta\Delta CT$ method. Values symbolized the average ± SD of three biological replicates of each reaction. ** Indicate significant differences at $p < 0.01$

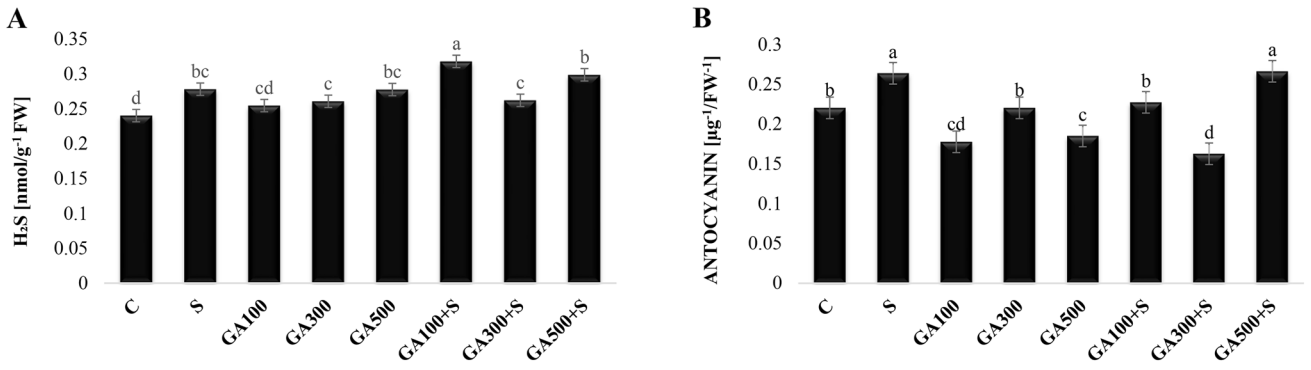


Fig. 7 The effects of gibberellic acid treatment on H_2S (**A**), antocyanin (**B**) content of leaves in maize (*Zea mays* L.) under salt stress. Control (C) (1), Salt (S); 350 mM NaCl (2), GA; 100 ppm (3), GA; 300 ppm (4), GA; 500 ppm (5), GA (100 ppm) + S; (6), GA (300 ppm) + S; (7), GA (500 ppm) + S; (8). Columns with different letters represent significantly different (P value < 0.05) values. One-Way Analysis of Variance (ANOVA) was performed to compare the values of the parameters studied in each group. The samples were repeated three times during the experimental period

and fourfold higher in all plants treated with GA_3 at different concentrations compared to salt stress. Consequently, GA_3 can also be used as a stimulant for *GST* gene expressions to induce defence responses in plants to adverse environmental conditions. More exhaustive molecular analysis is needed to identify the GA_3-responsive elements for GST in maize and other plants under stress conditions and bring to light the GA_3-GST interaction.

Author contributions BSD and HC designed the research. BSD, HC and ZS performed the analysis and wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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