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FULL-LENGTH RESEARCH ARTICLE

Effect of High-Temperature Stress on Ascorbate–Glutathione Cycle in Maize

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Abstract High-temperature stress induces cellular changes leading to over-production of highly reactive oxygen species (ROS) which damage macromolecules and cell organelles, eventually resulting in cell death. Antioxidative metabolism in plants comprising of enzymatic and non-enzymatic antioxidants imparts tolerance by scavenging or detoxification of excess ROS. We investigated the response of major H_2O_2-detoxifying system, the AsA–GSH cycle in four genotypes of maize differing in heat sensitivity. Stress was imposed by staggered sowing so that one set of plants faced high-temperature stress at their anthesis-silking stage. The concentrations of H_2O_2 increased across the genotypes by high temperature; however, the increase was lesser in tolerant genotypes: NSJ 189 and NSJ 221. High-temperature stress led to an increase in the level of GSH and GSSG in all the genotypes, whereas GSH/GSSG decreased in sensitive genotypes: PSRJ 13099 and RJR 270. The glutathione S-transferase activity increased significantly under heat stress. APX, MDHAR and DHAR activities decreased under heat stress in the sensitive group. Under high temperature, GR activity remained unchanged in sensitive genotypes, while it increased significantly in tolerant genotypes. Ascorbate levels increased in tolerant genotypes, while a decline was observed in sensitive genotypes. Isoforms of APX showed some new bands in tolerant genotypes as well as higher intensity of the existing ones as compared to sensitive genotypes under stress conditions. Isoforms of GR did not show any genotypic differences under heat stress. Findings emphasized the importance and complexity of the AsA–GSH system in fine-tuning the redox metabolism under heat stress in maize. The study also suggested that the antioxidative enzymes of AsA–GSH cycle play a key role in sustaining the ROS homeostasis in cells, thus minimizing the potential toxic effects of ROS.

Keywords Antioxidative enzymes · Heat stress · Lipid peroxidation · Non-enzymatic antioxidants

Introduction

High temperature is one of the most decrementing environmental constraints and is responsible for more than 50% yield reduction and crop loss in some major crops [1]. Following the recent trends of global warming and climatic variability, high-temperature response in crop plants becomes a major concern in the world [5, 22]. High-temperature stress results from the temperatures high enough to damage plant cellular homeostasis and plant tissues, substantially affecting the overall vegetative to reproductive growth and metabolism [4]. Temperatures producing heat stress are variable for different plant species and even for different genotypes. However, temperatures ranging between 35 and 45 °C are able to produce heat stress for most of the tropical plants [14]. At cellular level, high temperature causes oxidative stress [23, 41]. Oxidative stress is the particular situation in ROS metabolism when there is a substantial imbalance between ROS generation and their detoxification. In plants, high-temperature stress

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accelerates the production and reactions of ROS [37]. Major ROS produced under high-temperature stress include singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). The major effects of increased ROS level include autocatalytic peroxidation of biomolecules: lipids, proteins, nucleic acids and pigments; alterations in membrane characteristics; selective permeability and integrity [38].

To neutralize the ROS toxicity, a highly efficient and competent antioxidant defense system, comprising both enzymatic and non-enzymatic components, is present in all plants. The enzymatic antioxidant defense system comprises several antioxidative enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), glutathione reductase (GR), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Non-enzymatic antioxidants include the metabolites: ascorbate (AsA), carotenoids, glutathione (GSH), anthocyanins, tocopherol, flavanones, etc. [13, 27]. In consonance with ROS detoxification, the H_2O_2 concentrations must be kept under strict regulation because of its highly toxic and reactive nature. In plants, the main H_2O_2 detoxification system is the AsA–GSH cycle, which regulates majorly in chloroplasts [2]. Although the major site of AsA–GSH cycle operation is chloroplasts, it also plays a major role in ROS detoxification in mitochondria, peroxisomes and cytosol [25, 33]. AsA–GSH cycle, also known as Halliwell–Asada pathway, is the pathway responsible for recycling of reduced forms of ascorbate and glutathione. The change in ratio of reduced to oxidized form of ascorbate and glutathione is very crucial for the cell to sense oxidative stress and respond accordingly. The AsA–GSH cycle involves consecutive oxidation/reduction of ascorbate, glutathione and NADPH. These oxidation/reduction reactions are catalyzed by the enzymes APX, MDHAR, DHAR and GR.

Maize (*Zea mays* L.) is an important cereal crop in India and across the globe. High temperature during the reproductive phase (anthesis-silking and grain filling time) imposes severe yield loss of maize and other cereals [30, 39]. Taking into consideration the severe effects of extreme temperature, developing heat tolerance in maize plants is required to sustain the global agricultural productivity for the increasing population worldwide. The high-temperature stress tolerance is a complex phenomenon which involves an array of molecular, biochemical and physiological developments at whole plant as well as cellular and subcellular levels [36]. The present investigation was proposed to understand the response of tolerant and susceptible genotypes of maize to high-temperature stress with special emphasis on the role of activities of antioxidative enzymes of AsA–GSH cycle.

Materials and Methods

Plant Materials and Stress Treatments

Four maize (*Zea mays* L.) genotypes NSJ 189 and NSJ 221 (heat tolerant) and PSRJ 13099 and RJR 270 (heat susceptible) [40] were evaluated for various parameters related to antioxidative stress metabolism in response to high-temperature stress. The experiment was laid out in a randomized complete block design with three replications as described previously by Yadav et al. [39]. Sowing was spread over 30-days interval during January and February 2016 so that the reproductive stage of plants sown later was exposed to higher temperatures. Standard agronomic practices were followed to nullify their impact on growth. The maximum and mean day temperatures for the early sown crop were 37.2 and 33.5 °C, whereas for the late sown crop these were 43.6 and 38.7, respectively. The crop sown later faced high-temperature (HT) stress during fertilization and grain filling stage. Observations reported were recorded in triplicate on youngest fully expanded leaf of consistent size on three independent plants at their reproductive stage.

Determination of Lipid Peroxidation and H_2O_2

Lipid peroxidation in terms of MDA content and H_2O_2 determination was carried out as per procedure described previously [40]. Membrane lipid peroxidation, MDA, which is a by-product of predominantly polyunsaturated fatty acids (PUFA), was quantified by using thiobarbituric acid (TBA). H_2O_2 was extracted by homogenizing fresh leaf samples (0.5 g) with 3 mL of chilled 50 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged at $10{,}000\times g$ for 15 min. Three mL of extract was incubated for 10 min with 1 mL of 0.1% $TiCl_4$ in 20% H_2SO_4 at room temperature followed by centrifugation at $10{,}000\times g$ for 15 min. Optical density of the supernatant was recorded at 410 nm against respective blank. H_2O_2 content was calculated by using a molar extinction coefficient ($\epsilon = 0.28\ \mu M^{-1}\ cm^{-1}$) and expressed as nmol g^{-1} FW.

Extraction and Measurement of Ascorbate and Glutathione

The ascorbate and glutathione were extracted by homogenizing the fresh leaves (1 g) in 5 mL chilled 5% metaphosphoric acid amended with 1 mM EDTA. Homogenate was centrifuged at $12{,}000\times g$ for 10 min, and the supernatant was stored at 4 °C. To estimate ascorbate, method of Huang et al. [17] was followed with minor modifications

wherein the supernatant was neutralized with K-phosphate buffer (0.5 M, pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm using K-phosphate buffer (100 mM, pH 7.0) with 0.5 unit of ascorbate oxidase. Quantification was done by using standard curve made with AsA.

Total glutathione (pool) was assayed according to Yu et al. 2003 [42] with some small modifications. Five hundred µl of crude extract was added to 480 µl of reagent A [500 mM K-phosphate buffer (pH 7.0), 15 mM EDTA, 0.3 mM 5,5′-dithio*bis* (2-nitrobenzoic acid) (DTNB)] and 120 µl of reagent B (1 mM EDTA, 50 mM imidazole solution and 0.02% BSA) and 120 µl 5% Na_2HPO_4, 2 U of glutathione reductase and 80 µl $NADPHNa_4$. The observations were recorded at 421 nm. GSSG was measured in the similar manner as total glutathione, excepting that the crude extract was incubated with 40 µl 2-vinylpyridine for 1 h at 25 °C prior to adding in the assay mixture as above. Quantification was carried out by using standard curves of GSSG and GSH.

Determination of Protein Content

Soluble protein concentration was quantified according to Bradford assay method [6]. Bovine serum albumin (BSA) was used as a standard.

Enzyme Extraction and Assays

Enzyme extraction was performed by homogenizing 0.5 g fresh leaf tissue in 0.05 M potassium phosphate buffer, pH 7.0 amended with 0.1 M KCl, 0.001 M ascorbate, 0.005 M β-mercaptoethanol and 10% glycerol (w/v). The supernatant was collected after spinning the homogenate at 10,000×*g*. All the operations were carried out at 4 °C.

For APX activity assay, Nakano and Asada [26] method was followed. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.1 mM H_2O_2, 0.5 mM AsA, 0.1 mM EDTA and enzyme extract in a final volume of 3 mL. H_2O_2 was added to the above mixture just in the last to initiate the reaction. A linear decrease in A_{290} nm at every 15 s was recorded for 1 min. APX enzyme units were calculated by using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

MDHAR activity was determined according to Hossain et al. [16]. Assay reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 2.5 mM AsA, 0.5 unit of ascorbate oxidase (AO), 0.2 mM NADPH and enzyme extract in a final volume of 3 mL. Reaction was initiated by adding ascorbate oxidase, and a change in A_{340} was recorded at every 15 s for 1 min. Enzyme units were calculated according to a molar extinction coefficient (6.2 mM^{-1} - cm^{-1}), and activity was expressed as U mg^{-1} protein.

DHAR activity was assayed according to Nakano and Asada [26]. Assay reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM DHA and 2.5 mM GSH. The reaction was started by adding enzyme extract to the reaction mixture. DHAR activity was calculated by recording change in A_{265} for 1 min, and The enzyme unit was calculated by using the extinction coefficient of 14 mM^{-1} cm^{-1}.

GR activity was measured according to Hasanuzzaman et al. [15] with slight modifications. Reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 3.5 mM $MgCl_2$, 150 µM NADPH and 0.5 mM oxidized glutathione and crude extract. A decrease in A_{340} because of the oxidation of NADPH was recorded. A concentration of $NADP^+$ was calculated by using a molar extinction coefficient (€ = 6.2 mM^{-1} cm^{-1}). GR unit was defined as the amount of enzyme that produced 1 µmol min^{-1} $NADP^+$ and expressed as U mg^{-1} protein (nmol $min^{-1}mg^{-1}$ protein).

GST activity was determined by using the method of Hossain et al. [16] with some minor modifications. The reaction mixture contained 100 mM Tris–HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM CDNB and enzyme extract in a final volume of 3 mL. CDNB was added to initiate the reaction, and an increase in A_{340} was recorded for 1 min. GST units were calculated by using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

In gel Activity Assay of APX and GR

Crude enzyme extracts were used for *in gel* activity assays. Samples were applied with Laemmli [20] buffer without SDS. Electrophoretic separation of APX and GR isoforms was performed on 4% stacking and 10% resolving non-denaturing, non-reducing polyacrylamide gels by using ATTO mini-dual slab electrophoresis system (AE-6531M/P/MW) at a constant current of 30 mA per gel for 3 h at 4 °C. After electrophoresis, gels were stained according to the respective protocols for the enzyme.

APX isoforms were identified by following the method described by Mittler and Zilinskas [24], based on the inhibition of ascorbate-driven NBT reduction. Two mM ascorbate was added to the electrode buffer, and the gels were pre-run for 30 min. After electrophoresis, gels were equilibrated in 50 mM potassium phosphate buffers (pH 7.0) amended with 2 mM ascorbate for 30 min. The gels were again incubated for 20 min in 50 mM potassium phosphate buffer (pH 7.0) amended with 4 mM ascorbate and 2 mM H_2O_2. After incubation, gels were washed briefly in the same buffer for 1 min. After washing, the gels were submerged in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT with gentle shaking. The reaction was continued for

10–15 min until the appearance of achromatic bands against dark blue background.

GR isozyme activity staining was performed as per method described by Rao et al. [31]. Briefly, gels were incubated in Tris–HCl buffer (250 mM, pH 7.5) amended with 3 mM EDTA, 3.4 mM GSSG, 0.5 mM NADPH, 0.2 mg mL^{-1} of 2,6-dichlorophenolindophenol (DCPIP) and 0.2 mg mL^{-1} of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).

Statistical Analysis

Data were recorded in triplicates for individual genotypes. The analysis of variance (ANOVA) was performed with one- and two-factor [genotype and treatment (different date of sowing)] factorial analysis program of MSTAT program, and the critical difference (CD) values of genotypes X treatment were calculated at $P < 0.05$ and $P < 0.01$ significant level.

Results

The ANOVA results showed significant differences between diverse genotypes for all the parameters studied under high temperature. The combined statistical analysis of maize genotypes evaluated during summer 2015 showed that the parameters such as MDA, H_2O_2, AsA, GSH, GSSG, GSH/GSSG, APX, MDHAR, DHAR and GR were found highly significant at genotypes (G), different dates of sowing (T) and interaction (G × T) except DHAR activity (Table 1).

Lipid Peroxidation and H_2O_2 level

A sharp increase in lipid peroxidation in terms of MDA content was recorded in all the genotypes exposed to high temperature which was 46.9–177.1% higher as compared to the crop grown under normal temperatures (Table 2). The genotypes NSJ 189 and NSJ 221 exposed to high temperature showed the least increase in MDA content as compared to genotypes PSRJ 13099 and RJR 270. A significant higher level of H_2O_2 was observed in maize leaves in response to high temperature in all the genotypes, and compared to their respective control, the levels were 26.6–89.6% higher (Table 2). Importantly, the genotypes NSJ 189 and NSJ 221 maintained significantly lower levels of H_2O_2 content (26.3% and 43.9%, respectively), as compared to the genotypes PSRJ 13099 and RJR 270 (89.6% and 69.7%).

Table 1 Analysis of variance for oxidative metabolites and antioxidants (enzymatic and non-enzymatic) estimated in maize genotypes exposed to high-temperature stress during reproductive stage by sowing on two different dates

Source	df	MDA	H_2O_2	AsA	GSH	GSSG	GSH/GSSG ratio	APX	MDHAR activity	DHAR activity	GR activity	GST activity
Replications	2	25.29	1,098,377.55	8121.29	19.04	4.04	0.05	123.79	0.15	13.17	2.00	948.88
Treatments	7	1162.42**	18,272,969.52**	1,888,537.50**	122,870.76**	2452.10**	6.44**	28,980.83**	174.61**	2208.57**	321.73**	74,929.67**
Genotypes	3	235.82**	7,964,602.04**	1,637,088.72**	23,907.38**	357.44**	1.40**	17,202.07**	85.70**	88.04^{NS}	316.95**	21,196.03**
DOS	1	6370.04**	89,586,840.04**	3,727,240.17**	713,805.04**	14,800.67**	2.28**	122,079.87**	275.40**	145.04^{NS}	518.01**	392,389.56**
Genotypes X DOS	3	353.15**	4,810,046.82*	1,527,085.39**	24,856.04**	430.56**	12.88**	9726.58**	229.92**	5016.93**	261.09**	22,843.34**
Error	14	10.53	906,745.59	2480.05	32.19	15.61	0.12	312.93	8.05	49.12	2.06	116.03
Total	23	362.39	6,208,781.74	576,988.08	37,416.69	756.15	2.04	9021.50	58.05	703.22	99.35	22,957.82

df degree of freedom

^{NS}nonsignificant; **significant at 1% level; *significant at 5% level

Table 2 Mean, standard error and percent change for the characters evaluated in four maize genotypes sown on two different dates

Character	First date of sowing (NT)				Second date of sowing (HT)				% Change (with respect to NT)			
	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	PSRJ 13099	RJR 270	NSJ 189	NSJ 221
MDA (nmol g^{-1} FW)	29.3	27.7	28.0	32.7	70.7	76.7	52.7	48.0	140.9	177.1	88.1	46.9
H_2O_2 (nmol g^{-1} FW)	6104.7	7443.7	6381.0	7061.7	11,574.0	12,634.0	8076.7	10,162.7	89.6	69.7	26.6	43.9
AsA (nmol g^{-1} FW)	3398.0	3226.3	3233.7	3404.7	1804.0	1499.0	3306.7	3500.3	− 46.9	− 53.5	2.3	2.8
GSH (nmol g^{-1} FW)	249.0	243.3	251.7	221.3	563.3	419.7	712.3	649.7	126.2	72.5	183.0	193.5
GSSG (nmol g^{-1} FW)	41.0	32.3	51.3	48.7	113.3	84.3	84.7	89.7	176.4	160.8	64.9	84.2
GSH/GSSG ratio	6.1	7.6	4.9	4.5	5.0	5.0	8.4	7.2	− 18.0	− 34.4	70.3	59.6
APX activity (nmol min^{-1} mg^{-1} protein)	302.7	281.3	309.4	340.0	349.3	309.4	548.0	505.3	15.4	10.0	77.1	48.6
MDHAR activity (nmol min^{-1} mg^{-1} protein)	42.2	37.7	37.0	34.7	21.2	24.4	40.9	38.0	− 49.8	− 35.1	10.6	9.3
DHAR activity (nmol min^{-1} mg^{-1} protein)	191.0	202.7	154.7	150.7	154.3	149.7	209.0	205.7	− 19.2	− 26.2	35.1	36.5
GR activity (nmol min^{-1} mg^{-1} protein)	28.8	32.5	29.4	19.0	25.3	33.3	54.7	33.6	− 12.1	2.4	86.4	76.4
GST activity (nmol min^{-1} mg^{-1} protein)	225.2	225.1	224.5	219.0	435.2	331.3	545.1	605.2	93.2	47.2	142.8	176.4

'–' indicates % decrease

Non-enzymatic Antioxidants

Marked decreases in AsA contents were observed in genotypes PSRJ 13099 and RJR 270 (46.9% and 53.5%, respectively) in response to high-temperature stress, as compared to their respective control (Fig. 1). However, genotypes NSJ 189 and NSJ 221 had significantly higher AsA content, as compared to the plants subjected to high temperature. A drastic increase in GSH content was observed in response to high-temperature stress, as compared to the control in all the genotypes (Table 2). The genotypes NSJ 189 and NSJ 221 exposed to high temperature showed 183% and 193.5% increase, respectively, in GSH content compared to their respective control (Table 2). However, genotypes NSJ 189 and NSJ 221 maintained the GSSG levels lower than the genotypes PSRJ 13099 and RJR 270 under high temperature (Table 2). A significant decrease in the GSH/GSSG ratio was observed in genotypes PSRJ 13099 and RJR 270 in response to heat stress which was 18% and 34.4%, respectively, compared to their control (Table 2). However, genotypes NSJ 189 and NSJ 221 maintained the ratio significantly higher by 70.3% and 59.6% in response to heat stress as compared to their respective control.

Activities of Aantioxidative Enzymes

As given in Table 2, the APX activity of the maize leaves increased under high-temperature stress. The genotypes exposed to high temperature resulted in 10–77% increase in APX activity compared to their respective control. However, greater enhancement of APX activity was observed in genotypes NSJ 189 and NSJ 221, and compared to their respective control, the APX activities of genotypes NSJ 189, NSJ 221, PSRJ 13099 and RJR 270 were 77.1%, 48.6%, 15.4% and 10% higher, respectively.

Compared to the respective controls, the mean activities of MDHAR were 35.1% and 49.8% lower in the genotypes RJR 270 and PSRJ 13099, respectively, exposed to high temperature (Table 2). The DHAR activity also decreased in the same pattern and was 19.2 and 26.2% lower in PSRJ 13099 and RJR 270, respectively, at high temperature. More importantly, both the MDHAR and DHAR activities were recorded higher in genotypes NSJ 189 and NSJ 221 with very small changes in the activities when compared with their respective controls (Table 2).

The activity of GR remained almost unchanged in genotypes PSRJ 13099 and RJR 270, while it increased significantly in genotypes NSJ 189 and NSJ 221 under high-temperature stress (Table 2). Genotypes NSJ 189 and NSJ 221 showed 86.4% and 76.4% enhancement, respectively, in the GR activities at high temperature.

Among all of the enzymes studied, GST activity of maize leaves showed the highest up-regulation in response to high-temperature stress. Under high temperature, the mean activity increased by 114% compared to the plants grown under normal temperature conditions (Table 2). More importantly, the activity of genotypes NSJ 189 and

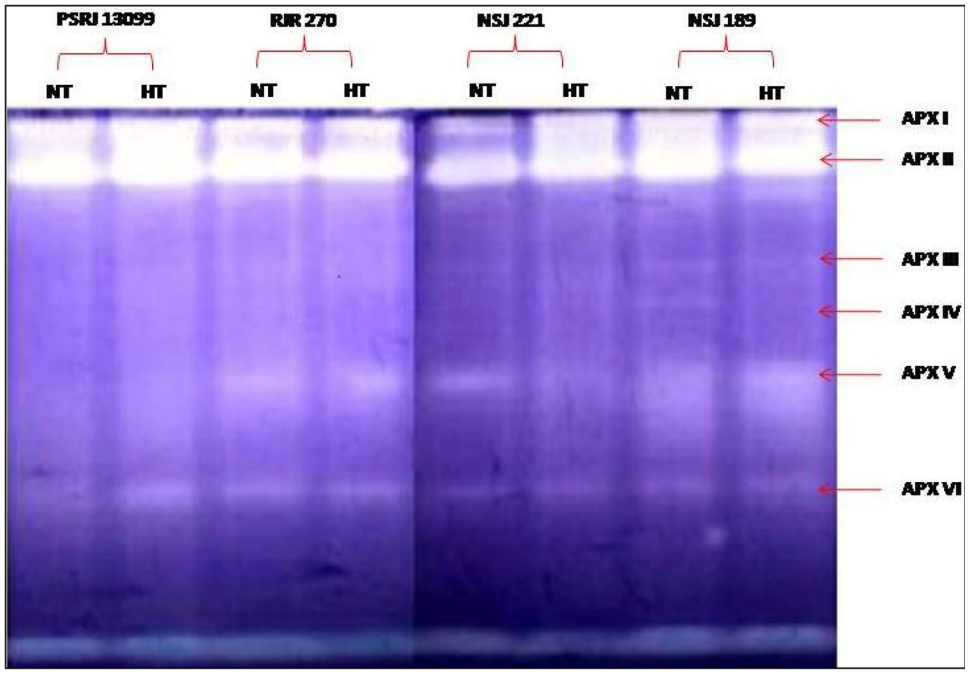

Fig. 1 Electrophoretic mobility profiling of ascorbate peroxidase (APX) isozymes in maize genotypes at reproductive stage under normal (NT) and high-temperature (HT) stress conditions

NSJ 221 (176.4% and 142.8%) was increased more as compared to genotypes PSRJ 13099 and RJR 270 (93.2% and 47.2%).

In gel Activity Assay of APX and GR

APX, the most important H_2O_2-degrading antioxidative enzyme in plants followed by CAT and POX, was found more heat stable in NSJ 189 and NSJ 221 than PSRJ 13099 and RJR 270. Diversity trends were observed in APX isozymes profile in the studied genotypes. Under normal temperature conditions, genotypes NSJ 189 and NSJ 221 showed some unique APX isozymes (APX III, IV and V). At higher temperature, seven isozymes of APX showed activity in both the genotypes (Fig. 1). Some APX isozymes (low molecular weight isoforms III, IV and V) which are unique in NSJ 189 and NSJ 221 showed an increase in activity under high-temperature conditions. High molecular weight APX isozymes (I and II) showed higher activity in all the genotypes under high temperature. APX isozymes VI and VII were of almost similar intensity in both growing conditions and across genotypes.

The native PAGE and *in gel* activity staining for GR revealed the presence of two isoforms in all the genotypes. Almost similar banding pattern and activity intensity appeared among all the samples irrespective of the variability observed in genotypes and growth conditions. Exposure to high temperature enhanced the intensity of both the isoforms to a very minimal extent (Fig. 2). However, no new GR isoform was observed under high-temperature stress.

Discussion

When plants face unfavorable conditions like high temperatures, the ROS production increased including $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ [23, 41], resulting in an imbalance in the redox equilibrium due to imbalance in the production and quenching of ROS. ROS may cause oxidative damage to chloroplasts and other subcellular structures [3, 34] by damaging membrane lipids. Lipid peroxidation (measured as MDA) via its generation of chemically reactive products contributes to the loss of cellular functions through the inactivation of membrane enzymes and even of cytoplasmic proteins [35]. Higher MDA levels decreased membrane fluidity, increased membrane permeability and damaged membrane embedded proteins, enzymes and ion channels [12]. Higher H_2O_2 levels resulted in peroxidation of membrane lipids [28], thus altering membrane structure and its functions. In the present study, both the MDA and H_2O_2 levels significantly increased by heat treatment. Genotypes having lower levels of H_2O_2 also showed the least increase in MDA content on exposure to high temperature. Similar trends have been observed between H_2O_2 and MDA content earlier [19, 40].

Ascorbate and reduced glutathione are the key non-enzymatic antioxidants [13]. Ascorbate is an effective ROS scavenger and cellular reductant in plants. Ascorbate can react with a range of ROS such as H_2O_2, $O_2^{\cdot-}$ and $^1O_2$, which makes it a potent antioxidant. Additionally, GSH plays a very vital role in alleviating heat-induced oxidative stress. GSH plays a key role in ROS neutralization by recycling other potential water-soluble antioxidant

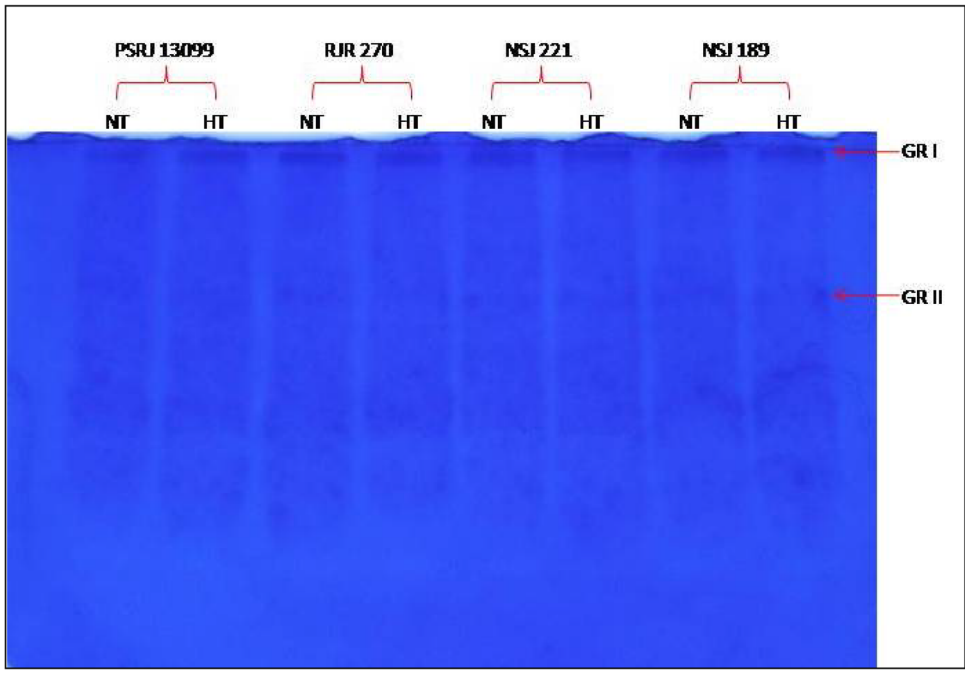

Fig. 2 Electrophoretic mobility profiling of glutathione reductase (GR) isozymes in maize genotypes at reproductive stage under normal (NT) and high-temperature (HT) stress conditions

metabolites via the ascorbate–glutathione cycle [13]. Our results from the present investigation demonstrated that AsA content significantly decreased by high-temperature stress (Table 1). This reduction in ascorbate pool was accompanied by the decrease in DHAR and MDHAR as well as increase in APX activity. On exposure to high temperature, glutathione accumulated in the leaves (Table 2). Kocsy et al. [18] and Yadav et al. [40] observed an increase in GSH and increase in GSH biosynthesis in plants under heat stress. Higher GSH content could be due to increased GR activity. Both AsA and GSH contents were observed to be significantly higher in genotypes NSJ 221 and NSJ 189 (Table 2). The formation of GSSG in heat-treated seedlings might be due to the reaction of GSH with oxy-radicals generated by oxidative stress or due to enhancement of GST activity that decomposes H_2O_2 and organic hydroperoxide. An increase in GSSG content under high temperature has been reported by Rivero et al. [32].

Ascorbate–glutathione cycle is the main metabolic pathway to detoxify the ROS and recycling of non-enzymatic antioxidants. The ascorbate–glutathione cycle contains four enzymes: APX, MDHAR, DHAR and GR which are systematically and coordinately involved in the H_2O_2 detoxification. Other than H_2O_2 detoxification, these enzymes also actively involved in the regeneration of non-enzymatic antioxidants like AsA and GSH. Results demonstrated that APX and GR activities significantly increased with high temperature in all the genotypes. The activities of MDHAR decreased markedly due to heat stress while a differential response was observed in respect of DHAR activities. These results were in partial agreement with Rivero et al. [32] but opposite to findings made by Ma et al. [21] and Dai et al. [8]. A slight increase in APX and GR activities was not enough to protect the plants from ROS-induced oxidative damages in susceptible genotypes. As MDHAR and DHAR both are equally vital in regulating the AsA level and its redox state [10, 11], decreases in the activities of these enzymes were followed by the decrease in AsA content. However, in genotypes NSJ 221 and NSJ 189 exposure to heat treatment significantly increased the activities of most of the enzymes resulting in increased AsA content (Table 2).

GSTs catalyze the conjugation reactions between electrophilic xenobiotic substrates and the GSH [9, 16]. In the present study, GST activity in maize seedlings profoundly increased in response to high temperature (Table 2). More importantly, heat-stressed seedlings of genotypes NSJ 189 and NSJ 221 showed increased GST activity. The enhanced GST activity might have reduced the levels of H_2O_2 and MDA content in maize seedlings under high-temperature stress (Table 2). Similar enhancements in the activity of GST have also been observed earlier [7, 15]. Polidoros and Scandolios [29] reported induction in GST1 gene expression in response to higher H_2O_2 levels.

AsA–GSH cycle is more efficiently operating in tolerant genotypes, thus imparting enhanced tolerance. The differential response of these genotypes to heat stress as a consequence of variation in the levels of non-enzymatic antioxidants and activities of enzymes suggests that manipulation of AsA–GSH pathway may lead to enhanced heat tolerance to maize.

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References

1. Ahmad P, Prasad MNV (2012) Abiotic stress responses in plants: metabolism, productivity and sustainability. Springer, New York
2. Asada K (1992) Ascorbate peroxidase—a hydrogen peroxide scavenging enzyme in plants. Physiol Plant 85:235–241
3. Asada K (1994) Production of active oxygen species in photosynthetic tissue. In: Foyer CH, Mullineaux PM (eds) Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, pp 77–101
4. Balla K, Bencze S, Janda T, Veisz O (2009) Analysis of heat stress tolerance in winter wheat. Acta Agron Hung 57:437–444
5. Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci 4:273
6. Bradford MM (1976) Arapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
7. Cetinkaya H, Tasci F, SeckinDinler B (2014) Regulation of glutathione S-transferase enzyme activity with salt pre-treatment under heat stress in maize leaves. Res Plant Biol 4:45–56
8. Dai AH, Nie YX, Yu B, Li Q, Lu LY, Bai JG (2012) Cinnamic acid pretreatment enhances heat tolerance of cucumber leaves through modulating antioxidant enzyme activity. Environ Exp Bot 79:1–10
9. Dixon DP, Skipsey M, Edwards R (2010) Roles of glutathione transferase in plant secondary metabolism. Phytochemistry 71:338–350
10. Eltayeb AE, Kawano N, Badawi G, Kaminaka H, Sanekata T, Morishia I (2006) Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. Physiol Plant 127:57–65
11. Eltayeb AE, Kawano N, Badawi G, Kaminaka H, Sanekata T, Shibahar T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Plants 225:1255–1264
12. Garg N, Manchanda G (2009) ROS generation in plants: boon or bane? Plant Biosyst 143:8–96
13. Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930
14. Hall AE (1992) Breeding for heat tolerance. Plant Breed Rev 10:129–168
15. Hasnuzzaman M, Fujita M (2011) Selenium pretreatment upregulates the antioxidant defense and methylglyoxal detoxification system and confers enhanced tolerance to drought stress in rapeseed seedlings. Biol Trace Elem Res 143:1758–1776
16. Hossain MZ, Hossain MD, Fujita M (2006) Induction of pumpkin glutathione *S*-transferase by different stresses and its possible mechanisms. Biol Plant 50:210–218
17. Huang C, He W, Guo J, Chang X, Su P, Zhang L (2005) Increased sensitivity to salt stress in ascorbate-deficient *Arabidopsis* mutant. J Exp Bot 56:3041–3049
18. Kocsy G, Szalai G, Galiba G (2002) Effect of heat stress on glutathione biosynthesis in wheat. Acta Biol Szeged 46:71–72
19. Kong W, Huang C, Chen Q, Zou Y, Zhang J (2011) Nitric oxide alleviates heat stress-induced oxidative damage in *Pleurotuseryngii* var. tuoliensis. Fungal Genet Biol 49:15–20
20. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophase T4. Nature 227:680–685
21. Ma YH, Ma FW, Zhang JK, Li MJ, Wang YH, Liang D (2008) Effects of high temperature on activities and gene expression of enzymes involved in ascorbate–glutathione cycle in apple leaves. Plant Sci 175:761–766
22. Meehl GA, Stocker TF, Collins WD, Friedlingstein P, Gaye AT, Gregory JM, Kitoh A, Knutti R, Murphy JM, Noda A, Raper SCB, Watterson IG, Weaver AJ, Zhao ZC (2007) Global climate projections. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds) Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, New York, pp 749–845
23. Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410
24. Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium. Anal Biochem 212:540–546
25. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490–498
26. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880
27. Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Ann Rev Plant Physiol Plant Mol Biol 49:249–279
28. Pastori GM, Trippi VS (1992) Oxidative stress induces high rate of glutathione reductase synthesis in a drought resistant maize strain. Plant Cell Physiol 33:957–961
29. Polidoros AN, Scandalios JG (1999) Role of hydrogen peroxide and different classes of antioxidants in the regulation of catalase and glutathione S-transferase gene expression in maize (*Zea mays* L.). Physiol Plant 106:112–120
30. Prasad PVV, Bheemanhalli R, Jagadish SVK (2017) Field crops and the fear of heat stress-Opportunities, challenges and future directions. Field Crops Res 200:114–121
31. Rao MV, Paliyath G, Ormrod DP (1996) UV-B- and ozone induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol 110:125–136
32. Rivero RM, Ruiz JM, Romero L (2004) Oxidative metabolism in tomato plants subjected to heat stress. J Hortic Sci Biotechnol 79:560–564
33. Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K (2002) Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot 53:1305–1319
34. Singh AK, Singhal GS (2001) Effect of irradiance on the thermal stability of thylakoid membrane isolated from acclimated wheat leaves. Photosynthetica 39:23–27
35. Stark G (2005) Functional consequences of oxidative membrane damage. J Memb Biol 205:1–16
36. Tiroli-Cepeda AO, Ramos CHI (2010) Heat causes oligomeric disassembly and increases the chaperone activity of small heat shock proteins from sugarcane. Plant Physiol Biochem 48:108–116
37. Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61:199–223
38. Xu S, Li J, Zhang X, Wei H, Cui L (2006) Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts

in two cool-season turfgrass species under heat stress. Environ Exp Bot 56:274–285
39. Yadav SK, Tiwari YK, Darala PK, Shanker AK, Jyothi Lakshmi N, Vanaja M, Maheswari M (2015) Genotypic variation in physiological traits under high temperature stress in maize. Agric Res 5:119–126
40. Yadav SK, Tiwari YK, Singh V, Patil AA, Shanker AK, Jyothi Lakshmi N, Vanaja M, Maheswari M (2016) Physiological and biochemical basis of extended and sudden heat stress tolerance in maize. Proc Natl Acad Sci India Sect B Biol Sci 88:249–263
41. Yin H, Chen QM, Yi MF (2008) Effects of short-term heat stress on oxidative damage and responses of antioxidant system in *Liliumlongiflorum*. Plant Growth Regul 54:45–54
42. Yu CW, Murphy TM, Lin CH (2003) Hydrogen peroxide induces chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. Funct Plant Biol 30:955–963