Australian Journal of Crop Science AJCS

AJCS 16(04):454-460 (2022) ISSN:1835-2707
doi: 10.21475/ajcs.22.16.04.p3376

Overexpression of the mitochondrial MT-sHSP23.6 gene during antioxidant activity in tomato cv. Micro-Tom subjected to flooding

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Abstract

Plants grown under flooding stress conditions form reactive oxygen species, both during oxygen deficiency and after reoxygenation. The tolerance of plants under these conditions depends on an efficient antioxidant system, which may be influenced by the higher expression of heat shock proteins, such as mitochondrial MT-sHSP23.6. The objective of the present study was to determine whether the overexpression of this gene increases the antioxidant activity in tomato cv. Micro-Tom plants subjected to flooding during the vegetative stage of four fully expanded leaves. The experiment was performed with three genotypes of tomato plants (*Solanum lycopersicum* Mill. cv. Micro-Tom), one non-transformed denominated "WT" and two modified for high and low expression of the MT-sHSP23.6 gene, subjected to normal irrigation conditions, 72 hours of flooding, and 24 and 192 hours of recovery after the removal of flooding, consisting a 3x4 split-plot factorial design with four replications. Plants were collected at the vegetative stage of 4 fully expanded leaves to determine superoxide dismutase, ascorbate peroxidase and catalase activities, hydrogen peroxide accumulation and lipid peroxidation. Overexpression of the mitochondrial MT-sHSP23.6 gene in the evaluated plants resulted in higher antioxidant system activity. However, it did not necessarily lead to less damage caused by oxidative stress. Low expression of this gene resulted in lower antioxidant system activity and greater damage caused by reactive oxygen species.

Key words: *Solanum lycopersicum* L., heat shock proteins, antioxidant system.
Abbreviations: ANOVA_analysis of variance; APX_ascorbate peroxidase; CAT_catalase activity; Cu_copper; DNA_desoxirribonucleic acid; EDTA_ethylenediaminetetraacetic; Fe_iron; Fe^{+2}_ferrous metal; GPOD_guaiacol peroxidase; H_2O_2_hydrogen peroxide; HO_2^+_ hydridodioxygen(1+); HSPs_heat shock proteins; MDA_malondialdehyde; Mn_manganese; NBT_nitrotetrazolium blue; O_2_oxygen; O_2^-_superoxide ion ; OH^-_hydroxide ion; ROS_reactive oxygen species; SOD_superoxide dismutases; TBA_thiobarbituric acid; TCA_trichloroacetic acid; WT_wild type; Zn_ zinc.

Introduction

Plants subjected to oxygen deficiency in roots often suffer damage to their cellular structure and, consequently, loss of cellular activity, resulting in reduced yield or even death.
Oxygen deficiency has been reported as the main problem associated with flooding. Thus, tolerance to hypoxia (low oxygen concentration) and/or anoxia (absence of oxygen) have been used as synonyms of tolerance to flooding stress. During anaerobiosis, there is a significant change in protein synthesis in the roots. Many of the anaerobic proteins identified are enzymes involved in glucose metabolism and fermentation. Consequently, this stress affects plant development (Fante et al., 2010). Moreover, a decrease in the photosynthetic capacity leads to the production of reactive oxygen species (ROS), such as superoxide anion radicals ($O2^{-\cdot}$), hydroxyl radicals ($\cdot OH$), hydrogen peroxide (H_2O_2), alkoxy radicals ($RO^{\cdot}$) and singlet oxygen (O_2^1) (Lukić et al., 2021; Fathi and Tari, 2016; Munne-Bosch and Penuelas, 2003).

Even when flooding conditions are removed, the plant remains susceptible to damage resulting from the accumulation of ROS. During electron transport in the chloroplasts and mitochondria, some electrons are lost and then captured by O_2, forming O_2^- (Leshen, 1988). At the end of a period of low O_2 concentration, i.e., when the plant returns to its normoxic condition, greater electron loss and, consequently, greater formation of O_2^- due to the malfunction of the electron transport chain occur.
The transfer of electrons to O_2 and the subsequent redox reactions in plant cells can cause the formation of ROS. In addition to the production of these more toxic species, reduction reactions can lead to the formation of other types of oxidants with a very positive redox potential, which can oxidize many essential cellular molecules. Under normal conditions, the cellular environment tends to maintain the redox potential in homeostasis, which, however, is disturbed by various intrinsic and extrinsic factors. A change in the

redox equilibrium state induced by various stresses creates an oxidative environment (Biswal and Biswal, 1999).

The accumulation of H_2O_2 and O_2^- results from the conversion of these molecules into even more reactive species. An example of this is the conversion of H_2O_2 in the presence of Fe^{+2} to OH^-, which is extremely toxic. These radicals can react with proteins and thus reduce enzyme activity, react with lipids (lipid peroxidation) and increase membrane permeability and react with DNA, causing mutations (Moller, 2001).

However, cells have several mechanisms that developed during the evolutionary process to efficiently detoxify these ROS in order to control the levels of these molecules and cancel the toxicity. Antioxidant molecules, simple enzymes and a more complex detoxification system may be involved in cellular protection against the accumulation of these substances, preventing the intoxication of plant cells (Anjum et al., 2011; Sairam et al., 2008). Examples include the superoxide dismutases (SODs), which catalyze the dismutation of O_2^- and HO_2^+ to H_2O_2 and may be bound to a metal (Cu/Zn, Mn and Fe); the ascorbate-glutathione cycle, which is the main ROS removal system in chloroplasts; and the catalases, which are enzymes that convert H_2O_2 into H_2O and O_2 (Jimenéz et al., 1997).

Cell homeostasis in all organisms, under optimal and adverse growth and development conditions, can be determined by a family of proteins that is highly conserved among species, namely, heat shock proteins (HSPs). HSP are molecular chaperones that regulate the folding, localization, accumulation and degradation of protein molecules, both in plants and in animals. Therefore, it is believed that these proteins play important roles in several cellular processes, which may have a generalized role in tolerance to multiple environmental stresses (Feder and Hofmann, 1999).

HSP expression is controlled by transcription factors (heat shock factor - HSF) and among the HSF classes, HSFA positively regulates plant tolerance to anoxia, heat, osmotic and oxidative stresses (Zhuang et al., 2018). HSFA1 found in tomato plants is considered a master regulator of signal perception, transduction and controlling the expression of stress-responsive genes, including HSPs (Guo et al., 2016; Mishra et al., 2002).

Although the interaction of HSPs with the antioxidant system has not been fully elucidated, several studies have sought to relate their expression with plant tolerance to oxidative stress (Krishna et al., 1995; Krishna and Gloor, 2001; Liu et al., 2006; Song et al., 2009; Reissig et al., 2018). Tomato plants (*Solanum lycopersicum* L.) of the Micro-Tom cultivar were modified for high and low mitochondrial MT-sHSP23.6 gene expression, as described by Huther et al. (2013). The aim of the present experiment was to determine whether the overexpression of this gene increases the antioxidant activity in tomato cv. Micro-Tom plants when subjected to flooding during the vegetative stage of four fully expanded leaves.

Results and discussion

Enzymatic activity

SOD, APX and CAT (Table 1) activities as well as lipid peroxidation (Table 2) were influenced by the interaction between the factors "flooding" and “tomato cv. Micro-Tom variety” (wild type (WT) and modified for high and low expression of the mitochondrial MT-sHSP23.6 gene). The accumulation of hydrogen peroxide varied only as a function of the flooding treatment and not as a function of the tomato cv Micro-Tom variety.

While water availability was maintained at field capacity, SOD activity was similar in WT plants and in those modified for high (High) and low (Low) mitochondrial MT-sHSP23.6 gene expression (Figure 1). When subjected to 72 hours of flooding, the WT and High varieties exhibited higher SOD activity than the Low variety. The large increase in SOD activity under anoxic conditions observed in flood-tolerant species was discussed as an evolutionary advantage of plants to prepare themselves for reoxygenation (Biemelt et al., 2000). VanToai and Bolles (1991) showed that high SOD activity under anoxic stress in soybean was accompanied by the ability to overcome post-anoxic stress.

Thus, higher SOD activity in WT tomato cv. Micro-Tom and that modified for high mitochondrial MT-sHSP23.6 gene expression after 24 hours and 192 hours of reestablishment of the normoxic condition suggests a higher tolerance capacity of these plants to damage caused by ROS formed when oxygen becomes available again as an electron acceptor for saturated redox chains (Biemelt et al., 2000). In addition, post-anoxic or post-hypoxic lesions may be caused by toxic acetaldehyde derived from catalase-dependent ethanol oxidation, which accumulates under oxygen deprivation conditions (Monk et al., 1987; Zuckermann et al., 1997).

APX activity under normal irrigation conditions was similar for the WT and High and Low varieties (Figure2). Immediately after 72 hours of flooding, APX activity increased in all tomato varieties, especially in the High variety. After 24 hours of restoration of the normal O_2 availability condition, APX activity decreased to less than half in all varieties, being higher in the Low variety, possibly due to the lower SOD activity, which, consequently, resulted in a longer time for dismutation of the superoxide anion (O_2^-) by SOD (EC 1.15.1.1) to produce H_2O_2, maintaining higher APX activity longer.

A similar result was found by Biemelt et al. (1998), who studied the activity of antioxidant enzymes in wheat roots during hypoxia and after reoxygenation. The authors found that the activities of both APX and glutathione reductase (GR) decreased progressively with the duration of anoxic stress and that, depending on the duration of this treatment, the restoration of both enzymatic activities was delayed after the plants were aerated again.

At 192 hours after restoration of the normoxic condition, the tomato cv. Micro-Tom variety with high expression of the mitochondrial MT-sHSP23.6 gene showed more than double the APX activity presented by the wild type and low expression varieties, which, together with the high SOD activity, indicates a greater ability to cope with both the superoxide (O_2^-) and the H_2O_2 formed after reoxygenation.

Reissig et al. (2018) studied tomato cv. Micro-Tom fruits modified for high MT-sHSP23.6 gene expression stored under hypoxic conditions (daily nitrogen flow rate of 0.098 MPa/10 min) for 8 days and normoxic conditions for 5 days and found higher activity for all antioxidant enzymes under hypoxic conditions (25, 20, 76 and 48% higher than the wild type for SOD, CAT, APX and guaiacol peroxidase (GPOD), respectively), as well as lower superoxide anion concentrations, suggesting that the overexpression of this gene results in higher antioxidant system activity throughout the plant.

Under normal irrigation conditions, CAT activity was also similar among the evaluated tomato varieties. However,

Table 1. Analysis of variance (ANOVA) for superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) as a function of the tomato cv. Micro-Tom genotypes – non-transformed (WT) and transformed for high and low mitochondrial MT-sHSP23.6 gene expression – and as a function of the flooding treatment (field capacity, 72 hours of flooding, and after 24 and 192 hours of recovery).

SOD						
	DF	SS	MS	Fc	Pr (<Fc)	
Flooding	3	18304699	6101566	1271.8	$2.2x10^{-16}$	***
Error a	12	57571	4798			
Variety	2	2727687	1363843	341.27	$2.2x10^{-16}$	***
Flooding*Variety	6	6852866	1142144	285.79	$2.2x10^{-16}$	***
Error b	24	95913	3996			
Total	47	28038736				
CV 1 (%)	11.8719					
CV 2 (%)	10.83					
APX						
	DF	SS	MS	Fc	Pr (>Fc)	
Flooding	3	657.25	219.083	394.66	$2.2x10^{-16}$	***
Error a	12	6.66	0.555			
Variety	2	85.96	42	52.39	$2.2x10^{-16}$	***
Flooding*Variety	24	126	21	25.6	$2.2x10^{-16}$	***
Error b	24	19.69	0.82			
Total	47	895.56				
CV 1 (%)	14.66					
CV 2 (%)	17.83					
CAT						
	DF	SS	MS	Fc	Pr (>Fc)	
Flooding	3	30	10.2071	72.545	$2.2x10^{-16}$	***
Error a	12	1	0.1407			
Variety	2	1	0.6852	5.707	0.009383	**
Floodplain * Variety	6	6	1.0763	8.966	$3.5x10^{-5}$	***
Error b	24	2	0.12			
Total	47	43.019				
CV 1 (%)	30.2016					
CV 2 (%)	27.89732					

***significant at 0.001%.**significant at 0.01%.

Table 2. Analysis of variance (ANOVA) for hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) as a function of the tomato cv. Micro-Tom genotypes – non-transformed (WT) and transformed for high and low mitochondrial MT-sHSP23.6 gene expression —– and as a function of the flooding treatment (field capacity, 72 hours of flooding and after 24 and 192 hours of recovery).

H_2O_2						
ANOVA	DF	SS	MS	Fc	Pr (>Fc)	
Flooding	3	104.029	34.676	14.5703	0.000264	***
Error a	12	28.559	2.38			
Variety	2	13.665	6.832	1.4512	0.25411	ns
Flooding*Variety	24	12.987	2.165	0.4598	0.830916	ns
Error b	24	112.989	4.708			
Total	47	272.228				
CV 1 (%)	11.67					
CV 2 (%)	16.42					
MDA						
ANOVA	DF	SS	MS	Fc	Pr (>Fc)	
Flooding	3	150.094	50.031	173.187	$2.2x10^{-16}$	***
Error a	12	3.467	0.289			
Variety	2	17.183	8.591	9.852	0.000752	***
Flooding*Variety	6	122.172	20.362	23.35	$2.2x10^{-16}$	***
Error b	24	20.929	0.872			
Total	47	313.845				
CV 1 (%)	7.54					
CV 2 (%)	13.10					

*** significant at 0.001%.ns = not significant.

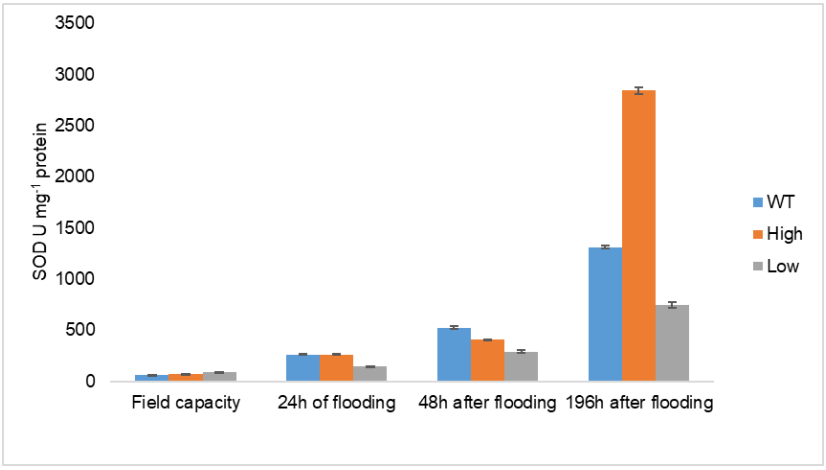

Figure 1. Superoxide dismutase (SOD) in tomato cv. Micro-Tom genotypes: non-transformed (WT) and transformed for high (High) and low (Low) mitochondrial MT-sHSP23.6 gene expression, subjected to 72 hours of flooding and after 24 and 192 hours of recovery.

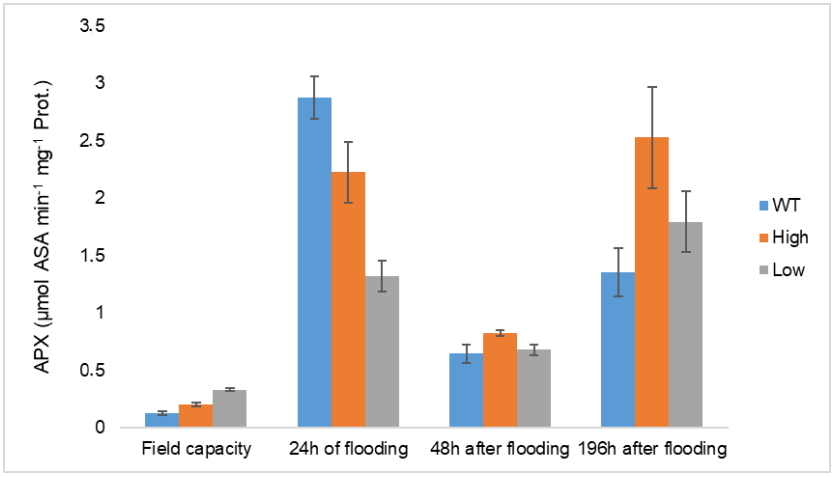

Figure 2. Ascorbate peroxidase (APX) activity in tomato cv. Micro-Tom genotypes: non-transformed (WT) and transformed for high (High) and low (Low) mitochondrial MT-sHSP23.6 gene expression, subjected to 72 hours of flooding and after 24 and 192 hours of recovery.

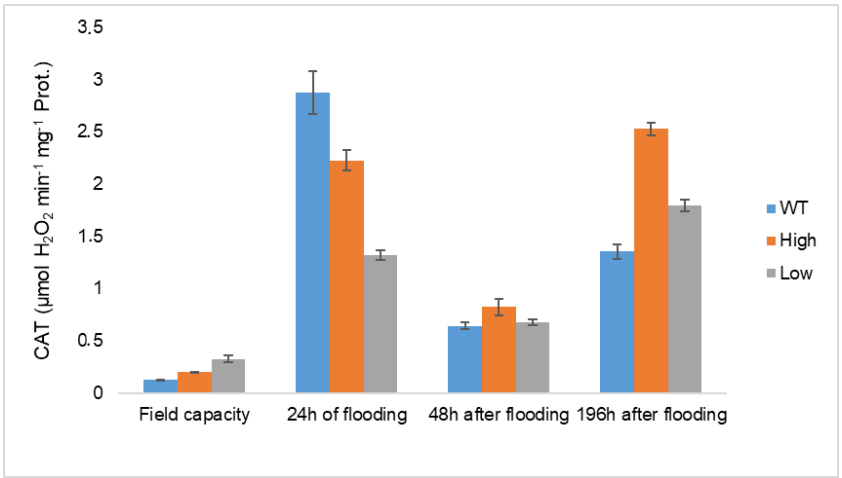

Figure 3. Catalase activity (CAT) in tomato cv. Micro-Tom varieties: wild type (WT) and transformed for high (High) and low (Low) expression of the mitochondrial MT-sHSP23.6 gene, subjected to 72 hours of flooding and after 24 and 192 hours of recovery.

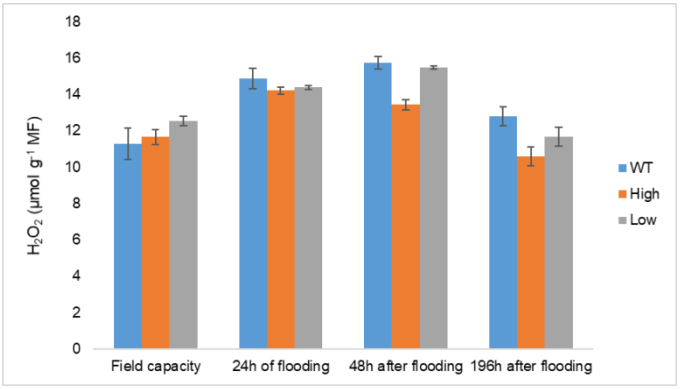

Figure 4. Hydrogen peroxide (H_2O_2) in tomato cv. Micro-Tom varieties: wild type (WT) and modified for high (High) and low (Low) expression of the mitochondrial MT-sHSP23.6 gene, subjected to 72 hours of flooding and after 24 and 192 hours of recovery.

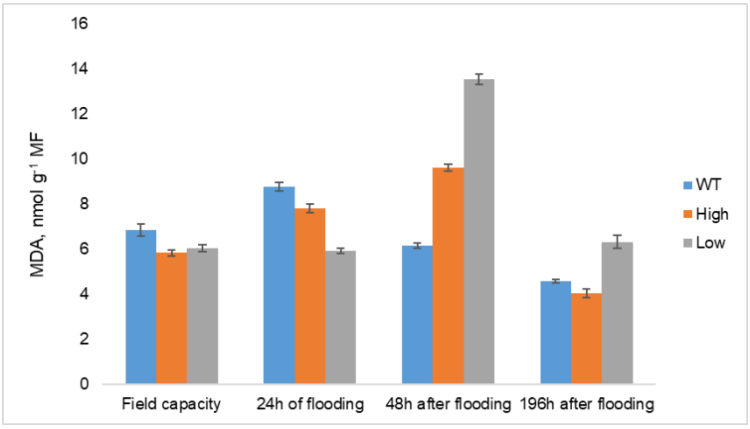

Figure 5. Lipid peroxidation (MDA) in the tomato cv. Micro-Tom varieties: wild type (WT) and modified for to high (High) and low (Low) expression of the mitochondrial MT-sHSP23.6 gene, subjected to 72 hours of flooding and after 24 and 192 hours of recovery.

immediately after 72 hours of flooding, higher activity was observed in the WT variety and lower activity in the Low variety (Figure 3). The same reduced APX activity was observed at 24 hours after restoration of the normoxic condition and increased activity at 192 hours after restoration was observed for CAT activity. The relationship between HSPs and the antioxidant system has been widely studied with respect to plant tolerance to oxidative stress; however, Song, Fan and Li (2009) concluded that the overexpression of the organellar and cytosolic AtHSP90 gene in *Arabidopsis thaliana* reduced the tolerance of the plants to oxidative stress, suggesting that these proteins are involved in different functional mechanisms during this type of stress. In the present study, there is evidence that the mitochondrial MT-sHSP23.6 gene is closely linked to the synthesis of antioxidant enzymes.

For both the wild variety and the modified varieties, the H_2O_2 concentration increased immediately after 72 hours of flooding, remained high at 24 hours after the restoration of the normoxic condition, and returned to normal concentrations at 192 hours after restoration (Figure 4). This result was consistent with the high SOD activity, especially at 192 hours after recovery of normoxia. A similar result was found by Biemelt et al. (2000) for wheat plants exposed to flooding and followed by re-exposure to air, in which the H_2O_2 concentration was below the control level at 16 hours after reaeration of anoxically treated plants; however, under hypoxic conditions, the H_2O_2 concentration in the wheat plants was reduced.

Lipid peroxidation and hydrogen peroxide

The lipid peroxidation level increased in the wild variety after 72 hours of flooding, reaching lower levels than the control (field capacity) after 24 and 192 hours of recovery (Figure 5). In the variety modified for high mitochondrial MT-sHSP23.6 gene expression, lipid peroxidation was highest after 24 hours of recovery, higher than the levels observed in the wild variety. After 192 hours of recovery, the lipid peroxidation levels in the variety modified for high expression were lower than those observed in the plants irrigated to field capacity; however, the peroxidation level was not lower than that of the wild variety. The variety modified for low MT-sHSP23.6 gene expression showed a higher level of lipid peroxidation after 24 hours of reoxygenation, higher than those presented by the WT and High varieties.

Materials and methods

Plant material and experimental conditions

The experiment was conducted at Eliseu Maciel Agronomy College, Federal University of Pelotas (Universidade Federal de Pelotas – UFPel), located in Capão do Leão, Rio Grande do Sul state, Brazil (31°48'02.69" S latitude). Seeds of three varieties of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom were used: a wild variety and varieties modified for high and low expression of the mitochondrial MT-sHSP23.6 gene, described in Huther et al. (2013).

The seeds were sown in 72-cell expanded polystyrene trays, and the seedlings were grown until the stage of 4 fully expanded leaves then transplanted to 20-liter black polyethylene trays. Soil collected from the A1 horizon of a Solodic Eutrophic Haplic Planosol (Streck et al., 2008), previously corrected according to soil analysis and based on the Manual of Fertilization and Liming for Soils of Rio Grande do Sul and Santa Catarina (CQFS-RS/SC, 2004), was used as substrate. The trays were kept in a greenhouse and irrigated until field capacity when necessary.

To facilitate excess water drainage and maintenance of the soil field capacity, the trays were perforated at the bottom and inserted into nonperforated trays to apply the flooding treatment. Field capacity was determined using the tension table method (EMBRAPA, 1997), which was used to define the volume of water required for the establishment of flooding over a period of 72 hours; the soil was flooded to 20 mm water depth above the soil surface.

Experimental design and data collection

Plants of each MT-sHSP23.6 gene expression variety of the cultivar used in normal irrigation conditions were collected immediately after the flooding period, at 24 hours of recovery after the removal of flooding and at 192 hours of recovery after the removal of flooding, when they were in the vegetative stage of four fully expanded leaves. Therefore, the experimental was arranged in a 3x4 split-plot factorial design, with the 'no' and 'with' flooding treatments allocated to the plot and MT-sHSP23.6 gene expression allocated to the subplot, with 4 replications.

Enzymatic activity

From each collected sample, 0.3 g of tissue was macerated in liquid nitrogen and homogenized in extraction buffer consisting of 100 mM potassium phosphate, pH 7.8, 0.1 mM EDTA and 10 mM ascorbic acid. After centrifugation at 13,000 *g* for 20 min at 4 °C, the supernatant was collected and desalted in Sephadex G-25 (PD-10 column). The eluate was used for enzymatic analysis of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Biemelt et al., 1998), and protein was quantified using the Bradford method (1976). SOD activity was determined by the ability of the enzyme to inhibit the photoreduction of nitrotetrazolium blue (NBT) (Giannopolitis and Ries, 1977) in an incubation medium composed of 50 mM potassium phosphate, pH 7.8, 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin. The samples and the control containing the reaction medium were illuminated with a 20 W lamp for 7 min, while the blank was kept in the dark. The readings were performed at 560 nm, and enzyme activity was calculated using the following equation: % inhibition = (A560 sample with enzyme extract – A560 control without enzyme)/(A560 control without enzyme). One unit of SOD corresponded to the amount of enzyme capable of 50% inhibition of NBT photoreduction under the assay conditions, and the result was expressed as U mg^{-1} protein.

CAT activity was quantified by the reduction in absorbance at 240 nm in incubation buffer containing 200 mM potassium phosphate, pH 7.0, and 12.5 mM H_2O_2, incubated at 28 °C, where the consumption of hydrogen peroxide was monitored (Havir and Mchale, 1987), and the result was expressed in µmol H_2O_2 min^{-1} mg $protein^{-1}$. APX activity was determined according to Nakano and Asada (1981) using 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM ascorbic acid and 0.1 mM H_2O_2, monitoring the rate of ascorbate oxidation at 290 nm, with the result expressed in µmol ascorbic acid min^{-1} mg $protein^{-1}$.

Lipid peroxidation and hydrogen peroxide

Lipid peroxidation was determined by quantifying thiobarbituric acid reactive species according to the method described by Buege and Aust (1978). Two hundred milligrams of plant tissue was macerated in liquid N_2 plus 20% PVP (w/v), homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 10 min. Aliquots of the supernatant were added to the reaction medium (0.5% (w/v) thiobarbituric acid (TBA) and 10% (w/v) TCA) and incubated at 95 °C for 30 min. The reaction was stopped by rapid cooling on ice, and the readings were determined in a spectrophotometer at 535 nm and 600 nm. The concentration of the malondialdehyde (MDA)/TBA complex was calculated using the following equation: [MDA] = (A535 – A600)/(ξ.b), where ξ (extinction coefficient) = 1.56 x 10^{-5} cm^{-1} and b (optical length) = 1. Peroxidation was expressed in nmol of MDA g^{-1} fresh weight. Quantification of hydrogen peroxide was determined by macerating 200 mg of plant tissue in liquid N_2, homogenization of the tissue in 5 ml of TCA and centrifugation at 12,000 *g* for 15 min at 4 °C. H_2O_2 was determined by measuring the absorbance at 390 nm in a reaction medium containing 100 mM potassium phosphate buffer, pH 7.0, 500 µL of the extract and 1 mL of potassium iodide (Velikova et al., 2000). The results were expressed in µmol H_2O_2 g fresh $weight^{-1}$.

Statistical analyses

The data were analyzed for normality using the Shapiro-Wilk test ($p \leq 0.05$) and subjected to analysis of variance (ANOVA) ($p \leq 0.05$). Significance was compared with Tukey's test ($p \leq 0.05$). The statistical program R ®, version 3.1.1, and the data package "ExpDes.pt" (Banzato and Kronka, 2006; R Core Team, 2014) were used.

Conclusion

The overexpression of the MT-sHSP23.6 mitochondrial gene in tomato cv. Micro-Tom plants results in higher antioxidant system activity but not necessarily less damage caused by oxidative stress. Low gene expression results in lower antioxidant system activity and greater damage caused by ROS.

Acknowledgments

The authors thank the CAPES (Brazilian Research Agency, Brazil) and CNPq (Brazilian Council for Scientific and Technological Development, Brazil) for financial support and grants.

Declaration of conflicting interests

The authors declare no competing interests.

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