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EFFECT OF SHORT-TERM SALT STRESS ON OXIDATIVE STRESS MARKERS AND ANTIOXIDANT ENZYMES ACTIVITY IN TOCOPHEROL-DEFICIENT *Arabidopsis thaliana* PLANTS

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Changes of carotenoids and anthocyanins content, lipid peroxidation, and activity of antioxidant enzymes were studied in wild type and tocopherol-deficient lines vte1 and vte4 of Arabidopsis thaliana subjected to 200 mM NaCl during 24 h. The salt stress enhanced the intensity of lipid peroxidation to different extent in all three plant lines. Salt stress resulted in an increase of carotenoid content and activity of catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase in wild type and tocopherol-deficient vte1 mutant. However, the increase in anthocyanins concentration was observed in vte1 mutants only. In vte4 mutant, which contain γ-tocopherol instead of α-tocopherol, the response to salt stress occurred via coordinative action of superoxide dismutase and enzymes of ascorbate-glutathione cycle, in particular, ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, and glutathione-S-transferase. It can be concluded, that salt stress was accompanied by oxidative stress in three studied lines, however different mechanisms involved in adaptation of wild type and tocopherol-deficient lines to salt stress.

Key words: Antioxidant enzymes; Arabidopsis thaliana; Oxidative stress; Salt stress; Tocopherols.

Salt stress is one of the most significant abiotic stresses and affects many aspects of plant physiology and homeostasis [1, 2]. The effects of high salinity on plants can be mainly classified as two different factors: osmotic stress induced by high salt concentration in the environment and the toxic effect of sodium accumulated in the cell. For most plants, these two effects are clearly separated in time. The first, osmotic stress, starts immediately after increase of salt concentration around the roots and leads to turgor loss and stomatal closure. The second stress is related with ion toxicity and starts when salt accumulates in plant cells to toxic concentrations [2]. High concentrations of sodium ions can inhibit activity of many essential enzymes, cell division and expansion, disorganize membrane, which finally can lead to death of old leaves and growth inhibition of young leaves [2]. Along with these primary effects, secondary stress, such as oxidative, occurs because high concentrations of ions disrupt cellular homeostasis and increase generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) [1]. The enhanced ROS production during stress can induce oxidative modification of lipids, nucleic acids, and proteins [3].

Plants possess several mechanisms to detoxify ROS which include non-enzymatic antioxidants as well as antioxidant enzymes [3]. Tocopherols are considered as important scavengers of singlet oxygen, hydroxyl radical and lipid peroxy radicals [4]. Among four forms of tocopherols (α-, β-, γ- and δ-), found in plants, α-tocopherol possesses the highest antioxidant activity and is the most abundant form in leaves, whereas γ-tocopherol dominates in seeds [5]. Numerous studies have reported the important role of α-tocopherols in salt stress tolerance of different plant species [6–8]. In *Arabidopsis* (Columbia ecotype) the level of α-tocopherol decreased, whereas the level of γ-tocopherol increased in response to short-term salt stress [9]. Function of tocopherols was studied in tobacco plant exposed to long-term salt stress [10]. It was shown that γ-tocopherol can't substitute α-tocopherol under salt-induced oxidative stress, but it improves the plant's physiological status under sorbitol-induced osmotic stress. Moreover, under salt stress α-tocopherol may indirectly better protect macromolecules than γ-tocopherol, which suggests a specific role of α-tocopherol in plants via its participation in cellular signaling as well [10]. In our previous work, we showed that tocopherol-deficient *vte1* and *vte4* lines of Arabidopsis were

resistant to long-term salt stress [11]. Recently Cela and colleagues [12] showed that *vte4* mutants had reduced jasmonic acid and ethylene signaling gene expression levels in mature leaves under salt stress conditions as compared to the wild type.

In this work, we have studied oxidative stress response of two tocopherol-deficient mutants of *Arabidopsis thaliana* subjected to short-term high salinity. The *vte1* mutant is deficient in tocopherol cyclase and consequently lacks all four tocopherols, as well as plastoquinone, but accumulates the redox active pathway intermediate dimethylphytylbenzoquinone (DMPBQ) [13, 14]. The *vte4* mutant is deficient in γ-tocopherol methyltransferase activity and lacks α-tocopherol, but instead accumulates γ-tocopherol in leaves [15]. The potential influence of tocopherol composition on carotenoids and anthocyanins content, lipid peroxidation, and antioxidant enzyme activities under salt stress was studied in the above mentioned plant lines.

Materials and Methods

Seeds of *Arabidopsis thaliana* wild type (Columbia) and mutant lines *vte4* (SALK_03676) and *vte1* (GABI_11D07), defective in *VTE4* and *VTE1* genes, respectively, were obtained from the Salk Institute [16] and GABI-Kat [17] and selected homozygote plants from the seeds at the Institute of Botany of Kiel University (Germany) were used in the present investigation. The plants were grown in hydroponic system using Rockwool supports as described by [18] at 28°C and naturally illuminated environmental conditions. The Gibeau nutrient solution [18] was used and changed every two weeks. Ten-week-old plants were subjected to salt stress by supplement of their nutrient solution with NaCl to final concentration 200 mmol/l for 24 h. Control plants were grown on nutrient solution without NaCl. After 24 h fully expanded leaves of plants were harvested and frozen with liquid nitrogen.

Contents of carotenoids and anthocyanins were measured spectrophotometrically in leaves as described by [19]. Tissues were homogenized in a Potter-Elvjeham glass homogenizer with ice-cold 96% ethanol (1 : 10, w/v) in the presence of $CaCO_3$ (for preventing of pheophytinization). The homogenates were centrifuged at 8000 g during 10 min (4 °C) using centrifuge OPN-8 (USSR), supernatants were collected and the pigments were repeatedly extracted two times from pellets with 1 ml ice-cold 96% ethanol. All supernatants were collected and concentrations of carotenoids were measured spectrophotometrically at 470 nm wavelength in the combined resulting extracts [19]. Carotenoids content was calculated as described by [11]. Anthocyanin content was determined after extract acidification with concentrated HCl to its resulting concentration 1%. The anthocyanin concentration was assayed spectrophotometrically at 530 nm wavelength and an absorption coefficient of 30 $mM^{-1}cm^{-1}$ was used [20].

To measure the level of lipid peroxidation and activity of antioxidant enzymes the frozen leaves were powdered in liquid nitrogen with mortal and pestle and mixed (1/5, w/v) with 50 mM potassium-phosphate buffer (pH 7.0) that contained 1 mM EDTA and 1 mM phenylmethylsulfonylfluoride (PMSF). Ascorbic acid (1 mM) was added to potassium-phosphate buffer in the case of ascorbate peroxidase (APX) assay. The homogenates were centrifuged at 13,000 g for 20 min at 4 °C in Eppendorf 5415R (USA) centrifuge. The supernatant obtained from each sample was collected and used for further assay.

Supernatants were mixed with an equal aliquot of 40% (w/v) trichloroacetic acid (TCA) and then centrifuged for 10 min at 5000 g. The supernatants were used for determination of lipid peroxide level. The degree of lipid peroxidation was evaluated as the level of thiobarbituric acid reactive substances (TBARS) as described by Heath and Packer [21].

The activity of superoxide dismutase (SOD; 1.15.1.1) was assayed as a function of its inhibitory action on quercetin oxidation [22]. One unit of SOD activity is defined as the amount of enzyme (per protein milligram) that inhibits quercetin oxidation reaction by 50% of the maximum value, which was calculated using 'KINETICS' program for non-linear inhibition [23].

Catalase (1.11.1.6) activity was measured spectrophotometrically at 240 nm [24]. The activity of ascorbate peroxidase (APX; 1.11.1.11) was monitored following the decrease of absorbance at 290 nm (ε = 2.8 $mM^{-1}cm^{-1}$) due the oxidation of ascorbic acid to dehydroascorbate [25]. Guaiacol peroxidase (GuPx; 1.11.1.7) activity was assayed spectrophotometrically following the increase in absorbance at 470 nm due to guaiacol oxidation (ε = 26.6 $mM^{-1}cm^{-1}$) [26]. Dehydroascorbate reductase (DHAR; 1.8.5.1) activity was determined by measuring the increase in absorbance at 265 nm due the formation of ascorbic acid (ε = 14 $mM^{-1}cm^{-1}$) [27]. Glutathione-S-transferase (GST; 2.5.1.18) activity was measured by monitoring the formation of adduct between GSH and 1-chloro-2,4-dinithrobenzene (CDNB) at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$) [22]. Glutathione reductase (GR; 1.6.4.2) activity was determined as the decrease in absorbance at 340 nm (ε = 6.22 $mM^{-1}cm^{-1}$) due to the oxidation of reduced NADPH [22].

One unit of CAT, APX, GuPx, DHAR, GR and GST activity is defined as the amount of the enzyme consuming 1 μmol of substrate or generating 1 μmol of product per minute; the activities were expressed as international units (or milliunits) per milligram of protein.

Protein concentration was determined with Coomassie brilliant blue G-250 according to Bradford's method [28] with bovine serum albumin as a standard.

All values were expressed as means ± S.E.M. of three independent experiments. For statistical analysis, the Student's t-test was used to compare values under stress conditions with their corresponding controls values, and to compare *vte4* and *vte1* mutant lines with the wild type.

Results and Discussion

It has been suggested that α-tocopherol and β-carotene cooperate in protection against singlet oxygen induced damage to photosystem II [5]. In our study, the concentration of carotenoids was increased by 43 and 38% in leaves of salt-treated wild type and *vte1* mutant line, respectively, but did not change in *vte4* mutants as compared with the control values (Table). It is possible, that not only α-tocopherol, but also its redox-active precursor DMPBQ could cooperate with carotenoids in scavenging of singlet oxygen during the stress. Previous studies indicated that deficiency in α-tocopherol resulted in enhanced anthocyanin level induced by stress [29]. Our data are in good agreement with that. Under salt stress conditions, anthocyanin content increased by 16% in *vte1* mutant line, but did not differ from control in the wild type and *vte4* mutants (Table).

It has been shown that ROS production, particularly $O_2^{\cdot-}$ and H_2O_2, is stimulated under salt stress conditions [30]. Free radical-induced peroxidation of lipids is one of the markers of stress-induced damage [31]. Decomposition of lipid hydroperoxides results in formation of diverse products including malondialdehyde (MDA) [31]. In this work the product of MDA condensation with thiobarbituric acid (TBA) was measured as thiobarbituric acid reactive substances (TBARS). In our study, the level of TBARS increased in salt stressed wild type, *vte4*, and *vte1* mutant lines by 79, 23 and 33%, respectively compared to the control values (Fig. 1). However, TBARS concentration in the *vte4* mutant was lower compared to wild type under salt stress. Similar tendency was observed in γ-TMT transgenic tobacco under both salt and sorbitol stresses [10]. It is likely, that during salt stress γ-tocopherol (present in *vte4* mutants) controls the extent of lipid peroxidation directly or indirectly via unknown mechanisms. Some studies

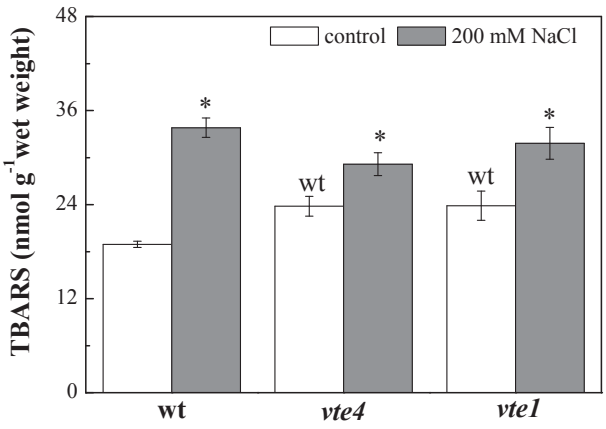

*Fig. 1. TBARS concentrations in leaves of wild type, vte4 and vte1 plants of A. thaliana after 24 h exposure to 200 mM NaCl. *Significantly different from respective control group (P < 0.01). [wt]Significantly different from respective control group of wild type plants (P < 0.05)*

*Effect of short-term salt stress on the carotenoids and anthacyanins content (μmol/gww) in the leaves of wild type, vte4, vte1 plants of A. thaliana. *Significantly different from the respective control group (P < 0.01). [wt]Significantly different from the respective group of wild type plants, [vte1] vte1 mutant line (P < 0.05)*

Carotenoids		Anthocyanins	
Control	200 mM NaCl	Control	200 mM NaCl
Wild type			
0.21 ± 0.02	0.30 ± 0.02*	0.44 ± 0.02	0.45 ± 0.02
vte4			
0.29 ± 0.02[wt,vte1]	0.28 ± 0.02	0.49 ± 0.02[wt,vte1]	0.46 ± 0.02
vte1			
0.16 ± 0.02[wt]	0.22 ± 0.02*	0.37 ± 0.02[wt]	0.43 ± 0.02*

suggested that in salt tolerant plant species a lower TBARS increase could be due to the higher activity of antioxidant enzymes, in particular SOD and APX, under the salt stress conditions [32–34].

SOD belongs to the first line of defense and catalyses the dismutation of $O_2^{\cdot-}$ to molecular oxygen and H_2O_2 [35]. This enzyme plays critical protective role against oxidative damage, since superoxide acts as a precursor for more cytotoxic or highly reactive oxygen derivatives, such as peroxynitrite or hydroxyl radical [3]. In our study, salt stress did not change SOD activity in wild type and *vte1* plants, but increased it by 26% in the leaves of *vte4* mutant line (Fig. 2, *A*). It can be supposed that substitution of α-tocopherol with γ-tocopherol compensates the increase of SOD activity and thereby provides the *vte4* mutants with better protection against salt-induced oxidative damage of lipids evidenced by a lower TBARS level. In salt stressed wild type and *vte1* mutants plants the lack of change in SOD activity may indicate that this enzyme is not crucial for ROS detoxification, and non-enzymatic routes for conversion of $O_2^{\cdot-}$ to H_2O_2 via antioxidants (glutathione and ascorbate) may compensate this.

Metabolism of H_2O_2 depends on various functionally interrelated antioxidant enzymes such as catalase, APX and peroxidases. Catalase has been found predominantly in leaf peroxisomes [36]. Treatment with 200 mM NaCl enhanced catalase activity in wild type, *vte4*, and *vte1* mutant plant lines by 47, 35 and 28%, respectively, in comparison with respective controls (Fig. 2, *B*). APX which uses ascorbate as a reductant in the first step of the ascorbate-glutathione (AsA-GSH) cycle is the most important plant peroxidase in H_2O_2 detoxification [3, 37]. In response to the stress, APX activity increased in wild type, *vte4*, and *vte1* mutant plant lines by 181, 66 and 27%, respectively, in comparison with control groups (Fig. 2, *C*). The fact, that the activity of APX was higher in salt stressed *vte4* plants than in wild type and *vte1* mutant plants, probably, indicates that this enzyme is responsible for elimination of SOD generated H_2O_2. In addition, it has been reported that higher APX activity correlated with lower levels of lipid

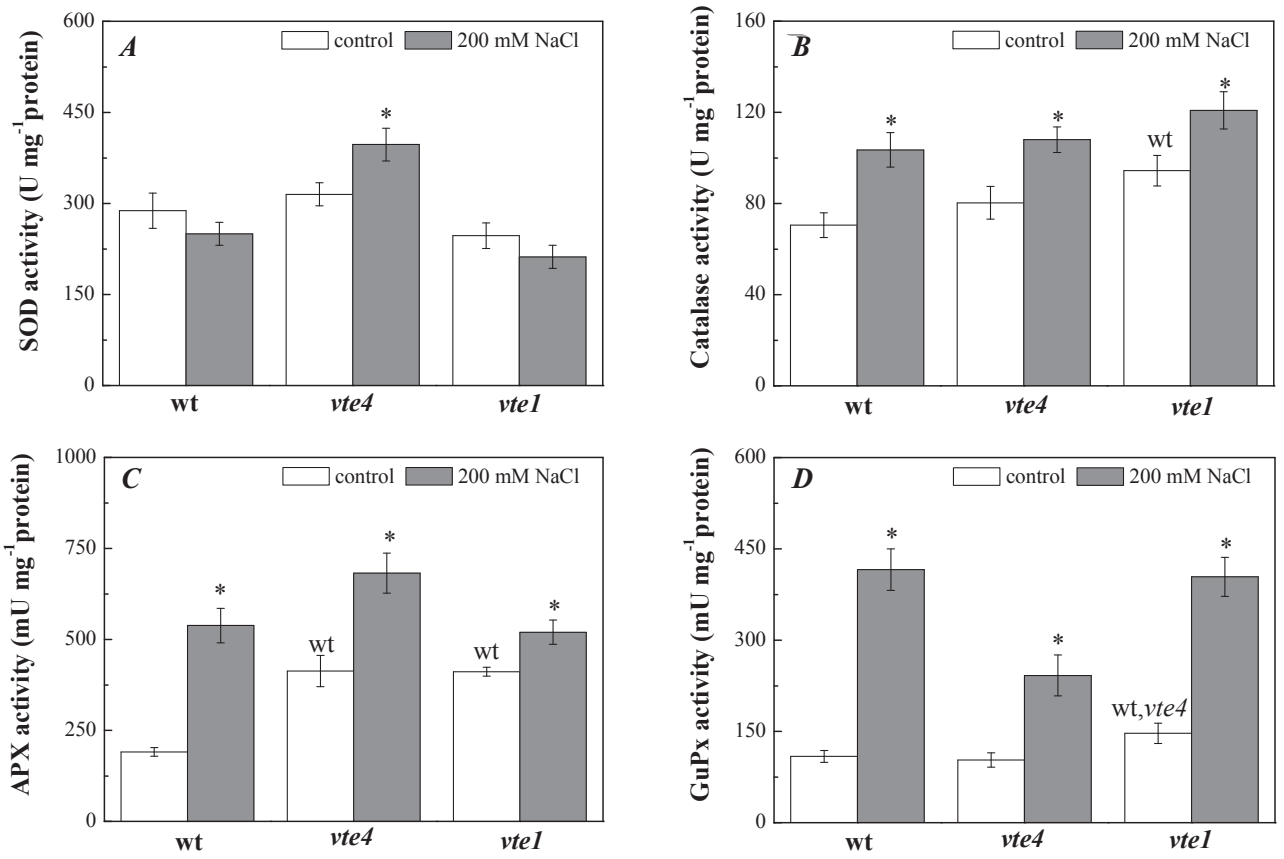

*Fig. 2. Activity of SOD (A), catalase (B), APX (C) and GuPx (D) in the leaves of wild type, vte4 and vte1 plants of A. thaliana after 24 h exposure to 200 mM NaCl. *Significantly different from the respective control group ($P < 0.025$). ^{wt}Significantly different from the respective control group of wild type plants, ^{vte4}vte4 mutant lines ($P < 0.05$)*

peroxidation under salt stress [38]. In addition to APX, guaiacol peroxidase (GuPx) is also involved in the scavenging of soluble hydroperoxides in plants [3]. Similarly to catalase and APX, the activity of GuPx increased 3.8, 2.4 and 2.8 times in the leaves of NaCl treated wild type, *vte4* and *vte1* plants, respectively, as compared to the respective controls (Fig. 2, *D*). The increase in activity of APX, catalase and GuPx in response to salt stress in wild type and both mutant lines, confirms previous results indicating that all three enzymes are involved in scavenging of H_2O_2 during salt stress [9, 32, 38].

Regeneration of ascorbate via AsA-GSH cycle requires the activity of MDAR, DHAR and GR [39]. The salt stress enhanced by 25% DHAR activity in *vte4* mutant plant leaves as compared to the control, whereas in the rest groups it was not affected (Fig. 3, *A*). The increase in DHAR activity may be required to sustain cycling of oxidized ascorbate when the flux through the AsA-GSH cycle is increased [40, 41]. That may occur in salt stressed *vte4* mutants, which posses the enhanced SOD and APX activities. The lack of changes in DHAR activity in wild type and *vte1* mutant can be associated with higher constitutive activity of this enzyme. GR is the last enzyme of ascorbate-glutathione cycle and catalyzes the NADPH-dependent reduction of oxidized glutathione [39]. In response to salt stress, the activity of GR increased by 19, 42 and 27% in leaves of wild type, *vte4* and *vte1* plants, respectively, in comparison with controls (Fig. 3, *B*). It seems that the increase of GR activity helps plants to maintain higher ratio of GSH/GSSG, which is required for ascorbate reduction.

Glutathione-S-transferase conjugates GSH with diverse electrophiles, resulting in their detoxification [3]. The response of this enzyme to salt stress treatment was observed in many plant species [7, 42]. In our experiments, treatment with 200 mM NaCl increased GST activity by 34% in the leaves of wild type and *vte4* mutant lines, as compared to the controls, whereas in *vte1* mutants no changes were found (Fig. 3, *C*). This indicates that wild type and *vte4* mutant lines possess effective mechanisms to conjugate with GSH and detoxify electrophiles when exposed to the salt stress conditions.

It can be concluded that salt stress may be accompanied by enhanced ROS generation, thereby leading to increased lipid peroxidation and activities of antioxidant enzymes, such as catalase, APX, GuPx and GR (Fig. 4). However, different mechanisms may be involved in adaptation of wild type and tocopherol-deficient lines to the salt stress. It can be suggested, that in *vte4* mutant,

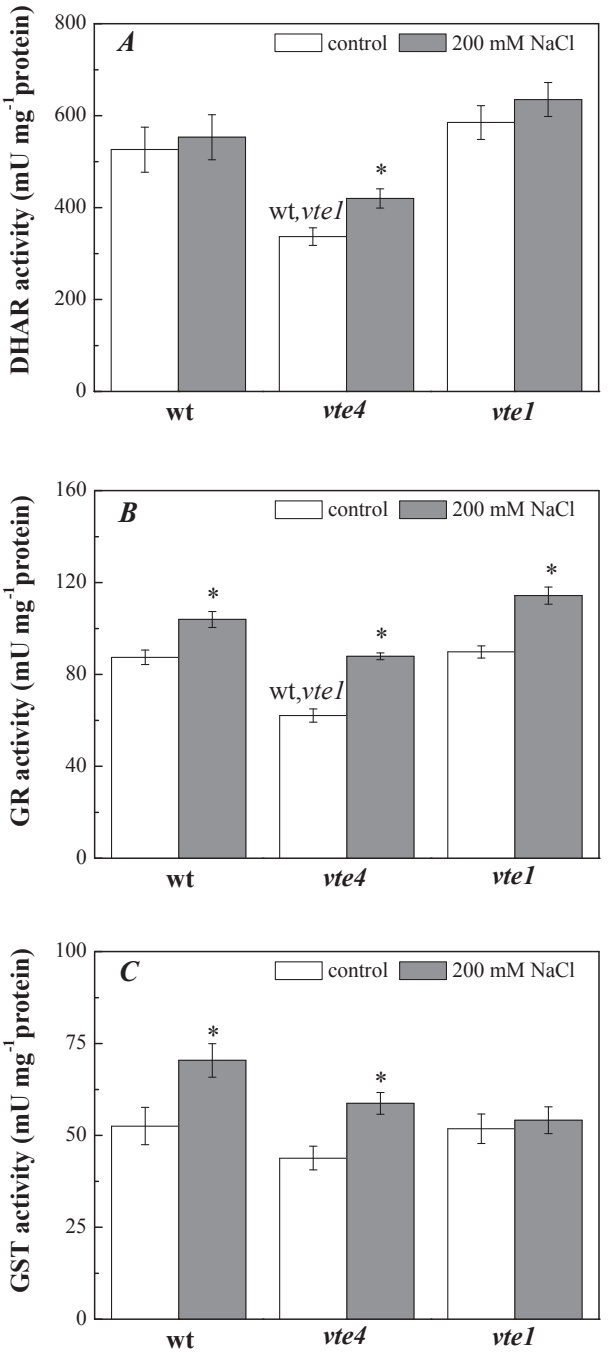

*Fig. 3. Activity of DHAR (A), GR (B) and GST (C) in leaves of wild type, vte4 and vte1 plants of A. thaliana after 24 h exposure to 200 mM NaCl. *Significantly different from respective control group ($P < 0.025$). ^{wt}Significantly different from respective control group of wild type plants, ^{vte1}vte1 mutant lines ($P < 0.05$)*

which contain γ-tocopherol instead α-tocopherol, co-operative action of SOD and enzymes of ascorbate-glutathione cycle provide better protection against lipid peroxidation. At the same

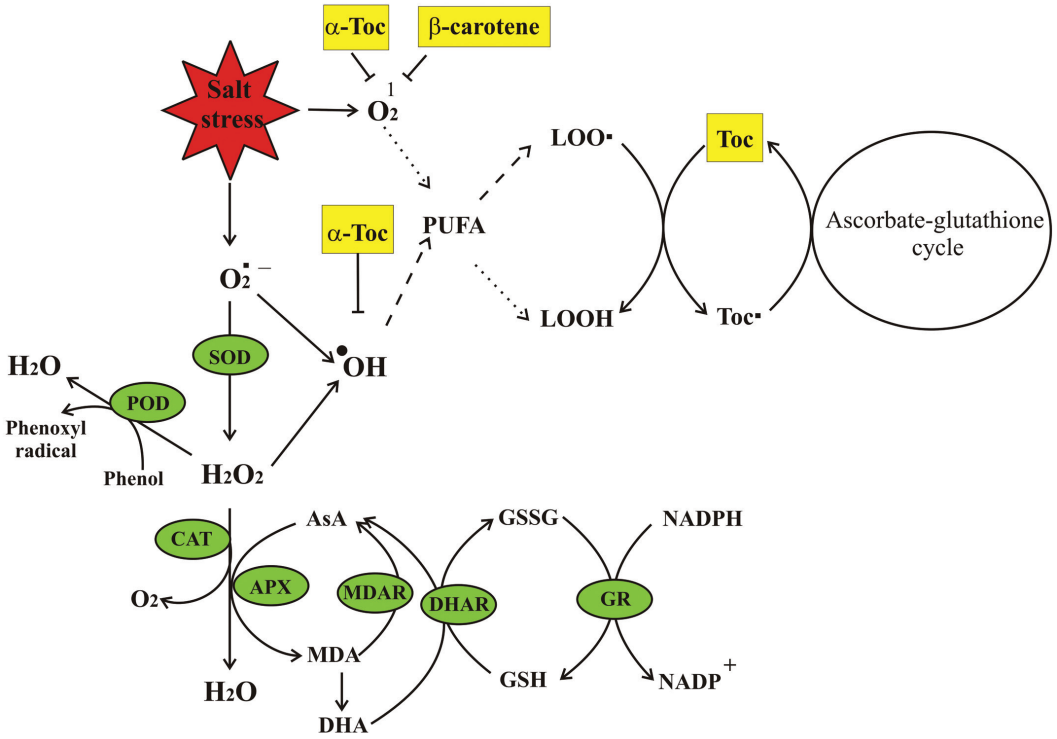

Fig. 4. Involvement of tocopherol in antioxidant system of plant. Abbreviation: AsA, ascorbate; MDA, monodehydroascorbate; DHA, dehydroascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; Toc, tocopherol; Toc•, tocopheryl-radical; LOOH, lipid hydroperoxide; LOO•, lipid peroxyl radical. Enzymes involved: APX, ascorbate peroxidase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; MDAR, monodehydroascorbate reductase, CAT, catalase; SOD, superoxidedismutase; POD, peroxidase

time, the lack of tocopherols in *vte1* mutant plants may be compensated by an increase of carotenoid and anthocyanin concentrations in response to the salt stress.

ВПЛИВ КОРОТКОТРИВАЛОГО СОЛЬОВОГО СТРЕСУ НА МАРКЕРИ ОКСИДАТИВНОГО СТРЕСУ ТА АКТИВНІСТЬ АНТИОКСИДАНТНИХ ЕНЗИМІВ У ТОКОФЕРОЛ-ДЕФІЦИТНИХ РОСЛИН *Arabidopsis thaliana*

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Досліджено вміст каротиноїдів, антоціанів, рівень пероксидного окислення ліпідів та активність антиоксидантних ензимів у рослин *Arabidopsis thaliana* дикого типу і дефектних за біосинтезом токоферолу лініях *vte1* та *vte4* за дії 200 мМ NaCl протягом 24 годин. Сольовий стрес призводив до зростання інтенсивності пероксидного окислення ліпідів у всіх трьох досліджуваних ліній рослин. За дії сольового стресу концентрація каротиноїдів та активність каталази, аскорбатпероксидази, гваяколпероксидази та глутатіонредуктази зростали у рослин дикого типу та токоферол-дефіцитної лінії *vte1*, проте, підвищення концентрації антоціанів спостерігалося тільки у рослин мутантної лінії *vte1*. У рослин мутантної лінії *vte4*, яка містить γ-токоферол замість α-токоферолу, відповідь на сольовий стрес відбувалася через узгоджену дію супероксиддисмутази та ензимів аскорбат-глутатіонового циклу, а саме аскорбатпероксидази, дегідроаскорбатредуктази, глутатіонредуктази та глутатіон-S-трансферази. Можна дійти висновку, що сольовий стрес супроводжується оксидативним стресом у трьох досліджуваних ліній рослин, разом з тим різні механізми задіяні в адаптації рослин дикого типу та токоферол-дефіцитних ліній.

Ключові слова: антиоксидантні ензими, *Arabidopsis thaliana*, оксидативний стрес, сольовий стрес, токофероли.

ВЛИЯНИЕ КРАТКОВРЕМЕННОГО СОЛЕВОГО СТРЕССА НА МАРКЕРЫ ОКСИДАТИВНОГО СТРЕССА И АКТИВНОСТЬ АНТИОКСИДАНТНЫХ ЭНЗИМОВ В ТОКОФЕРОЛ-ДЕФИЦИТНЫХ РАСТЕНИЯХ *Arabidopsis thaliana*

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Исследовано содержание каротиноидов, антоцианов, уровень пероксидного окисления липидов и активность антиоксидантных энзимов в растениях *Arabidopsis thaliana* дикого типа и дефектных по биосинтезу токоферола линиях *vte1* и *vte4* при воздействии 200 мМ NaCl в течение 24 часов. Солевой стресс повышал интенсивность пероксидного окисления липидов во всех трех исследуемых линиях растений. При солевом стрессе концентрация каротиноидов и активность каталазы, аскорбатпероксидазы, гваяколпероксидазы и глутатионредуктазы повышались в растениях дикого типа и токоферол-дефицитной линии *vte1*, однако, увеличение концентрации антоцианов наблюдалось только в растениях мутантной линии *vte1*. В растениях мутантной линии *vte4*, содержащей γ-токоферол вместо α-токоферола, ответом на солевой стресс было согласованное действие супероксиддисмутазы и энзимов аскорбат-глутатионового цикла, а именно аскорбатпероксидазы, глутатионредуктазы, дегидроаскорбатредуктазы и глутатион-S-трансферазы. Можно сделать вывод, что солевой стресс сопровождается оксидативным стрессом у трех исследуемых линий растений, вместе с тем различные механизмы задействованы в адаптации растений дикого типа и токоферол-дефицитных линий.

Ключевые слова: антиокислительные энзимы, *Arabidopsis thaliana*, окислительный стресс, солевой стресс, токоферолы.

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