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DOUBLE-STRANDED RNA-SUPPRESSOR OF PROLINE DEHYDROGENASE GENE IMPROVES DROUGHT RESPONSE OF PHOTOSYNTHETIC APPARATUS OF SUNFLOWER PLANTS

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The biochemical and physiological peculiarities of the photosynthetic apparatus and its response to drought in transgenic sunflower plants of the VK-121 line (T_3 generation) harboring a double-stranded RNA suppressor of the proline dehydrogenase (ProDH) gene were studied in comparison with the wild type line VK-121. Under optimal water conditions, the transgenic plants did not differ from the wild type in total chlorophyll content, quantum efficiency of PSII, or CO_2 assimilation and transpiration rates; however, they exhibited significantly higher activities of the antioxidant enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) in chloroplasts. Soil drought caused a smaller decline in chlorophyll content, maximum quantum efficiency of PSII photochemistry (F_v/F_m), effective quantum yield of PSII in the light (ϕPSII), as well as respiration and transpiration rates in the transgenic plants, although the net CO_2 assimilation rate was equally inhibited in both lines. At the same time, the plants with the double-stranded RNA suppressor of ProDH also demonstrated a smaller reduction in CO_2 assimilation after an abrupt increase in leaf temperature. Under drought conditions, non-photochemical quenching (NPQ) of absorbed light energy was lower in transgenic VK-121 T_3 plants than in the wild type VK-121 line. This was accompanied by an increase in the degree of xanthophyll cycle pigment de-epoxidation in the wild type line, while remaining constant in the leaves of the transgenic plants. Drought induced a stronger increase in SOD and APX activity in the leaves of the wild type line compared to the transgenic line. The results indicate that the VK-121 T_3 transgenic sunflower plants with partial suppression of the proline dehydrogenase gene exhibit enhanced tolerance of the photosynthetic apparatus compared to the wild type VK-121 line, owing to the pronounced activity of protective and regulatory mechanisms under soil drought.

Key words: *Helianthus annuus* L., sunflower, genetic transformation, double-stranded RNA-suppressor of proline dehydrogenase gene, photosynthesis, superoxide dismutase, ascorbate peroxidase, drought.

The frequency and intensity of the most adverse weather event — water scarcity — are expected to increase globally due to the ongoing rise in

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atmospheric greenhouse gas concentrations and the resulting negative trend of climate change [1, 2]. This challenge is compounded by the declining availability of new arable land and the degradation of existing soils [3, 4]. In many regions, reduced agricultural productivity and quality driven primarily by drought are anticipated to be among the most significant future threats [5—7]. Consequently, developing strategies to mitigate the detrimental effects of water stress on crop yield and quality is critical for maintaining global food security [8].

It is important to note that under natural conditions, drought is often accompanied by high air temperatures, and moreover, the temperature of photosynthesizing organs exceeds the ambient temperature due to a decline in the transpiration rate caused by stomatal closure, which significantly reduces the leaves' ability to self-cooling [9]. Thus, tolerance to high temperature could be considered as important constituent of drought-tolerance for photosynthetic apparatus.

Genetic engineering opens up broad prospects for the improvement of plants and the development of new stress-tolerant forms [11]. One of the key directions is transgenesis based on RNA interference technology and homology-dependent gene silencing [11, 12]. Epigenetic gene silencing mediated by short interfering siRNAs of 20—24 bp, which can function as regulatory molecules, provides new opportunities for controlling plant adaptive processes. Particular attention is given to genes regulating the metabolism of a key stress-protective compound, proline [13, 14].

It is well established that the reciprocal regulation of genes encoding enzymes of proline biosynthesis (Δ^1-pyrroline-5-carboxylate synthetase, P5CS) and catabolism (proline dehydrogenase, ProDH) plays a decisive role in maintaining proline levels under abiotic stress. A crucial task is the choice of strategy to enhance the content of this amino acid to physiologically significant levels. This can be achieved either by introducing additional copies of the P5CS cDNA or by partial suppression of endogenous ProDH genes. The latter is accomplished by integrating ProDH gene fragments in antisense orientation or as inverted repeats, which leads to post-transcriptional RNA silencing and reduced expression of this gene [15].

Biotechnological studies in this area have enabled the development of genetically modified lines of various crops with enhanced tolerance to stress factors. In particular, using *Agrobacterium*-mediated transformation, we obtained sunflower lines with reduced ProDH expression, which exhibited increased tolerance to osmotic stress [16]. This trait of transgene functionality was stably inherited in subsequent seed generations. In T_3 and T_4 transgenic sunflower plants, the presence of a double-stranded RNA suppressor of the ProDH gene resulted in elevated proline accumulation under both optimal and stress conditions (2.5—5.0-fold), which contributed to improved tolerance to water deficit and salinity, and correlated with biomass accumulation. Both under normal irrigation and drought conditions, the shoot and root weight in transgenic plants exceeded that of the wild type variants [17, 18]. Similar results were also obtained in spring and winter wheat [19, 20].

As known, the basis of plant productivity is the carbon dioxide photosynthetic fixation [21]. Yield losses during drought are related to a decrease in the photosynthetic apparatus functional activity as a result of

damages and protective reactions [22, 23]. Drought decreased the CO_2 assimilation activity due to a reduction in stomatal conductance, a slowdown in the diffusion of CO_2 from the leaf intercellular spaces to the carboxylation centers (mesophyll conductance), as well as impaired ATP synthesis in chloroplasts, reduced activity and deteriorate synthesis of photosynthetic enzymes, and increased photorespiration rate [24].

At elevated temperature, in addition to these factors, significant constituents of the photosynthesis decrease are the impairment of Rubisco activase's activity and worsening Rubisco's substrate specificity [25]. In bright sunlight under stress conditions suppression of CO_2 assimilation sharply increases the risk of oxidative damage to photosynthetic membranes by reactive oxygen species (ROS), which are formed due to the accumulation of excess electrons in the chloroplast electron transport chain (ETC) and subsequent reduction of molecular oxygen [26]. The most important systems for protecting the photosynthetic apparatus from oxidative damage caused by ROS are the enzymatic antioxidant systems of chloroplasts [27].

To obtain new crop genotypes through genetic engineering that can produce high yields under unfavorable conditions, a deep understanding of the relationships between the introduced genetic changes and the regulatory systems of key physiological functions in plants, primarily photosynthesis, is necessary [28]. Therefore, the combination of biotechnological approaches to the creation of new stress-resistant forms of cultivated plants and their phenotyping in terms of physiological and biochemical parameters is of particular relevance [10].

The aim of our work was to compare the functional characteristics of the photosynthetic apparatus in the sunflower plants of wild type and transgenic line harboring a double-stranded RNA suppressor of the proline dehydrogenase gene, under optimal water supply and soil drought conditions, to identify physiological changes associated with enhanced drought tolerance.

Materials and methods

In this study, transgenic sunflower (*Helianthus annuus* L.) plants of the VK-121 line T_3 generation were used. These plants were obtained through *Agrobacterium*-mediated *in planta* transformation of the inbred line VK-121 (developed at the Institute of Oilseed Crops, National Academy of Agrarian Sciences of Ukraine, Zaporizhzhia region). The transformation was performed using *Agrobacterium tumefaciens* strain LBA4404 carrying the binary vector pBi2E with a double-stranded RNA suppressor of the proline dehydrogenase gene. The construct was designed based on fragments of the first exon and intron of the ProDH1 gene from *Arabidopsis thaliana* L. Integration of these gene fragments in antisense orientation resulted in the suppression of ProDH expression via post-transcriptional RNA silencing [16].

The presence of the ProDH1 exon and intron fragments from *Arabidopsis* in the transgenic sunflower plants was confirmed by molecular genetic analysis. The T_3 plants were characterized by reduced proline dehy-

drogenase activity and elevated proline levels compared with the original line [17].

The wild type and transgenic T_3 plants were grown in pots with 10 kg of soil fertilized by NPK (5 g per pot). 4—5 seeds were sown in each pot. At the first true leaf stage, some of the plants were removed leaving 2 typical well-formed plants. The soil moisture in all pots was maintained at a level 70 % of the field capacity (FC) and controlled by the gravimetric method.

After the full development of the third true leaf, soil drought was induced by withholding water from half of the pots with both transgenic and wild type plants, until the soil moisture in these pots reached the target level of 30 % FC. This moisture level was maintained for 5 days. The control plants remained at 70 % FC. The maximum daytime air temperature varied in the range 26—30 °C, and the minimum nighttime temperature — 13—18 °C. The all measurements were taken using third true leaf on the first and/or fifth days of drought at 30 % FC.

Relative water content in the leaf was determined according to the standard procedure [29]. Freshly picked leaves were immediately weighted to get fresh weight (FW). The leaves were incubated in distilled water in darkness at 4 °C for 24 h until fully turgid to determine the turgid weight (TW). The fully turgid leaves were dried in an oven at 105 °C until a constant dry weight was achieved (DW). The RWC was calculated by the following formula:

$$\text{RWC } (\%) = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100.$$

The chlorophyll and carotenoids contents in the leaf lamina was assayed spectrophotometrically using Specord 200 (AnalyticJena, Germany) following extraction with dimethyl sulfoxide and calculated according to the protocol of Wellburn [30].

The indices of photosynthetic activity in leaves of control and stressed plants were measured on the fifth day of drought at 30 % FC. The net CO_2 assimilation (A_N) rate was recorded under controlled conditions by an infrared gas analyzer GIAM-5M using widely accepted principles [31]. A section of intact third true leaf was placed in a temperature-controlled leaf chamber with window frame 3 × 7 cm and illuminated 1800 μmol/($m^2 \cdot s$) PAR by the TA-11 50W LED spotlight with a color temperature of 5200 K. The temperature-controlled assimilation chamber used by us allows regulating the leaf temperature within wide range with accuracy ±0.2 °C. Most measurements were taken at 25 °C, if not indicated other. Atmospheric air was blown through the chamber at a speed of 1 L/min. The dark respiration rate (R_d) was determined 30 min after turning off the light. The transpiration rate (T_r) was measured by a thermoelectric micropsychrometer based on the difference in air humidity at the inlet and outlet of the chamber.

To assess the thermotolerance of the photosynthetic apparatus, the CO_2 assimilation rate in the leaves of plants of the wild type and transgenic line grown under conditions of optimal soil moisture was determined at 25 °C, then the temperature in the chamber was increased at a rate of 1 °C/min to 45 °C, after that the A_N was measured immediately, then the

temperature was lowered to 25 °C at the same rate and the measurement was recorded again. Gas exchange parameters were calculated according to standard protocols [31].

Chlorophyll fluorescence was measured using FL2LP PAM fluorometer (Qubit system Inc., Canada). The parameters of PSII photochemistry in dark- and light-adapted leaves were routinely measured using a standard protocol [32]. Leaves were adapted to dark for 30 min before measuring F_0. A saturating pulse of white light having approximately 5000 μmol/(m^2 · s) PAR was given to obtain F_m. Fluorescence parameters on the light were recorded after 30 min adaptation to actinic light of 750 μmol/(m^2 · s) PAR and value of the actual quantum efficiency of PSII in the light (ϕPSII) and non-photochemical quenching (NPQ) were calculated as described in [32].

Leaf samples for the xanthophylls content estimation were taken in the morning (10—11 AM) under bright sun light (1600—2000 μmol/(m^2 · s) PAR) and quickly frozen in liquid nitrogen. Xanthophylls were extracted with 100 % acetone in the cold and quantitatively determined by high performance liquid chromatograph Agilent 6890N/5973 inert (Agilent Technologies, USA) in a mixture of acetonitrile, methanol, and distilled water in a ratio of 70 : 9.6 : 3, respectively [33]. The determination was carried out on a 3 × 150 mm column packed with Separon TM SG XC18 with a particle size of 5 μm. The wavelength at which the passage of the pigments detected was 436 nm. The xanthophyll content was calculated using a calibration curve. The state of xanthophyll cycle pigments de-epoxidation was determined as the ratio of the zeaxanthin content and half of the antheraxanthin content to the sum of the violaxanthin, zeaxanthin and antheraxanthin content [34].

For the determination of antioxidant enzymes activity, chloroplasts were isolated mechanically at a temperature of 0—4 °C. The sample (2 g) of fresh leaves was homogenized in a 7-fold volume of buffer solution of the following composition: 0.33 M sorbitol, 5 mM $MgCl_2$, 0.1 % BSA, 4 mM ascorbic acid and 50 mM Tris-HCl (pH 7.5). The homogenate was filtered through two layers of nylon fabric and centrifuged in a centrifuge K-24D at 80 g and a temperature of 0—4 °C for 5 minutes to precipitate heavy particles. The supernatant was poured into other pre-cooled centrifuge tubes and centrifuged at 2000 g for 10 minutes to obtain a fraction of chloroplasts. The chloroplasts sediment was resuspended in isotonic medium with 4 mM ascorbic acid, 50 mM *Tris*-HCl (pH 7.5) in a volume of 2 mL and subsequently used to determine the activity of superoxide dismutase (SOD), and ascorbate peroxidase (APX).

The superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically by the ability to inhibit the photochemical reduction of nitroblue tetrazolium according to Giannopolitis and Ries [35]. The ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by measuring concentration of ascorbate in the presence of hydrogen peroxide by Chen and Asada method [36].

The determination of lipid peroxidation products was carried out by the method of their reaction with thiobarbituric acid (TBA) [37].

Repeatability of determinations of the relative water content and photosynthetic pigments content was 3-fold, determination of gas exchange

and fluorescence parameters, enzymes activity and products of lipid peroxidation — 4-fold. The obtained data were processed by using Microsoft Excel. The figures and the tables show the arithmetic mean and standard error of the mean. The significance of the difference between controls and treatments were evaluated using ANOVA. Differences were considered significant at $p < 0.05$.

Results and discussion

The water cessation caused little disturbances in water relations in plants on the first day of soil moisture reaching 30 % FC. The relative water content in the third leaf of the stressed VK-121 plants tended to decrease compared to well-watered control (Fig. 1). At the same time, it was practically no change for transgenic VK-121 plants. The value of relative water content in stressed and control transgenic VK-121 plants did not differ significantly from the level in wild type plants of the VK-121 line.

Under prolonged drought conditions, on the fifth day at 30 % FC, the relative water content in leaf tissues of both the wild type and transgenic lines declined by 13—15 % to approximately the same values however the differences between control and stressed plants of transgenic line were statistically insignificant at $p \leq 0{,}5$.

There were no significant differences in the leaf chlorophyll contents between the plants of wild type and transgenic line under optimal watering conditions (Table 1). However, drought caused different decline in the green pigments content in leaves of the lines. Chlorophyll content in the leaves of the wild type line decreased compared to the control by about 19 % on the first day of drought, and by 45 % on the fifth day of drought, while in transgenic plants — by 8 and 36 %, respectively. Under prolonged drought, the total chlorophyll content in the leaves of drought-stressed transgenic plants was significantly higher (by almost 20 %) than in the

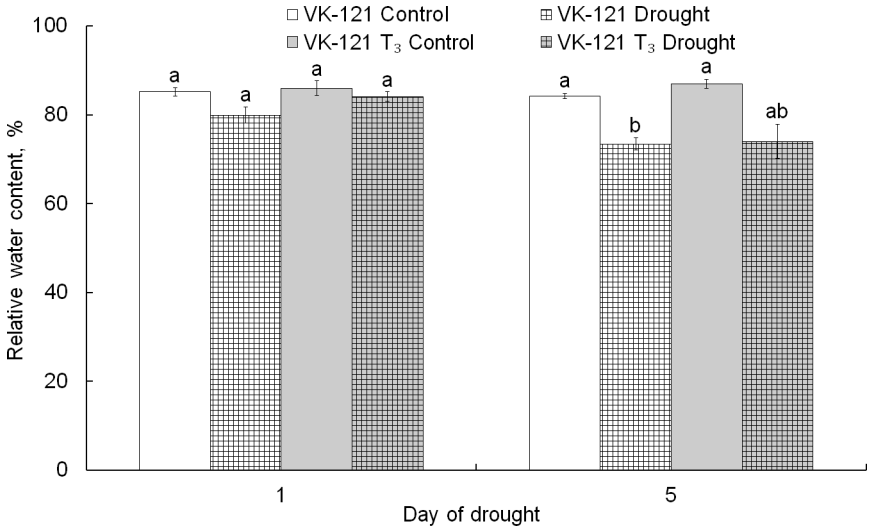

Fig. 1. Relative water content in the tissues of sunflower third leaf of the wild type line (VK-121) and transgenic plants (VK-121 T_3) under optimal and soil drought conditions (different letters indicate significant differences at $p < 0.05$)

TABLE 1. Photosynthetic pigments content in the tissues of sunflower third leaf of the wild type line (VK-121) and transgenic plants (VK-121 T_3) on the 1st and 5th days of drought (mg/g dry weight)

Genotype	Treatment	Chlorophyll (*a*+*b*)		Carotenoids	
		1	5	1	5
VK-121	Control	11.94±0.08[a]	11.17±0.04[a]	3.07±0.04[a]	2.62±0.08[a]
	Drought	9.69±0.08[b]	6.10±0.13[b]	2.54±0.11[b]	1.64±0.03[b]
VK-121 T_3	Control	11.45±0.11[a]	11.44±0.41[a]	2.86±0.14[ab]	2.80±0.09[a]
	Drought	10.57±0.05[c]	7.28±0.26[c]	2.93±0.14[a]	1.90±0.02[c]

Note. Here and in Tables 2—4: different letters indicate significant differences within the columns at $p < 0.05$.

plants of the wild type line. Drought-induced changes in the carotenoids content in both lines were similar but slightly lower comparing to chlorophyll content. The carotenoids content in wild type line diminished by about 17 % on the first day of drought, and by 45 % on the fifth day of drought whereas in transgenic line, the carotenoids content did not change at the onset of drought and decreased by 32 % under prolonged drought.

On the fifth day of drought, any significant differences between lines in CO_2 assimilation and transpiration rates for well-watered plants were detected while dark respiration rate was a bit lover in transgenic ones (Table 2). Insufficient watering caused significant and approximately identical reduction in CO_2 assimilation in leaves of the wild type and transgenic plants. On the fifth day of drought, the degree of photosynthesis inhibition was about 20 % for both transgenic and non-transgenic plants, maintaining no differences in the A_N between the lines studied.

Noticeable differences between the plants of the wild type and transgenic lines were found in the drought-induced changes of dark respiration and transpiration rates. On the fifth day of drought, the R_d in drought-stressed plants decreased relative to the well-watered control by 55 % and 37 % in plants of the wild type VK-121 and transgenic VK-121 T_3, respectively and its value tended to be 22 % higher in transgenic plants compared to non-transgenic. This may be considered as evidence of better energy provision for metabolism of the transgenic plants, including their protective systems.

The differences in drought-response for the transpiration rate were much significant. On the fifth day of drought, the T_r decreased in the stressed plants compared to control by 36 % for wild type VK-121 line, and by 15 % for transgenic VK-121 T_3 one. As a result, T_r in the stressed plants

TABLE 2. Gas exchange rate in the sunflower third leaf of the wild type line (VK-121) and transgenic plants (VK-121 T_3) on the 5th day of drought

Genotype	Treatment	Net assimilation, μmol $CO_2/(m^2 \cdot s)$	Dark respiration, μmol $CO_2/(m^2 \cdot s)$	Transpiration, mmol $H_2O/(m^2 \cdot s)$
VK-121	Control	19,28±1,20[a]	1,58±0,06[a]	4,33±0,18[a]
	Drought	14,99±1,32[b]	0,71±0,08[b]	2,77±0,28[b]
VK-121 T_3	Control	18,46±0,50[a]	1,39±0,06[c]	4,62±0,43[a]
	Drought	14,49±1,32[b]	0,87±0,11[b]	3,91±0,37[c]

of the VK-121 T_3 was 40 % times higher than in the wild type VK-121 line. This indicates that, under drought conditions, the transgenic plants were able to maintain a higher stomatal conductance.

Recent studies suggested that, in sunflower, stomatal conductance is governed primarily by ABA signaling rather than by hydraulic regulation (osmotic adjustment) [38]. Such predominance of ABA control may underlie the observed disparity between substantial changes in transpiration rates and the relatively minor alterations in leaf relative water content.

Although, there were no significant differences between the wild type line and transgenic sunflower plants in the A_N at normal temperature (25 °C), heating leaf under 45 °C caused much stronger inhibition of CO_2 assimilation in wild type VK-121 line plants than in transgenic VK-121 T_3 ones (Fig. 2). A_N in heated leaves of wild type line decreased by 49 % while reduction in A_N for transgenic line was 28 %. Heat-induced inhibition of CO_2 assimilation was mainly reversible as A_N, measured immediately after leaf temperature was returned to 25 °C, restored to the levels of more than 80 % of initial ones in plants of both lines.

The smaller drought effects on respiratory metabolism and transpiration as well as higher heat-tolerance in transgenic plants probably are related to the increase in the free proline content previously revealed in transgenic plants as compared to the wild type line [17].

There are many evidences that proline is not only an osmotically active substance, but also it fulfills a range of other protective functions under stress conditions, including membrane stabilization, chaperone-like activity, antioxidant defense, redox regulation, regulation of specific genes expression etc. [15, 39, 40, 41]. Therefore, the increased proline content, constitutively inherent in transgenic sunflower plants under normal conditions, can increase the thermotolerance of their photosynthetic apparatus.

Efficiency of photosynthetic light conversion in PSII reaction centers assayed by parameters of chlorophyll fluorescence did not differ signifi-

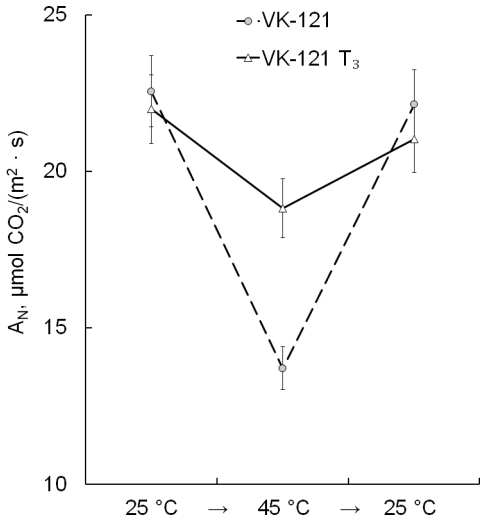

Fig. 2. Effect of high temperature on net CO_2 assimilation rate (A_N) in sunflower leaves of the wild type line (VK-121) and transgenic plants (VK-121 T_3)

cantly in transgenic and non-transgenic plants under well-watered conditions (Table 3). However, under drought, transgenic plants had slightly higher maximum quantum efficiency of PS II photochemistry (F_v/F_m) and significantly superior effective quantum yield of PSII (ϕPSII). The last parameters, which represents intensity of linear electrons transfer through the PSII reaction center [42], in the drought-stressed plants of the wild type VK-121 line was 44 % lower than in stressed plants of T_3 transgenic VK-121 line. The drought-induced decline compared to the well-watered control amounted 39 and 13 % in non-transgenic and transgenic plants, respectively.

The small differences in F_v/F_m between well-watered and drought-stressed plants indicate minor irreversible damage to PSII reaction centers caused by five-days drought. Thus, the decrease in ϕPSII in stressed plants was largely related to down-regulation due to reduction in CO_2 assimilation. The discrepancy between equal drought-responses of A_N in both lines and essentially smaller decline in ϕPSII in stressed transgenic plants suggested different changes in an energy use efficiency for the CO_2 assimilation, i.e. the number of electrons used per unit of assimilated CO_2. The increased energy requirements for CO_2 assimilation in drought-stressed transgenic plant may be brought about by higher photorespiration activity, the increased rate of pseudocyclic electron transport (water-water cycle) in chloroplasts, metabolic changes associated with the regulation of redox balance in electron transport chain and entire photosynthetic cell, that all are considered as protective mechanisms facilitating safe operation under unfavorable environmental conditions [26].

In contrast to the effective quantum yield of PSII, non-photochemical quenching (NPQ) in the transgenic line was lower than in the wild type line by 22 % and 26 % under optimal and limited watering, respectively. These data indicate a higher energy demand and lower non-photochemical energy dissipation in the transgenic T_3 VK-121 line compared to the wild type VK-121 line. Taking into account the absence of differences in A_N between well-watered plants of both lines, one can suggest that electron utilization for the above-mentioned protective mechanisms in the chloroplasts of transgenic plants is higher than in those of wild type plants even under optimal watering conditions.

The drought increased NPQ in plants of wild type line by 27 % and by 21 % in transgenic plants. This raise in NPQ was related to CO_2 assimilation inhibition and reflected increase in dissipation of excess absorbed energy as heat in drought-stressed plants of both genotypes. It is considered

TABLE 3. Chlorophyll fluorescence parameters in the sunflower third leaf of the wild type line (VK-121) and transgenic plants (VK-121 T_3) on the 5th day of drought

Genotype	Treatment	F_v/F_m	ϕ PSII	NPQ
VK-121	Control	0,839±0,007[a]	0.223±0.024[a]	2,33±0,11[a]
	Drought	0,812±0,007[b]	0.137±0.023[b]	2,97±0,08[b]
T_3VK-121	Control	0,840±0,003[a]	0.281±0.021[a]	1,82±0,07[c]
	Drought	0,829±0,006[a]	0.244±0.028[a]	2,21±0,12[ac]

as protective mechanism preventing damage to the photosynthetic machinery under stress-induced inhibition of CO_2 assimilation [43].

The protective dissipation of excess energy under high-light conditions is driven in part by xanthophyll cycle [44]. The deepoxidation of violaxanthin to zeaxanthin, catalyzed by violaxanthin de-epoxidase occurs to prevent photoinhibition of the photosynthetic apparatus. An increase in the degree of xanthophyll pool de-epoxidation promotes an increase in the thermal dissipation of absorbed light energy and a decrease in its use in photochemical (photosynthetic) processes.

In our experiments, under optimal soil moisture, the total xanthophyll cycle pigments pool content per unit of chlorophyll content in T_3 plants of the transgenic VK-121 line was approximately 20 % higher than in the wild type VK-121 line (Table 4). The reduction in water supply caused an increase in the zeaxanthin content (by 33 %) and the degree of de-epoxidation (by 13 %) compared to the control only in plants of the wild type line. In transgenic plants, no significant changes in these parameters were recorded under drought conditions: they were almost the same at optimal and low soil moisture levels and close to the control values in the plants of the wild type line.

Changes in the de-epoxidation degree in the leaves of wild type line and transgenic plants under drought conditions are consistent with the data on the quantum efficiency of PSII and non-photochemical quenching. The absence of changes in the de-epoxidation degree and an insignificant decrease in the light energy photochemical utilization level in the leaves of transgenic plants under drought conditions is consistent with the data given above on a smaller decrease in the transpiration rate (see Table 2).

Chloroplast antioxidant enzymes SOD and APX, in addition to the function of protection against ROS, also play an important regulatory role in the homeostasis of photosynthetic metabolism under changing environmental conditions [27] participating in the signaling systems of photosynthetic cells through control over the level of key signaling compounds — superoxide anion radical and hydrogen peroxide [45, 46]. For photosynthetic metabolism, the participation of these enzymes in pseudocyclic electron transport, which allows to regulate the ratio of the NADPH and ATP formation rates, as well as the redox balance in chloroplasts particularly under sharp changes in the illumination level, is critically important [47].

TABLE 4. The xanthophyll cycle pigment content and the degree of their de-epoxidation in the sunflower third leaf of the wild type line (VK-121) and transgenic plants (VK-121 T_3) on the 5th day of drought

Genotype	Treatment	Xanthophyll pigments content, mg/g chlorophyll				De-epoxidation degree
		Violaxanthin	Antera-xanthin	Zeaxanthin	Total content	
VK-121	Control	40.6±5.0[a]	1.4±0.5[a]	20.6±0.9[a]	62.6±4.0[a]	0.32±0.02[a]
	Drought	39.0±2.2[a]	1.3±0.4[a]	26.0±2.2[b]	66.2±2.3[a]	0.40±0.01[b]
VK-121 T_3	Control	48.2±3.1[b]	1.5±0.5[a]	25.6±2.0[b]	75.4±6.1[b]	0.35±0.02[a]
	Drought	50.3±3.7[b]	1.0±0.5[a]	25.7±2.6[b]	77.0±6.8[b]	0.34±0.01[a]

It was found that, under optimal watering, the SOD activity in the leaves of T_3 VK-121 transgenic sunflower plants was approximately 2 times, and the APX activity was 1.5 times higher than in the plants of the wild type line VK-121 (Fig. 3). The water supply reduction caused a sharp increase in the antioxidant enzymes activity in the leaves of both forms. On the first day of drought, the SOD and APX activities increased relative to the control by 86 and 63 %, respectively, in the plants of the wild type line, and by 62 and 69 % in transgenic T_3 plants. However, the further development of the response of chloroplast antioxidant system key enzymes to drought in the T_3 and wild type VK-121 plants was different. In plants of the wild type line under severe water stress on the fifth day of drought, the differences between the control and treatment in SOD and APX activity increased by 125 and 84 % respectively, while in the transgenic plants, on the contrary, decreased by 22 and 31 %, respectively.

The increase in enzymes activity in transgenic plants relative to the wild type line under normal conditions, as well as under the influence of drought, was not proportional — the activity of SOD increased more strongly than APX. As a result, the ratio of SOD/APX activities under optimal moisture in the leaves of transgenic plants was approximately 35 % higher than in the plants of the wild type line (see Fig. 3). Under the drought, this ratio increased in plants of the wild type line, but practically did not change in the transgenic plants.

Although the mechanisms of the proline effect on the antioxidant enzymes activity are still very poorly understood, many studies showed that exogenous proline application enhanced the antioxidative enzymes activity in several plants under stress condition [40]. It was shown the rise in proline content in the transgenic citrus plants carrying heterologous gene P5CS112A leads to increase in the transcription of cytosolic APX and chloroplast Cu/Zn SOD isoforms [48], that implies functioning proline as a signaling molecule. Wheat transformants containing double-stranded RNA suppressor of the proline dehydrogenase gene and high proline content had increased activity of antioxidant enzymes, SOD and ascorbate peroxidase, in chloroplasts [19].

In this study, the transgenic sunflower plants harboring a double-stranded RNA suppressor of the ProDH and exhibiting elevated proline content demonstrated increased activities of chloroplastic SOD and APX, even under optimal watering conditions. This may be interpreted as pre-acclimation of photosynthetic apparatus to oxidative stress and could, in part, account for the enhanced tolerance of the transgenic plants to abrupt increases in leaf temperature. The elevated activities of chloroplastic SOD and APX, and consequently a potentially more powerful pseudocyclic electron flux provide an improved capacity for ROS scavenging and energy balancing during photosynthesis.

Despite the significantly higher antioxidant enzymes activity in chloroplasts of transgenic plants at optimal soil moisture, the level of TBA-active products in their leaves tended to be slightly higher than in the wild type line, although these differences were statistically insignificant (Fig. 4). Under drought conditions, the TBA-active products content increased significantly in plants of the wild type line by 50 %, and in transgenic plants — by 37 %.

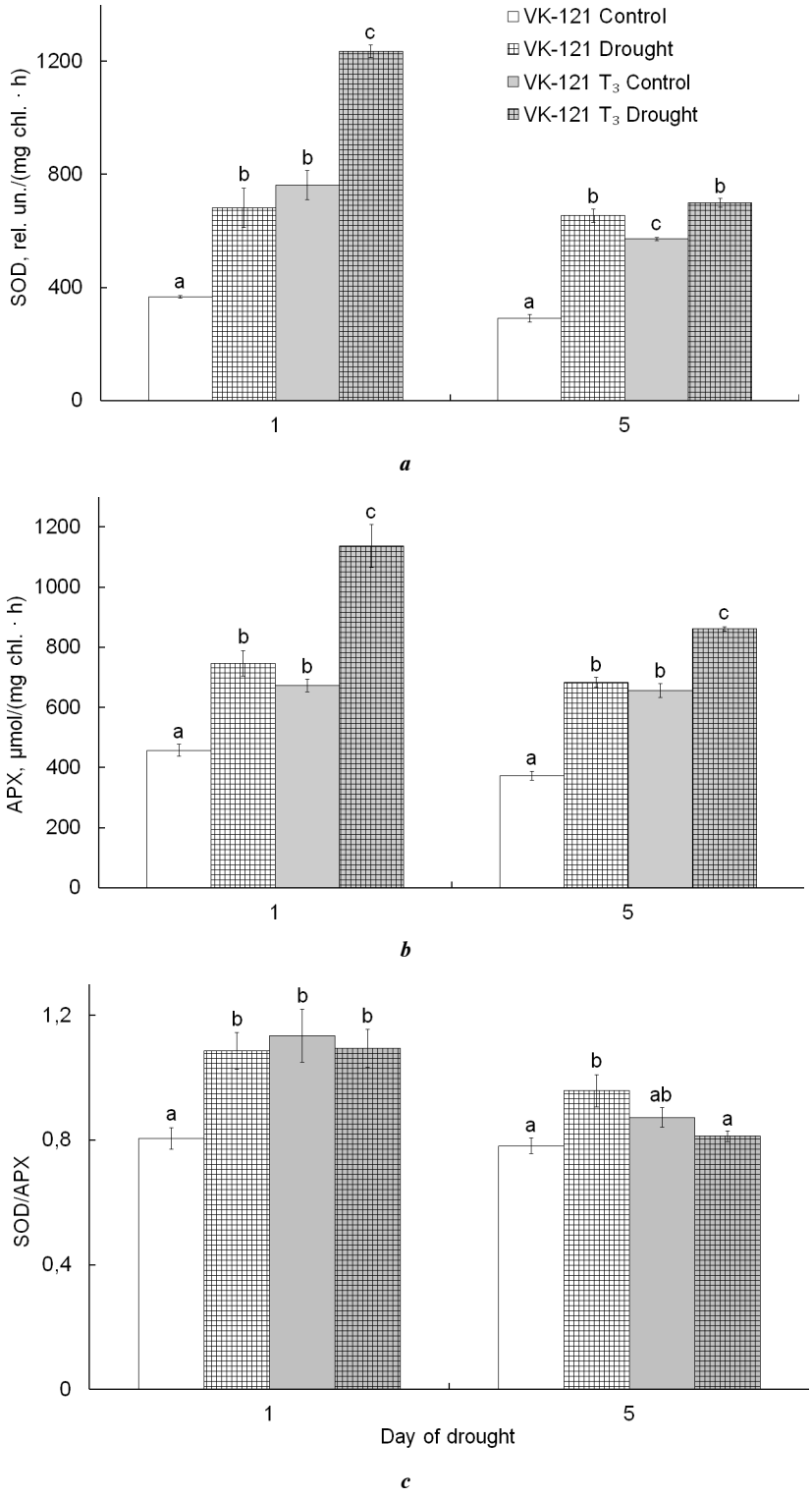

Fig. 3. Chloroplastic superoxide dismutase (*a*), ascorbate peroxidase (*b*) activity and their ratio (*c*) in sunflower leaves of the wild type line (VK-121) and transgenic plants (VK-121 T_3) (different letters indicate significant differences inside the parameters and for separate day, the same letters indicate no difference at $p < 0.05$)

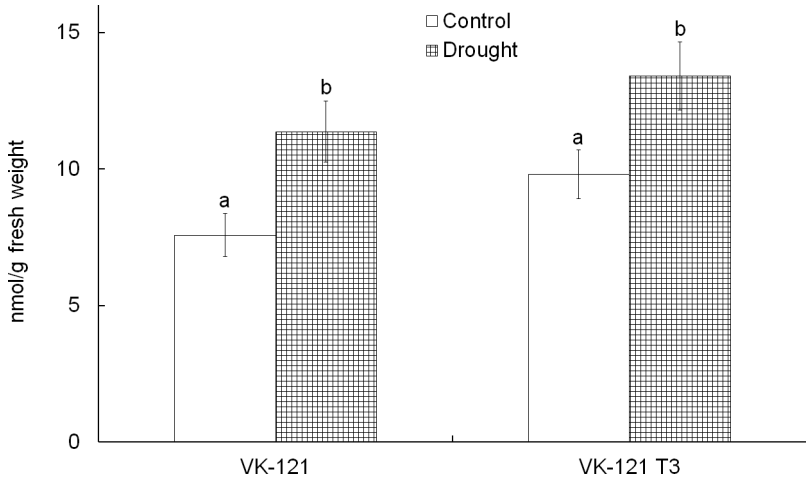

Fig. 4. TBA-active products content in sunflower leaves of the wild type line (VK-121) and transgenic plants (VK-121 T_3) (different letters indicate significant differences for separate genotype, the same letters indicate no difference at $p < 0.05$)

Thus, the obtained data indicated that, under optimal conditions, transgenic sunflower plants with a double-stranded RNA-suppressor of the proline dehydrogenase gene did not differ in CO_2 and H_2O gas exchange rates from the plants of wild type line; however, they had a significantly higher activity of SOD and APX in chloroplasts. Soil drought inhibited the net CO_2 assimilation rate in both forms to the same extent, but in transgenic plants under drought conditions, the transpiration rate decreased less. Plants with double-stranded RNA suppressor of the ProDH gene also showed a higher thermotolerance of the photosynthetic CO_2 assimilation.

Under conditions of drought, in transgenic plants the chlorophyll content and the quantum efficiency of PSII photochemical reactions decreased to a lesser extent. Moreover, the non-photochemical losses of absorbed light energy in the light-harvesting complexes of chloroplast membranes were less than in the wild type line. These changes in the distribution of absorbed light energy in photosynthetic membranes were associated with an increase in the degree of xanthophyll cycle pigments de-epoxidation in the wild type line leaves and its retention at a constant level in the transgenic plants. Drought much stronger increased the superoxide dismutase and ascorbate peroxidase activities in the chloroplasts of the wild type line leaves than in transgenic plants, while in the latter the relative increase in the content of lipid peroxidation products was lower.

These findings demonstrate that partial suppression of the proline dehydrogenase gene enhances drought resistance of the sunflower photosynthetic apparatus by reinforcing its protective and regulatory functions.

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ДВОЛАНЦЮГОВИЙ РНК-СУПРЕСОР ГЕНА ПРОЛІНДЕГІДРОГЕНАЗИ ПОЛІПШУЄ СТІЙКІСТЬ ФОТОСИНТЕТИЧНОГО АПАРАТУ РОСЛИН СОНЯШНИКА ДО ПОСУХИ

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Досліджено фізіолого-біохімічні особливості фотосинтетичного апарату і його реакція на посуху у трансгенних рослин соняшника покоління T_3 з дволанцюговим РНК-супресором гена проліндегідрогенази порівняно з вихідною лінією VK-121. Встановлено, що в оптимальних умовах вологозабезпечення трансформовані рослини не відрізнялися за вмістом суми хлорофілів, квантовою ефективністю фотосистеми II (ФС II), інтенсивністю асиміляції CO_2 і транспірації, однак мали істотно вищу активність ферментів антиоксидантного захисту — супероксиддисмутази (СОД) і аскорбатпероксидази (АПО) — у хлоропластах. Ґрунтова посуха спричинювала менше зниження вмісту хлорофілу, максимальної квантової ефективності ФС II (F_v/F_m), ефективного квантового виходу ФС II на світлі (ϕФС II), а також інтенсивності дихання та транспірації у трансгенних рослин, хоча нетто-інтенсивність асиміляції CO_2 була однаково пригнічена в обох ліній. Водночас у трансгенних рослин менше знижувалася інтенсивність асиміляції CO_2 у відповідь на швидке підвищення температури листка. В умовах посухи нефотохімічне гасіння (NPQ) поглиненої світлової енергії у трансгенних рослин було меншим, ніж у вихідної лінії, що супроводжувалося збільшенням ступеня деепоксидації пігментів віолаксантинового циклу у вихідної лінії і збереженням його на незмінному рівні в листках трансгенних рослин. Посуха значно сильніше підвищувала активність СОД і АПО в листках вихідної лінії, ніж у трансгенних рослин. Отримані дані свідчать, що трансгенні рослини соняшника з частковим пригніченням гена проліндегідрогенази мають підвищену толерантність фотосинтетичного апарату порівняно з вихідною лінією VK-121 завдяки більшій активності захисних і регуляторних механізмів за умов ґрунтової посухи.

Ключові слова: *Helianthus annuus* L., соняшник, генетична трансформація, дволанцюговий РНК-супресор гена проліндегідрогенази, фотосинтез, супероксиддисмутаза, аскорбатпероксидаза, посуха.

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