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Responses of Transgenic Tobacco Plants with Increased Proline Content to Drought and/or Heat Stress

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ABSTRACT

Transgenic tobacco plants (*M*51-1) *constitutively over-expressing a modified gene for the proline biosynthetic enzyme* Δ^2*-pyrroline-5-carboxylate synthetase* (*P5CSF*129*A*) *and the corresponding wild-type plants* (*WT*) *were compared during drought or heat stress and under combination of both stresses. The proline content in M*51-1 *was several times higher than in WT plants. Under optimal conditions, the transpiration rate and stomatal conductance of M*51-1 *plants were lower than those in WT plants. The differences in net photosynthetic rate were not significant and water use efficiency and contents of chlorophyll and xanthophyll cycle pigments were higher in M*51-1 *than in WT plants. Drought induced by cessation of watering for 7 d resulted in decrease of all gas exchange parameters and chlorophyll content, but in an increase of the content of xanthophyll cycle pigments and degree of their de-epoxidation. After application of heat stress* (40˚C/60 *min*) *to control or water-stressed plants the gas exchange parameters decreased considerably. Short-term heat stress alone, however, did not affect pigment contents. The responses of M*51-1 *and WT plants to the tested stresses did not differ significantly. Therefore, a decisive contribution of elevated proline content to drought or heat stress tolerance of tobacco was not proved.*

***Keywords*:** *Carotenoids, Chlorophyll, Net Photosynthetic Rate, Stomatal Conductance, Transpiration Rate, Xanthophyll Cycle Pigments*

1. Introduction

In many plant species, osmotic adjustment (lowering of the osmotic potential in order to maintain the pressure potential) occurs in response to water stress induced by drought, salinity or low temperature. The composition of solutes contributing to osmotic adjustment differs according to the plant species or genotype, duration as well as severity of water stress [1,2]. One of the well-known osmotically active compounds is proline (for review see e.g. [3]). It may serve not only as osmoprotectant, but also as a molecular chaperone, antioxidant, regulator of redox homeostasis or source of carbon and nitrogen (for review see [4]). Therefore, its accumulation occurs not only under water stress but also under other abiotic and biotic stresses.

In higher plants, proline is synthesized mainly from glutamate. The oxidation of glutamate to glutamic-γ-semialdehyde (GSA) is catalysed by a Δ^1-pyrroline-5-carboxylate synthetase (P5CS). GSA undergoes spontaneous cycling to Δ^1-pyrroline 5-carboxylate (P5C). The next step involves the reduction of P5C to proline by Δ^1-pyrroline-5-carboxylate reductase (P5CR). Proline biosynthesis steadily occurs in the cytosol, while it is augmented to the chloroplasts during stress conditions [4]. As proline biosynthesis requires NADPH, the enhanced rate of its biosynthesis in chloroplasts maintains the low NADPH: $NADP^+$ ratio, resulting in reduction of photoinhibition under high irradiance [5]. Thippeswamy *et al*. [6] suggested that the proline synthesis from glutamate has been limited by P5CS activity. Proline degradation by proline dehydrogenase (PHD) occurs in mitochondria [4]. Intracellular proline content thus depends on its biosynthesis, degradation and transport from other plant parts [7]. For example, P5CS activity in *Arabis stelleri* was increased by mannitol and sorbitol, but not by NaCl, while activity of PHD was decreased by mannitol and NaCl, which resulted in an increase of free proline amount under all,

above mentioned, treatments [8]. During stress conditions, elevation of proline content coincided with modulation of the enzyme activities (increase in case of P5CR and decrease in PDH, respectively), as well as with changes in expression of the corresponding genes [6,9,10]. Recently, two closely related genes, *P5CS*1 and *P5CS*2, were identified in *Arabidopsis* [11]. The former one is involved in regulation of development, while the latter one is stress-responsible [4]. Similarly, two genes for proline degradation, *MsPHD*1 and *MsPHD*2 were distinguished in *Medicago sativa* [9].

The correlation between proline accumulation and plant stress tolerance is not always clear (for review see [4,12]). For example, with increased NaCl concentration, salt resistant rice cultivars accumulated less proline than the salt sensitive ones [13]. With the aim to contribute to the elucidation of the role of proline in response to drought and heat stress alone or in combination, transgenic tobacco plants (M51-1) constitutively over-expressing a modified gene (*P5CSF*129*A*) for the proline biosynthetic enzyme Δ^2-pyrroline-5-carboxylate synthetase and the corresponding wild-type plants (WT) were compared. While previous papers focused on M51-1 plants described predominantly proline and phytohormone contents [14,15], in the present paper net photosynthetic rate, transpiration rate, stomatal conductance and pigment contents were followed.

2. Materials and Methods

2.1. Plants and Cultivation

The seeds of transgenic tobacco plants were kindly donated by Dr. Jozef Gubis. The transformation was described in detail by Gubis *et al.* [14]. Wild type (WT) and transgenic (M51-1) tobacco (*Nicotiana tabacum* L.) seedlings were grown in *Perlite* with nutrient solution in a growth chamber at a 16-h photoperiod, an irradiance of 250 μmol (photon) $m^{-2}\cdot s^{-1}$ (400 - 700 nm), day/night temperature of 25˚C/20˚C, and relative humidity of about 50%. The below mentioned parameters were followed in control plants sufficiently supplied with water, after cessation of watering for 7 days and again after rehydration for 7 days. Heat shock (40˚C, 60 min) was applied either to control plants or to water-stressed plants and measurements were done immediately after treatment.

2.2. Measurements of Gas Exchange and Photosynthetic Pigments

Net photosynthetic rate (P_N), transpiration rate (E) and stomatal conductance (g_s) were measured on attached leaves using the commercial gas exchange system *LCA*-4 (*ADC Bio Scientific*, Hoddesdon, UK). All measurement were done at a temperature of 25˚C, saturating irradiance of 750 μmol $m^{-2}\cdot s^{-1}$, CO_2 concentration of 350 μmol·mol^{-1}, and relative humidity of about 30%. Contents of photosynthetic pigments were determined in acetone extracts of leaf discs by HPLC (*ECOM*, Prague, Czech Republic) using a reverse phase column (*Watrex Nucleosil* 120-5-*C*18, 5 μm particle size, 125 × 4 mm). The solvent system was acetonitrile:methanol:water (80:12:10) followed by methanol:ethylacetate (95:5), the gradient was run from 2 to 6 min. The flow rate was 1 $cm^3\cdot min^{-1}$, the detection wavelength 445 nm. The pigment analyses were performed using software *Clarity* (*DataApex*, Prague, Czech Republic). Contents of proline and abscisic acid were measured as described previously by Dobra *et al*. [14].

2.3. Data Analysis

Presented results are means ± standard error of 9 (gas exchange) or 3 (pigments) independent samples. Significance of differences between WT and M51-1 as well as between control and treated plants was evaluated by *t*-test (**Tables 1** and **2**). The experiments were repeated twice with similar results.

3. Results

The characteristic feature of transgenic tobacco plants M51-1 was several times higher proline content in comparison with WT [14,15]. In both genotypes, prolonged water stress resulted in a highly significant increase in proline content (**Figure 1**), while no significant changes in proline content were observed after heat stress [15].

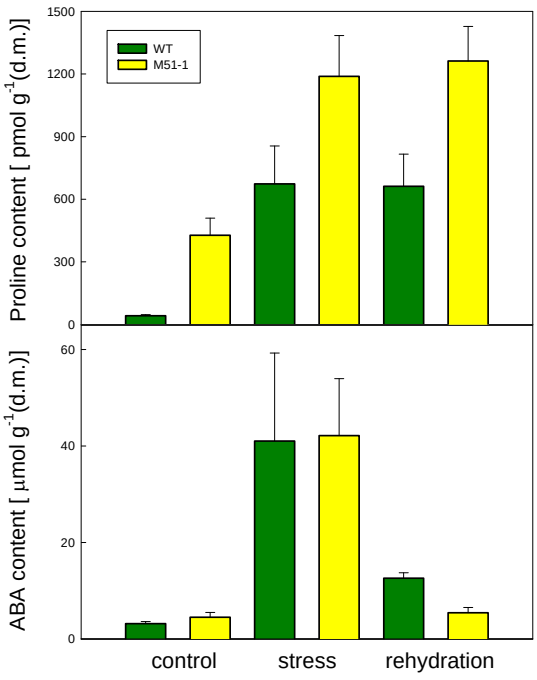

Figure 1. Contents of proline and abscisic acid in wild type (WT) and transgenic (M51-1) tobacco plants during water stress and rehydration (both lasted 7 days).

3.1. Comparison of Transgenic and Wild Type Plants under Sufficient Water Supply

In non-stressed plants, transpiration rate (E) and stomatal conductance (g_s) of M51-1 were lower than those of WT, which was in agreement with higher abscisic acid content in M51-1 (**Figure 1**). Net photosynthetic rate (P_N) was not significantly different between the plant types (**Figure 2**). Water use efficiency (WUE = P_N/E) in M51-1 and WT plants was 5.95 and 5.08 mmol (CO_2) mol^{-1} (H_2O), respectively. Contents of chlorophyll (Chl *a* + *b*) or xanthophyll cycle pigments (Xan = zeaxanthin + anteraxanthin + violaxanthin) were significantly higher in M51-1 than WT (**Figure 3**).

3.2. Response of Transgenic and Wild Type Plants to Drought and Rehydration

Cessation of watering for 7 days induced water stress and decreased P_N, E and g_s, the response being similar in M51-1 and WT plants. All gas exchange parameters were partially recovered after rehydration in both plant types (**Figure 2**). The water stress slightly decreased Chl *a* + *b* content, similarly in both plant types. The content of Car was slightly increased under drought, this increase was higher in M51-1 than in wild type. The Xan content and the degree of their deepoxidation [DEPS = zeaxanthin + 0.5 antheraxanthin)/(antheraxanthin + violaxanthin + zeaxanthin)] were markedly increased under drought in both plant types. These changes were partially reversed after rehydration (**Figure 3**).

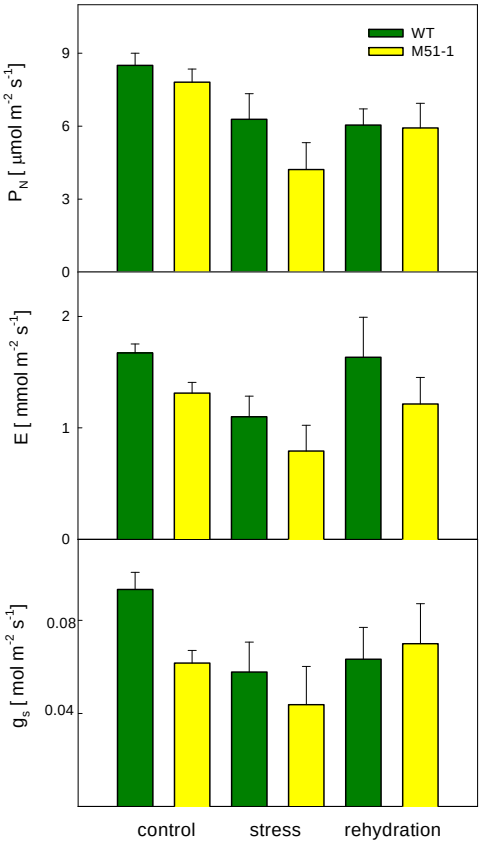

Figure 2. Net photosynthetic rate (P_N), transpiration rate (E) and stomatal conductance (g_s) in wild type (WT) and transgenic (M51-1) tobacco plants during water stress and rehydration (both lasted 7 days).

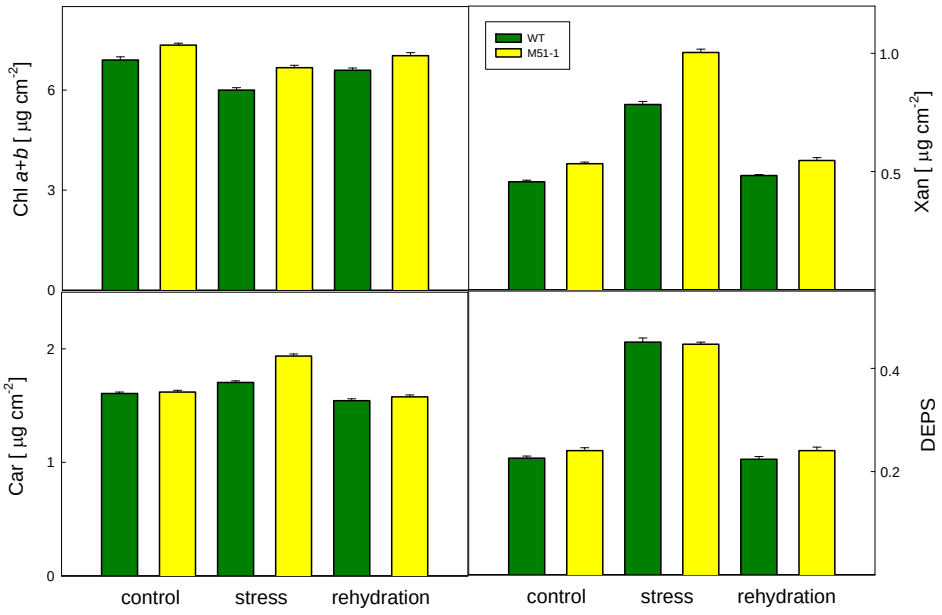

Figure 3. Contents of chlorophylls (Chl *a* + *b*), carotenoids (Car) and xanthophyll cycle pigments (Xan) and Xan degree of deepoxidation (DEPS) in wild type (WT) and transgenic (M51-1) tobacco plants during water stress and rehydration (both lasted 7 days).

Table 1. Results of statistical evaluation (*P* values) of the effects of material (WT × M51-1) under control conditions, 7-d water stress and 7-d rehydration and effect of treatments (control × stress or rehydration) measured in different materials.

Parameter	Materials	Treatments					
	Control	Stress	Rehydration	Stress		Rehydration	
				WT	M51-1	WT	M51-1
P_N	0.355	0.194	0.926	0.040	0.003	0.010	0.086
E	0.007	0.317	0.329	0.003	0.021	0.881	0.656
g_s	0.001	0.014	0.765	0.016	0.197	0.043	0.262
Chl *a* + *b*	0.017	0.003	0.019	0.002	0.002	0.061	0.044
Car	0.530	0.001	0.252	0.007	0.001	0.041	0.137
Xan	0.001	0.001	0.007	<0.001	<0.001	0.023	0.387
DEPS	0.097	0.681	0.130	<0.001	<0.001	0.770	0.971

Table 2. Results of statistical evaluation (*P* values) of the effects of material (WT × M51-1) under control conditions, heat and water stress + heat and effect of treatments (control × heat or stress + heat) measured in different materials.

Parameter	Materials	Treatments					
	Control	Heat	Stress + Heat	Heat		Stress + Heat	
				WT	M51-1	WT	M51-1
P_N	0.831	0.380	0.008	0.012	0.012	<0.001	<0.001
E	0.389	0.137	0.270	<0.001	<0.001	<0.001	<0.001
g_s	0.145	0.061	0.033	<0.001	<0.001	<0.001	<0.001
Chl *a* + *b*	0.017	0.001	0.002	0.148	0.786	0.001	0.002
Car	0.530	0.336	0.001	0.530	0.336	0.009	<0.001
Xan	0.001	0.020	<0.001	0.012	0.005	<0.001	<0.001
DEPS	0.097	0.240	0.026	0.002	0.031	<0.001	<0.001

3.3. Response of Transgenic and Wild Type Plants to Heat Stress and Combined Heat and Water Stress

After heat stress (40˚C/60 min) applied to control or water-stressed plants P_N, E and g_s decreased considerably in both M51-1 and WT plants (**Figure 4**). These effects were much more pronounced at the combined drought and heat stress than at drought or heat alone. The lowest values of all gas exchange parameters were found in M51-5 plants under combined drought and heat stress. Short-term heat stress alone, however, did not affect significantly pigment contents. The pigment contents, however, were affected by combined heat and water stress in pattern similar to that of water stress alone (**Figure 5**). Under combined heat and drought, the pigment contents as well as DEPS were higher in M51-1 than in WT plants.

4. Discussion

Accumulation of proline during drought was repeatedly reported in many species. The pathways of proline biosynthesis and degradation were recently described [4], but its biological functions have not been fully elucidated yet. The unsolved question is whether proline accumulation is a sign of stress tolerance or only consequence of the stress (for review see [12]). The reply to this question was searched by determination of changes in proline content during different stresses, by application of proline with the aim to ameliorate negative effects of stress (for review see e.g. [3,12]) and recently by using transgenic plants with increased endogenous proline content. The last approach has been based either on increased of proline biosynthesis achieved by over-expression of *P5CS* gene [16-18] or on decrease of proline degradation by re-

pression of *PDH* genes [9]. In addition, it has been found that antisense *P5CS* transgenic *Arabidopsis* plants, showing significantly lower proline accumulation than respective wild type, were hypersensitive to osmotic stress [19].

General feature of transgenic plants over-expressing *P5CS* gene was high accumulation of proline, especially under stress conditions. In *indica* rice accumulation of proline was accompanied by better biomass production and growth performance under drought or salt stress [18,20]. It was rather surprising that in sugarcane, *Vigna aconitifolia* and tobacco the increase in proline accumulation was not accompanied by markedly higher osmotic adjustment in transgenic plants than in respective WT plants [15,20,21].

In our experiments, transgenic tobacco plants M51-1 had a little lower E and g_s that WT plants under sufficient water supply, which might be caused by slightly higher ABA content in M51-1 than in the WT plants. Lower E might led to lower water consumption, more conservative water use and better growth as observed by Molinari *et al*. [20], Vendruscolo *et al*. [21] and Dobra *et al*. [15].

Chl content was slightly higher in M51-1 than in WT plants. Due to the higher Chl content but lower g_s in M51-1 than in WT, P_N was similar in both genotypes. Due to similar P_N and lower E, WUE was higher in M51-1 than in WT plants. This can be a positive feature of M51-1 plants leading to better utilization of water sources. On the other hand, transpiration efficiency (measured as accumulation of biomass per amount of water transpired) in chickpea plants was similar in transgenic plants with high proline content to that in WT plants [22].

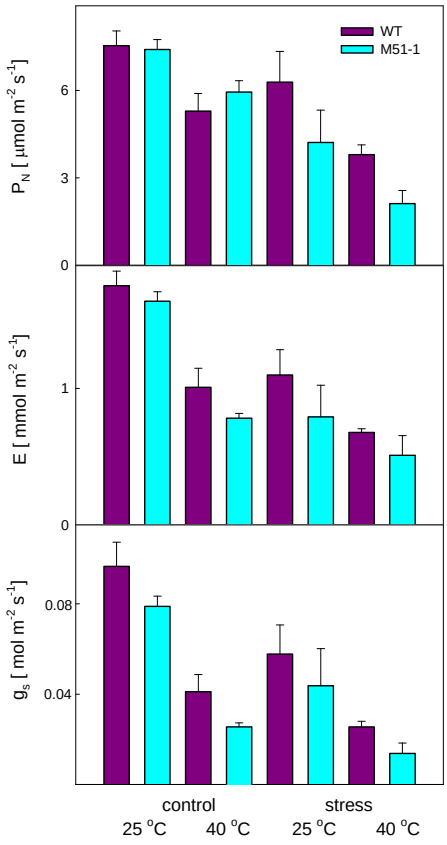

Figure 4. Net photosynthetic rate (P_N), transpiration rate (E) and stomatal conductance (g_s) in wild type (WT) and transgenic (M51-1) tobacco plants after heat shock (40˚C/60 min) imposed to plants sufficiently supplied with water or to water-stressed plants.

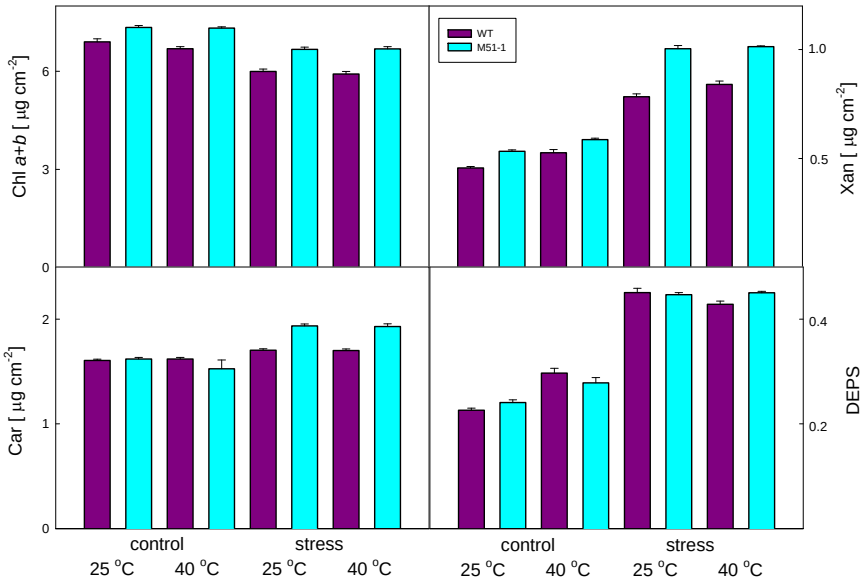

Figure 5. Contents of chlorophylls (Chl *a* + *b*), carotenoids (Car) and xanthophyll cycle pigments (Xan) and Xan degree of deepoxidation (DEPS) in wild type (WT) and transgenic (M51-1) tobacco plants after heat shock (40˚C/60 min) imposed to plants sufficiently supplied with water or to water-stressed plants.

However, no marked difference in the responses to water stress, including decrease in E, g_s, P_N and Chl content and increase in Xan content and DEPS, was observed between the genotypes. The same holds for the recovery after water stress. When *P5CS* gene was introduced under stress-inducible promotor, Chl content and variable to maximum Chl fluorescence ratio (F_v/F_m) in transgenic and WT sugarcane plants did not differ under sufficient water supply but, in contrast to our results, these characteristics remained higher in transgenic plants that in WT plants under water stress [20]. During simultaneous drought and heat stress, dissociation of the oxygen-evolving complex was bypassed by proline feeding electrons into photosystem 2, maintaining the acceptable NADPH level in transgenic soybean plants [23]. Under drought stress, the cumulative daily transpiration was higher only in some lines of transgenic chickpea plants than that in WT plants. Also g_s in these transgenic chickpea plants was slightly higher than of WT at both control and stress conditions [22].

Short-term heat stress affected only gas exchange parameters but not pigment contents. Application of heat stress to previously water-stressed plants led to further decrease in E, g_s and P_N. Similar results were recently mentioned in two pepper cultivars [24] and *Ceratonia siliqua* [25]. Our data indicate only relatively minor differences between M51-1 and WT in responses to heat or combined stresses.

All the above-mentioned results did not prove clear correlation between proline accumulation and stress tolerance. However, in our experiments, the induced stresses were rather mild than severe. Maybe, that under severe stress, the proline plays more important role, especially as protectant against oxidative stress as suggested, e.g., Molinari *et al*. [20]) and Vendruscolo *et al*. [21].

5. Conclusions

The increased content of proline in transgenic tobacco plants M51-1 was accompanied by slightly lower E and g_s and slightly higher Chl and Xan content under sufficient water supply, which might suggest more conservative water regime in these transgenic plants than in respective WT plants. The changes of all parameters induced by mild water stress or/and heat shock reflect more similarities than differences in response of M51-1 and WT tobacco plants. Therefore, the clear correlation between proline content and stress tolerance was not proved in our experiments.

6. Acknowledgements

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