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SHORT COMMUNICATION

PHOTOSYNTHETIC EFFICIENCY OF TRANSGENIC TOBACCO PLANTS (*NICOTIANA TABACUM* L.) OVER-EXPRESSING *mtlD* GENE UNDER DROUGHT AND PARAQUAT STRESS

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The objective of this study was to examine the performance of genetically transformed tobacco plants over-expressing *mannitol 1-phosphate dehydrogenase* (*mtlD*) in maintaining better photosynthetic activity than the untransformed wild plants during water deficit stress and in combination with paraquat stress. Inhibitions in the rates of net CO_2 assimilation (P_N) and the non-cyclic photosynthetic electron transport across photosystem II (ETR) due to water deficit stress were much smaller in the *mtlD* transformed plants (22% and 9%, respectively) than in the untransformed wild ones (55% and 52%, respectively). These differences were even more marked when the plants experiencing water deficit stress were treated with paraquat, which blocks the photosynthetic electron transfer chain and diverts the excitation energy into producing reactive oxygen species (ROS). The minimal inhibitions in the photochemical activity (9-10%) of *mtlD* transformed plants resulting from the environmental stresses agree with their expected efficient use of photosynthetic electrons. Results of the present study thus suggest that *mtlD* transformed tobacco plants tolerated the stress better than the untransformed wild plants which is noteworthy for further attention.

Key words: **CO_2 assimilation rate; photochemical activity; rate of photorespiration**

Under normal conditions, most of the light energy absorbed by the leaves is used for photosynthetic CO_2 assimilation and other useful processes such as nitrate assimilation etc. A part of the absorbed energy is dissipated through photorespiration and also as heat, which are safety mechanisms to avoid accumulation of excess energy in the photosynthetic apparatus. Under conditions of environmental stresses, presence of high light intensity aggravated the stress situation by diverting excitation energy and photosynthetic electrons away from photosynthetic carbon metabolism to molecular oxygen leading to irreversible oxidative damage of senescence (Asada 1999, Halliwell 1996, Noctor *et al*. 2002, Alam and Jacob 2002, Jacob and Karaba 2000, Alam *et al*. 2005). Thus, proper utilisation of the absorbed energy is important in tiding over environmental stresses. Increasing the efficient use of the excitation energy, including the reductant (NADPH), which is a direct output from energized thylakoids, will help to ease the energy load on the photosynthetic apparatus and thus production of ROS (reactive oxygen species) (Kozaki and Takeba 1996, Fryer *et al*. 1998, Savitch *et al*. 2000). In the present study genetically transformed tobacco (*Nicotiana tabacum* L. cv. Wisconsin 31) plants over expressing *mannitol 1-phosphate dehydrogenase* (*mtlD*) and untransformed tobacco plants were used.

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The enzyme *mtlD* triggers the synthesis of mannitol from Fructose-6-P via mannitol-1-P and it is coupled with the oxidation of NADPH to NADP. Therefore, over-expression of *mtlD* should result in increased use of photosynthetic electrons and thus avoid over-excitation of the photosynthetic apparatus. It is, therefore, hypothesized that tobacco plants over-expressing *mtlD* would be able to tolerate the environmental stresses better than their wild counter parts.

Two types of tobacco (*Nicotiana tabacum* L. cv. Wisconsin 31) plants were used for this experiment. One was transformed to overexpress *mannitol-1-phosphate dehydrogenase* (*mtlD*) gene and the other was untransformed wild (WLD) plants. Transformation of tobacco leaf discs to over-express the *mtlD* gene and the regeneration of *mtlD*-transformed plants were done by Karakas *et al*. (1997). The transgenic plants and the wild plants of tobacco were supplied by Dr. Mark Rieger from the department of Horticulture of the University of Georgia, Georgia, U.S.A. The plants were grown in pots in a greenhouse of the University of Georgia in Athens, Georgia, U.S.A. (34°N, 84°W) following standard agronomic practices followed by the university. One month old plants with similar growth and size were used in this study during the months of January and February 2003. Greenhouse temperature was maintained 30° C during day and 16° C in night and the plants received about 70% of full sun during the day over a photoperiod of 12 hours. Both plant types (WLD and transformed) were grown under well-watered conditions for one month. Afterwards, the plants were arranged in two sets from each type for regular watering (control) and withholding of watering (water deficit stress). For water deficit stress, watering was withheld for ten days to impose progressive drought stress in one set of plants belonging to each plant types. To further aggravate the harmful effects of water deficit stress, on the tenth day since withholding irrigation, 0.5 mM paraquat in a suitable surfactant was smeared on the top surface of three fully mature leaves per plant. Leaves smeared with surfactant alone were used as controls.

Two Li-6400 portable photosynthesis systems each attached with a leaf chamber fluorometer (LCF) (Licor, 6400-40, U.S.A.) were used at the same time for simultaneous measurements of photosystem II (PS II) chlorophyll fluorescence and gas exchange rates in control and treated plants. Each leaf was dark adapted for about 35 min in the sample chamber and at this point CO_2 efflux from the leaf was taken as rate of dark respiration (Rd). Following the dark measurements photosynthetic light response curves were made at different levels of photosynthetic photon flux densities (PPFD) using an LED source attached to the leaf chamber fluorometer. The apparent quantum yield of CO_2 fixation (Φ_c) was estimated as the slope of the linear portion of the light response curve. To obtain various levels of CO_2 during measurement, the system's external CO_2 injector (model 6400-01, Licor) was used.

To perform the measurements under non-photorespiratory condition, a gas mixture of 2% O_2 / 98% N_2 was fed to the air intake line of the LI-6400 instead of normal atmospheric air. The flow of the gas mixture from a pressurized tank was regulated to an optimum level by the flow meter of a dew-point generator (Licor, U.S.A.) using a 'T' connector bypassing the excess flow. The CO_2 concentration of the air stream was adjusted to a 360 μmol mol^{-1} or 1000 μmol mol^{-1} by the CO_2 injector when the measurement was made under photorespiratory (normal atmospheric O_2 concentration of about 21%) or non-photorespiratory (a gas mixture of 2% O_2 / 98% N_2) conditions, respectively.

To evaluate the comparative photosynthetic performance of the transformed and WLD plants under control or stressed conditions, all the measurements were made at 1500 μmol m^{-2} s^{-1} PPFD, leaf temperature 28°C and leaf-air VPD of 2 to 3 kPa.

Various chlorophyll fluorescence parameters namely maximal fluorescence under dark (Fm) and light exposure (Fm′), steady state fluorescence at any given time (Fs) and minimal fluorescence immediately under dark (Fo) and after light exposure (Fo′) which were required for estimation of different components of photochemical and non-photochemical events of the leaf were recorded following the standard techniques (Schreiber *et al.* 1986, Genty *et al*. 1989, Earl and Tollenaar 1998). CO_2 assimilation, stomatal conductance

and fluorescence (Fs) were constantly monitored to ensure that they reached a steady-state before a reading was taken.

Estimation of effective PS II quantum yield ($\Phi_{PS\ II}$) was made by using multiple pulse/ linear regression technique (Earl and Ennahli 2004). $\Phi_{PS\ II}$ and coefficients of photochemical (qP) and non-photochemical (qN) quenching of chlorophyll fluorescence were estimated as follows : $\Phi_{PS\ II}$ = (Fm′-Fs)/Fm′, qP= (Fm′-Fs)/(Fm′-Fo′) and qN= (Fm-Fm′)/(Fm-Fo′). The rate of non-cyclic electron flow across PS II (ETR) was calculated from the chlorophyll fluorescence data as ETR = $\Phi_{PS\ II}$ x PPFD x α_l x 0.5 where α_l is the leaf fractional absorptance of incident PPFD and 0.5 is the fraction of absorbed PPFD which is absorbed by the light harvesting complex of PS II as accepted for C_3 species (Genty *et al*. 1989, Schreiber *et al*. 1998). After the simultaneous measurements of gas exchange and fluorescence, the same leaf surface was used for the measurements of α_l by a spectrometer (PP system, U.S.A.) and an integrating sphere (Licor, U.S.A.) and this was used for estimating ETR. Rate of photorespiration (Rp) was computed following the formula as follows: Rp = 0.5 x Vo where Vo is the rate of RuBP oxygenation and Vo is estimated as Vo = (ETR-4 P_G)/6 where P_G is the gross photosynthetic CO_2 assimilation rate (Yoshimura *et al*. 2001). Mitochondrial respiration of illuminated leaf samples was assumed to be equal to R_d, and P_G was therefore calculated as $P_N + R_d$. Statistical analysis was done by analysis of variance, critical difference (CD) for test of significance of the means and standard errors of the means estimated.

Comparative photochemical efficiency when measured in non-photorespiratory (at 2% O_2) and photorespiratory (21% O_2) situations were also similar in both the plant types under unstressed condition (Table 1). Rates of net photosynthetic CO_2 assimilation (P_N) and electron transport across PS II (ETR) in both the transformed and untransformed (WLD) plants were almost comparable when measured in unstressed (well watered) plants. But when the plants were exposed to environmental stress, such as water deficit or paraquat application, either alone or in combination, there was considerable inhibition in the photosynthetic rates in the WLD plants as compared to the transformed plants (Table 2a). Water deficit stress as well as paraquat application led to far greater reductions in P_N and ETR in WLD than the transformed plants and the reductions were more marked when water deficit stress was coupled with paraquat application (Table 2a). Paraquat blocks photosynthetic electron transport and diverts excitation energy into producing ROS and thus causes

Table 1. Comparative photochemical efficiency of the wild (WLD) and transformed (*mtlD*) tobacco plants under unstressed conditions as measured in photorespiratory (PR) and non-photorespiratory (NPR) environments. [PR conditions = photorespiratory conditions as measured under normal atmospheric O_2 concentration (about 21%) and the CO_2 concentration was maintained at 360 µmol mol^{-1} by the external CO_2 injector of the system (please see text for details). NPR = non-photorespiartory conditions as measured with a gas mixture of 2% O_2/ 98% N_2 and the CO_2 concentration was maintained at 1000 µmol mol^{-1} by the external CO_2 injector. (n = 3 ± s.e)].

Plant types/ Measuring conditions	P_N [net photosynthetic rates (µmol CO_2 m^{-2} s^{-1})]	Gs [stomatal conductance to water vapour (mol m^{-2} s^{-1})]	Ci [CO_2 concentration in leaf intercellular spaces (µmol mol^{-1})]	$\Phi_{PS\ II}$ (effective PS II quantum yield)	ETR [electron transport rate across PS II (µmol m^{-2} s^{-1})]	qP (photochemical quenching of chlorophyll fluorescence)	qN (non-photochemical quenching of chlorophyll fluorescence)	Φ_C [apparent quantum yield for CO_2 assimilation {mol $(CO_2)mol^{-1}$ (PPFD)}]
WLD/PR	23.79±0.82	0.509±0.031	268.6±3.6	0.335±0.068	208.9±14.5	0.53±0.06	0.80±0.03	0.037±0.002
WLD/ NPR	37.1±4.9	0.183±0.011	668.1±27.7	0.267±0.039	165.6±26.1	0.47±0.05	0.84±0.01	0.044±0.005
Transformed/ PR	24.81±2.80	0.436±0.049	249.8±6.1	0.367±0.030	238.1±22.1	0.50±0.01	0.78±0.01	0.036±0.006
Transformed/ NPR	39.5±1.13	0.193±0.026	606.9±54.7	0.275±0.007	177.1±5.7	0.46±0.01	0.824±0.01	0.048±0.001

Table 2a. Comparative photosynthetic parameters and photochemical activity under normal and stress conditions in wild (WLD) and transformed (*mtlD*) tobacco plants. [Measurements made in photorespiratory (PR) conditions. PR conditions = measured with 21% O_2 and CO_2 concentration was maintained at 360 µmol mol^{-1} by the external CO_2 injector of the system (please see text for details). Rp = rate of photorespiration (µmol CO_2 $m^{-2}s^{-1}$). The other abbreviations be read as in Table 1.

Plant types/Measuring conditions	P_N	$\Phi_{PS\ II}$	ETR	qP	qN	Rp
WLD/ irrigated control	20.73	0.243	150.63	0.444	0.870	5.10
WLD/ irrigated and treated with paraquat	4.35	0.077	47.83	0.195	0.901	2.15
WLD/ droughted	9.25	0.116	72.63	0.236	0.851	2.52
WLD/ droughted and treated with paraquat	1.71	0.040	24.64	0.105	0.895	1.15
Transformed/ irrigated control	17.60	0.238	154.26	0.435	0.844	6.14
Transformed/ irrigated and treated with paraquat	10.94	0.169	108.63	0.330	0.857	4.70
Transformed/ droughted	13.73	0.217	139.95	0.425	0.856	6.27
Transformed/ droughted and treated with paraquat	12.15	0.215	138.76	0.377	0.852	6.69
CD at 5%	3.08	0.04	25.26	0.07	0.02	1.14

oxidative stress (Varadi *et al*. 2000). Given the fact that both WLD and transformed plants, which were subjected to water deficit stress were uniform in appearance and age with almost similar stomatal conductance (Table 2b), the leaf water status is expected to be similar in both the plant types. Still the transformed plants maintained greater P_N which is also reflected in their better $\Phi_{PS\ II}$. The absolute rates of ETR in the control plants of both transformed and WLD were not different under normal conditions (Table 2a). But with drought, paraquat application or both together, ETR remained significantly more in the transformed than in the WLD types (Table 2a) reflecting the increased use of photosynthetic electrons in the former. It is clear that in case of the transformed plant, water stress and paraquat reduced P_N without strong reduction in Ci which suggests possible involvement of non-stomatal restrictions (Table 2b). This indicates probably mesophyll limitations (Flexus *et al*. 2008).

Under stress conditions maintenance of better P_N, $\Phi_{PS\ II}$ and ETR also reflected in higher levels of qP and smaller levels of qN in transformed than WLD types (Table 2a). Under control conditions photorespiration (Rp) was comparable in the WLD and transformed types. But under stress conditions Rp was remarkably more in the transformed plants (Table 2a) which may be a reflection of better maintenance of photosynthetic apparatus.

Table 2b. Stomatal conductance to water vapour (Gs) and leaf intercellular CO_2 (Ci) in wild (WLD) and transformed (*mtlD*) tobacco plants under normal and stress conditions (n=3 ± s.e.).

Plant types/ Measuring conditions	Gs [stomatal conductance to water vapour (mol m^{-2} s^{-1})]	Ci [CO_2 concentration in leaf intercellular spaces (µmol mol^{-1})]
WLD/ irrigated control	0.62±0.01	294±1.9
WLD/ irrigated and treated with paraquat	0.31±0.04	324±8.6
WLD/ droughted	0.29±0.04	294±15.0
WLD/ droughted and treated with paraquat	0.22±0.02	330±2.6
Transformed/ irrigated control	0.45±0.03	285±2.4
Transformed/ irrigated and treated with paraquat	0.32±0.04	293±6.2
Transformed/ droughted	0.33±0.01	280±0.2
Transformed/ droughted and treated with paraquat	0.36±0.02	293±3.4

Our data categorically show that both photosynthetic CO_2 assimilation and photochemical activity were maintained at higher rates in intact leaves of the transformed plants than in the wild types when they were subjected to water deficit or paraquat stress, either one at a time or in combination. Mannitol that accumulated inside the chloroplast acted as a scavenger of hydroxyl radicals in the chloroplasts (Shen *et al*. 1997). Given the increased capacity of the *mtlD* transformed plants for better use of photosynthetic electrons it is possible that the *mtlD* transformed plants would be able to tolerate environmental stresses and the associated problems better.

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