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Responses to water withdrawal of tobacco plants genetically engineered with the AtTPS1 gene: a special reference to photosynthetic parameters

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Abstract We have previously obtained several lines of tobacco transformed with a trehalose-6-phosphate synthase gene of plant origin (*Arabidopsis thaliana*), involved in the first step of the biosynthesis of trehalose, a known osmoprotectant. Two showed distinct intensity of expression: high (B5H) and low (B1F). Such lines were analyzed for trehalose-6-phosphate content and the obtained results demonstrated to be in accordance with the expression results. In order to study the responses of photosynthesis to water deficit of transgenic lines in comparison to wild type (WT), three experiments were performed under different conditions: (1) Relative water (2) Leaf gas exchange (3) Modulated Chlorophyll *a* Fluorescence. Different responses in RWC of plant lines to water withdrawal were detected, with transgenic line B5H indicating less water loss after the water withdrawal period. Similar responses to water deficit regarding the leaf gas exchanges were recorded for the three lines. When subjected to water deficit stress situations, higher F_v/F_m, Φ_{PSII} and qP were detected for the transgenic lines. Under a SWC of 20% where higher values for such parameters were detected with special relevance for the B5H line, indicating a possible higher ability to withstand severe drought stress and to resist to prolonged periods without water than the B1F and WT lines.

Keywords Trehalose · Photosynthesis · Water deficit

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Introduction

Three important factors are believed to be determinant for world agricultural production in the 21st century: increase in world population, continuous scarcity of water accessible for irrigation

and deterioration of arable land. These factors suggest a need to contribute to the improvement of abiotic stress tolerance of plants (Siedow 2001). Transgenic approaches to improve stress tolerance have been proposed and are frequently considered less time consuming and laborious than classical breeding (Bajaj et al. 1999). Regarding osmotic stress tolerance, several genetic engineering approaches have been applied towards the increase of osmoprotectant compounds such as glycine betaine, sorbitol and trehalose (Nuccio et al. 1999). Accumulation of trehalose is considered as a major way to confer protection to multiple abiotic stresses (Penna 2003).

Trehalose (α,α-trehalose or α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide of glucose that occurs in several organisms such as bacteria, fungi, nematodes and crustaceans (Elbein 1974). Trehalose, in such organisms, has the ability to stabilize proteins and membranes under stress conditions, especially desiccation and heat, preventing denaturation of proteins and the fusion of membranes (Wingler 2002). In plants, trehalose accumulation seems to be related with the ability of several resurrection plant species such as *Selaginella lepidophylla* to withstand severe desiccation (Scott 2000). Engineering trehalose accumulation into agronomical important plants is considered to be a significant way of increasing their drought and salinity tolerance (Romero et al. 1997) as described using genes of microbial origin in the transformation of tobacco, potato, tomato and rice (Holmström et al. 1996; Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000; Garg et al. 2002; Jang et al. 2003, Cortina and Maciá 2005) that lead to improved tolerance to several abiotic stresses.

Trehalose and trehalose-6-phosphate synthase, play a major role in plant carbohydrate metabolism (Eastmond et al. 2003). Evaluation of sugar content can therefore be considered an aspect of capital importance in the study of the metabolism of transgenic plants transformed with trehalose biosynthesis genes.

Genetic engineering of plants with genes involved in trehalose synthesis towards the accumulation of this sugar has led to changes in photosynthesis of transgenic plants when compared to wild type. Pilon-Smits et al. (1998) evaluated chlorophyll *a* fluorescence of transgenic tobacco transformed with the otsA and otsB genes from *E. coli* under drought stress. Higher photochemical quenching and a higher ratio of variable fluorescence over maximal fluorescence were registered leading to the conclusion that transformed plants had a more efficient photosynthesis than wild type plants. The same plants were also evaluated for rates of CO_2 fixation using an infrared gas analyzer as described by Pellny et al. (2004). Chlorophyll *a* fluorescence was evaluated in rice plants transformed with a fusion of the otsA and otsB genes by comparison with non-transformed plants, under drought stress (Garg et al. 2002; Jang et al. 2003). After 100 h under drought stress the quantum yield of PSII photochemistry in wild type plants had decreased by 68%, while the best performing transgenic lines had decreases of only 29–37 %. According to these authors, similar results were obtained for the measure of accumulated photo-oxidative damage to photosystem II (PSII). The authors also report that similar responses were recorded for salt and cold stress.

Of the several AtTPS genes discovered, AtTPS1 is considered to be the main gene involved in trehalose biosynthesis (Leyman et al. 2001), it is therefore the first choice for plant genetic engineering with trehalose biosynthesis genes. We have described the methodology by which we obtained transgenic tobacco plants transformed with the *Arabidopsis thaliana* AtTPS1 gene that accumulate the protein trehalose-6-phosphate synthase as revealed by northern and western blots (Almeida et al. 2005). Three lines had different levels of expression (higher in B5H and B5A and lower in B1F) and showed better responses to salt and temperature stresses in what concerns seed germinations.

The objective of this work is to describe the study of the photosynthetic response of transgenic plants to water withdrawal in comparison with non-transformed wild type plants. Measurements of chlorophyll *a* fluorescence and gas exchange, were recorded. This paper will also include the quantification of trehalose-6-phosphate (T6P), sucrose and reducing sugars in the leaves of two transgenic plant lines, as well as wild type plants.

Material and methods

Plant material and generation of transgenic tobacco lines

Methodology regarding the establishment of transgenic lines transformed with the AtTPS1 gene has been described in detail (Almeida et al. 2005). Briefly, a cassette harboring the *AtTPS1* gene (trehalose-6-phosophate synthase from *Arabidopsis thaliana*) under the control of the CaMV35S promoter and the Bialaphos resistance gene was inserted in the binary plasmid vector pGreen0229 and used for *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum*) using the method described by Horsch et al. (1985). Three homozygous transgenic T1 lines (B5A, B5H, and B1F) were used for gene expression assays through northern and western blots and several abiotic stress tolerance tests under the conditions described (Almeida et al. 2005). As reported, transgenic lines showed absence of abnormal phenotypes such as lancet-shaped leaf, frequently reported in transgenic plants transformed with trehalose biosynthesis genes. They had, however, smaller leaf area than wild type plants and similar leaf thickness. Nevertheless, higher leaf thickness of the third upper expanded leaf was found for the transgenic plants (data not shown).

In the studies subsequently described, non-transgenic wild type tobacco (*Nicotiana tabacum*, cv. Petit Havana) controls were used as well as two homozygous transgenic lines (B5H and B1F) that displayed respectively a higher and a lower level of AtTPS1 expression (Almeida et al. 2005).

Extraction and quantification of trehalose-6-phosphate

Seeds of lines WT, B5H and B1F were germinated and transferred to 250 ml pots with standard ''*terra de Montemor*'' commercial soil (Horto do Campo Grande, Lisboa, Portugal) and grown in a cabinet chamber with controlled photoperiod (16 h light/8 h dark) and temperature (18–25°C). Three different plants per line were used. After 8 weeks, leaf disks (9 mm of diameter) from the 3rd to 5th leaf were collected and frozen in liquid nitrogen.

T6P was acid-extracted and quantified by HPLC as described (Paul et al. 2000). Briefly, frozen leaf disks was ground to a fine powder in liquid Nitrogen with a pestle and mortar and extracted with 3.5% (v/v) trifluoracetic acid (1:10 plant fresh weight/volume of extraction). After complete homogenization, samples were clarified by centrifugation. Supernatant was removed and passed through a column containing 1 g of Mega Bond Elut C_{18} (Varian, UK) followed by column washing with water. The resulting solution was evaporated to dryness *in* speed *vacuo*. The residue was dissolved in water and passed through a column of Dowex-50 (H^+) cation-exchange resin and eluted with water. This was then dried again in speed vacuo and re-dissolved in water. T6P was then quantified through HPLC based on an adaptation of methods described by Paul et al. (2000) using a carbopac PA1 analytical column (250 mm long, 4 mm i.d.) with a carbopac PA1 guard column (50 mm long, 4 mm i.d.). Samples (75 µl) prepared as described were injected onto the column. Samples were run during HPLC with 100 mM NaOH and eluted over a Sodium acetate gradient between 0 mM and 100 mM NaOH. Peaks obtained from each leaf extracts was compared to similar sample containing additional concentration of pure commercial T6P standards (Sigma). This method has been used to validate other methodologies as described (Pellny et al. 2004; Schluepman et al. 2004).

Results of T6P are presented by leaf area. Statistical comparison was done by Anova-Single factor.

Extraction and quantification of soluble reducing sugars and sucrose

Seeds of lines WT, B5H and B1F were grown as described in the previous section. Four, 6-week-old, well-irrigated plants per line were used. Leaf disks (9 mm of diameter) of the last fully expanded leaf of each plant were used. Per sample, a total of 0.003–0.007 g of leaf dry weight was used (4–6 leaf disks).

Extraction of soluble sugars was conducted as reported by Arrabaça (1981). Briefly, samples were placed in 10 ml of 80% (v/v) ethanol for 10 min at 80°C. Extracts were cooled in ice,

evaporated in nitrogen (N_2) flow and resuspended in 1 ml of ultra-pure water. Quantification of soluble reducing sugars was performed according to the modified DNS (3,5-dinitrosalicilic acid) method (Bernfeld 1955). Equal volumes (100 µl) of both sugar extract and DNS reagent (0.25 g of 3,5-dinitrosalicil acid was dissolved in 50 ml of 2N NaOH + 75 g of Sodium and Potassium tartarate dissolved in 150 ml of water; to a final volume of 250 ml) were mixed and incubated at 100°C for 10 min. Samples were subsequently cooled on ice and absorbances read at 492 nm, using a MPR-A4 microplate reader (Du Pont, Belgium). The application of this method to solutions with pre-determined concentrations of glucose allowed us to establish a regression trendline and hence the quantification of reducing soluble sugars in sample solutions.

Sucrose quantification was determined following the resorcinol method (Roe 1934). Briefly, 100 µl of sugar extract were added to 0.25 ml of resorcinol solution (1% diluted in 95% ethanol (w/v)) and to 0.75 ml of a solution of 30% HCl (w/v), mixed and incubated at 80°C for eight minutes. Samples were cooled on ice and absorbances read in the microplate reader at the same wavelength as described for the extraction of reducing sugars. The application of this method to solutions with pre-determined concentrations of sucrose allowed us to establish a regression trendline and hence the sucrose quantification in sample solutions.

Results of both reducing sugars and sucrose are presented by leaf area as well as dry weight. Statistical comparison was done by Anova-Single factor.

Analysis of relative water contents (RWC)

Seeds of B1F, B5H and wild type lines were germinated in standard MS medium (Murashige and Skoog 1962) solidified with gelrite® at a 0.4% concentration and supplemented with 20 g/l sucrose in standard Petri dishes and in standard growth chambers at 24°C and a 16 h light per 8 h dark photoperiod. When plants reached the top of the dish they were transferred to the greenhouse under natural light and at 24°C and placed in pots with 700 g of fully dried standard ''*terra de Montemor*'' commercial soil (Horto do Campo Grande, Lisboa, Portugal) and allowed to grow until 12 weeks of age. At this stage, they were used in the experiments.

Four, eleven-week-old, plants per line were used. At day 0 the, plants were watered to saturation. Total stress period lasted 21 days. At days 0 and 21 of experiment, the first fully expanded leaves of two plants per line were detached from the plant. Each leaf was divided into four equal sections, from which a sample (0.2–0.3 g of leaf fresh weight), were weighted, hence calculating fresh weight (FW). Samples were later hydrated for 12 h in distilled water in order to determine turgid weigh (TW) and later dried at 80°C until constant weight for the estimation of dry weight (DW). These three parameters allow the calculation of RWC of the leaf section, according to the formula: RWC (%) = ((FW–DW)/(TW–DW)) * 100.

Statistical comparison between lines was calculated by Anova-Single factor between the same line and at the two different ages and between the three lines at the same age.

Leaf gas exchange experiment

Seeds of B1F, B5H and wild type (WT) lines were germinated as described in the analysis of RWC. When plants reached the top of the dish, they were transferred to pots with 200 g of fully dried standard peat-perlite-vermiculite 2:1:1 (v:v:v) and allowed to grow until 10 weeks of age when they were used in the experiments.

At day 0 of the experiment, all plants were watered extensively until all soil in the pot had become fully wet. Excessive water was allowed to drain out completely from the pot and experiment begun. The combination soil + pot + plant were weighted at day 0, as well as every day when measurements were performed. At the end of the experiment, the plant was removed, and the soil was dried in an oven at 80°C until constant weight, which allowed us to calculate the soil water content (SWC) in each date of measurement, according to the formula: SWC (%) = ((WSDM–WDS)/WSDM) * 100. Where WSDM is the weight of the soil at the day of measurement and WDS is the weight of dry soil. Plant weight was considered to

be negligible. Such system allows to gradually obtaining a wide range of soil water availabilities and hence provides a broad view of the plant response to water deficit as described (Pena-Rojas et al. 2004; Llorens et al. 2004).

At each day of weighting, leaf gas exchange measurements were performed per plant and a chart was plotted based on the data collected throughout the experimental period of 10 days. The evolution of the parameters allowed the establishment of third grade polynomial trend lines that were drawn based on such data. The option of presenting photosynthesis parameters in relation to SWC allows a clear identification of the evolution patterns of such parameters as a consequence of water deficit stress.

Assays were conducted in mid spring (April–May) in the greenhouse of the Faculty of Biology of the University of Barcelona (Barcelona, Spain) between 09.30 h and 13.00 h and at least two measurements were performed per day and plant line.

On each day of measurements, plants were allowed to equilibrate for 30 min under artificial light from standard greenhouse illumination lamps (model 64702, Osram, Madrid, Spain). Measurements were conducted randomly in the three varieties in order to reduce bias due to time of measurement. The first fully expanded leaf of each plant was used and pots were placed in a regulated tripod that allowed plants to be at the distance of one meter from the 500W illumination lamp. Irradiance (PAR) ranged from 900 μmol m^{-2} s^{-1} to 1,100 μmol m^{-2} s^{-1}. Photosynthesis, transpiration and stomatal conductance to water vapor were measured using a gas exchange system with automatic data handling: the LI-6200 portable photosynthesis (LI-COR 1990) system was used, with a LI-6250 CO_2 analyzer (Lincoln, Nebraska, USA). All measurements were done in the terminal part of the first fully expanded leaf of each plant. Leaf area was marked and calculated. The same leaf area was used throughout the experiment as changes in growth were considered to be irrelevant. Photosynthesis, stomatal conductance to water vapor and transpiration are calculated directly at the apparatus using system definitions based on the measurements of the balances of CO_2 and water in the LI-6200 system and corrected in function of the leaf area used (LI-COR 1990).

Modulated chlorophyll *a* fluorescence

Plants of B1F, B5H and wild type (WT) lines were germinated in Petri dishes and grown in the greenhouse under the same conditions to those previously reported for the analysis of RWC. When plants reached the top of the dish they were transferred to the greenhouse under natural light at 24°C and placed in pots with approximately 700 g of fully dried standard ''*terra de Montemor*'' commercial soil (Horto do Campo Grande, Lisboa, Portugal) and allowed to grow until 12 weeks of age. At this stage, they were used in the experiments.

At day 0 of the experiment, all plants were watered to saturation as described. Soil water content for each day of measurement was equally calculated using the same method described. At each day of weighting, chlorophyll fluorescence measurements were performed per plant (first fully expanded leaf) and charts were plotted based on the data collected throughout the experimental period of 35 days. Observation of the evolution of the parameters suggested the existence of several different phases, so for each line and within each parameter analyzed; data were divided into one, two or three phases accordingly with the tendency verified at each case. Subsequently, a lineal trendline was drawn for each one of the phases intending to maximize the correlation factor (R^2) of each trendline.

Assays were conducted in early spring (March–April) in the laboratory at the Instituto de Tecnologia Química e Biológica (Oeiras, Portugal) between 09.30 h and 14.00 h and two measurements were made per line and day.

To conduct these assays, a modulated pulse fluorometer PAM 210 (Heinz Walz, Effeltricht, Germany) was used. Measurement, saturating and far-red lights used had respectively intensities of 6, 10 and 8 as defined in the PAM 210 fluorometer. Determination of minimal fluorescence yield of a leaf adapted to darkness (Fo) was done under measurement light of intensity 6. The maximal photosystem II (PSII) quantum yield (F_v/F_m) was determined through the exposition to

a saturating light pulse of intensity 10 and duration 0.9 s as defined in the PAM fluorometer. The effective quantum yield of PSII (Φ_{PSII}), the coefficient of photochemical quenching (qP) and the coefficient of non-photochemical quenching (qN) were determined through the exposition to a saturating light pulse in a leaf successively adapted to three different levels of actinic light: 110 (low), 590 (medium) and 1,850 (high) μmol photons m^{-2} s^{-1}. Leaf adaptation to each level of actinic light lasted 5 min and after each pulse of saturating light, a 3 s pulse of far-red light of intensity 8 was applied. Studied parameters are automatically calculated by the apparatus using adequate formulae (Schreiber 1997).

Results

Extraction and quantification trehalose-6-phosphate

In Fig. 1, results obtained for the quantification of T6P are presented. Significantly higher amounts of T6P were obtained in the transgenic lines, when compared to wild type. Such increase represented a three-fold increase in B5H plants and a two-fold increase in B1F line regarding the levels found in WT plants.

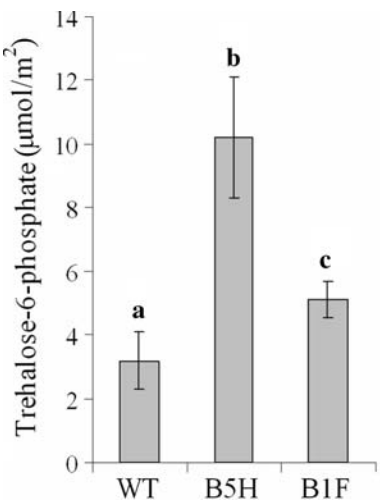

Fig. 1 Quantification of Trehalose-6-phosphate per leaf area of non-stressed wild type tobacco (WT) and transgenic lines B5H and B1F. Bars with different superscripts indicate statistical significance ($P < 0.05$)

Extraction and quantification of soluble reducing sugars and sucrose

In Fig. 2, the results obtained for the concentration of soluble reducing sugars are presented. No significant difference was recorded between the three lines.

In Fig. 3 the results obtained for sucrose concentration are presented. Both transgenic lines had significantly higher concentrations of sucrose (two- to three-fold increase) per unit of leaf area and leaf dry weight.

Analysis of RWCs

The results for RWC are depicted in Fig. 4. At the beginning of the experiment, all plant lines had similar RWCs (65–70%). After 21 days of non-watering, RWCs of lines B1F and WT had decreased to nearly 30%, whereas in the B5H line the decrease was only of 10%.

Leaf gas exchange experiment

Results obtained for photosynthesis, stomatal conductance and transpiration in gas exchange experiment are presented respectively in Figs. 5, 6 and 7. Trendline equations and regression coefficients are presented in Table 1.

The three parameters in study decreased as soil water content diminished. Regression trend lines obtained for the three plant lines were very similar in what concerns photosynthesis, respiration and stomatal conductance.

Modulated chlorophyll *a* fluorescence

Quantum yields of a leaf adapted to darkness (F_v/F_m) are depicted in Fig. 8. Results tend to maintain the initial level registered at the beginning of the experiment (SWC of about 55%) until soil water content of 12–15% is attained. At such a point, a decrease is registered for the three plant lines. However decrease seems to be more pronounced for WT and B1F plants than the one registered for B5H lines as can be seen in the slope of the trendline (see Table 2).

For Φ_{PSII} at the lower light level (Fig. 9), the two transgenic lines show a very slight decrease

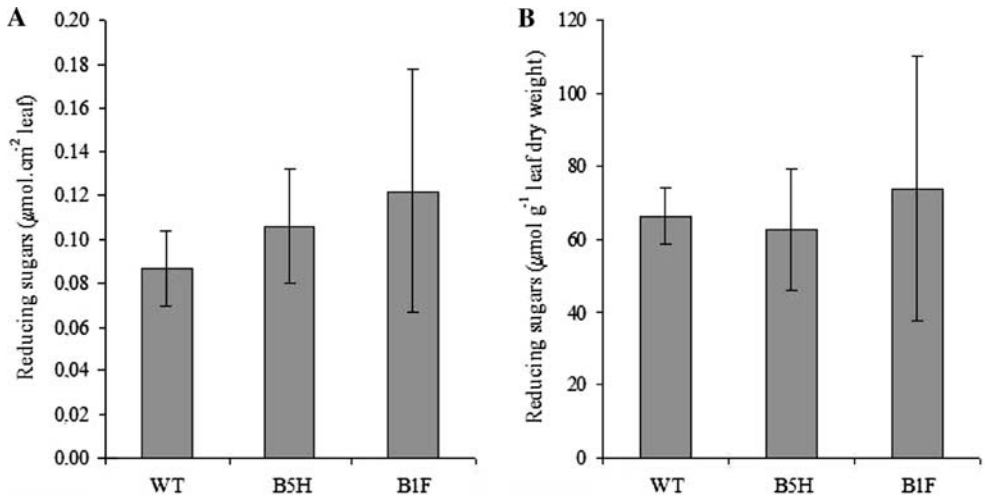

Fig. 2 Quantification of reducing sugars in leaves of non-stressed wild type tobacco (WT) and transgenic lines B5H and B1F. Results are presented per leaf area (**A**) and weight of leaf dry weight (**B**)

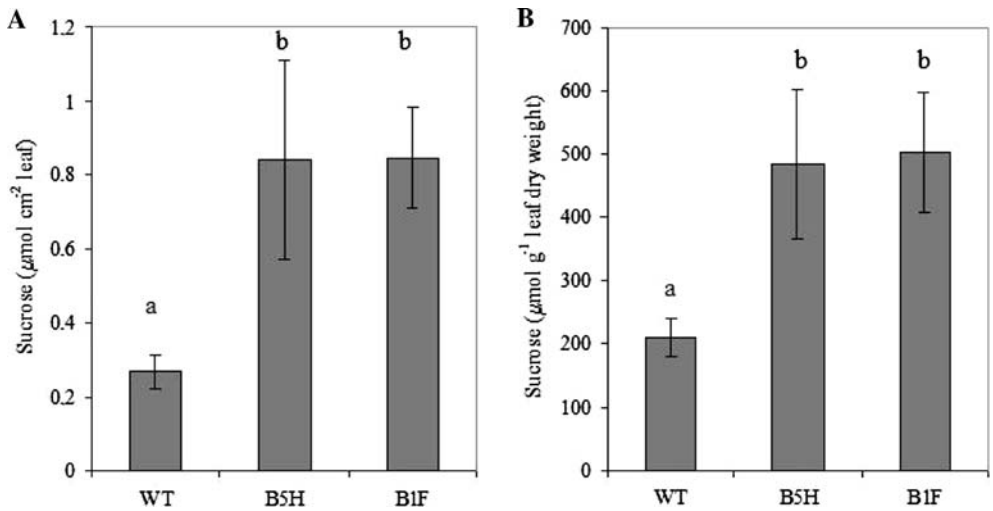

Fig. 3 Quantification of Sucrose in leaves of non-stressed wild type tobacco (WT) and transgenic lines B5H and B1F. Results are presented per leaf area (**A**) and weight of leaf dry weight (**B**). Bars with different superscripts indicate statistical significance ($P < 0.05$)

starting above 0.6 until SWC reaches around 15%. A different trend was found for the WT line: between 55% and 40% there is a steep decrease and then a stabilization at 0.5 until SWC reaches around 15%. When the SWC is lower than 15% the decrease of the Φ_{PSII} of the WT and B1F plants is higher than for the B5H plants. For values registered at a medium actinic light intensity (respectively Figs. 10 and 11) three distinct tendencies can be observed: WT plants display an initial decrease until a SWC of 35%, followed by stabilization until a 12% SWC is attained and subsequent marked decrease; transgenic line B1F shows a constant decrease throughout the initial phases of the experiment followed by a marked decrease, very similar to the one verified for WT plants and the B5H line shows a pattern of initial decrease (until 42% SWC), followed by stabilization until SWC of 15% and decrease. However, two characteristics can be pointed out, the

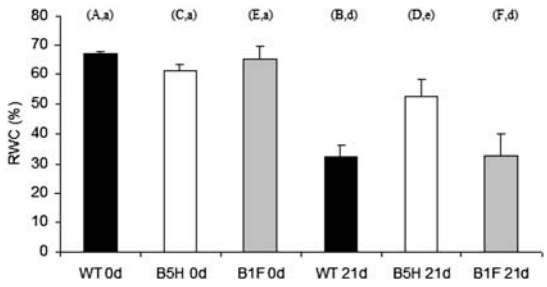

Fig. 4 Determination of Relative Water Content (RWC) in leaves of wild type tobacco (WT—black bar) and transgenic lines B5H (white bar) and B1F (gray bar) before (0d) and after 21 days of water withdrawal (21d). Black bars with different major superscripts (A and B) indicate significant difference between WT plants at 0 and 21 days of stress. White bars with different major superscripts (C and D) indicate significant difference between B5H plants at 0 and 21 days of stress. Gray bars with different major superscripts (E and F) indicate significant difference between B5H plants at 0 and 21 days of stress. Bars with different minor superscripts at day 0 (a,b,c) indicate significant difference between the three lines at that age. Bars with different minor superscripts at day 21 (d,e,f) indicate significant difference between the three lines at that age. Results were considered statistically different when $P < 0.05$

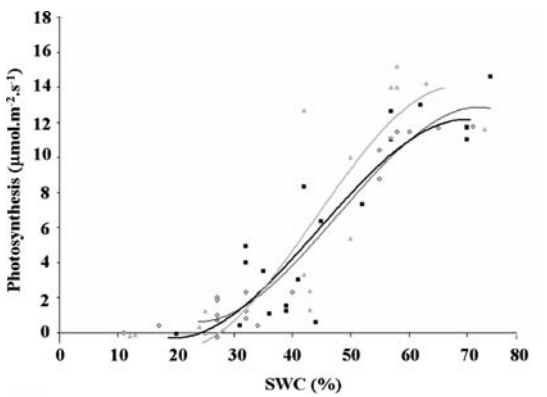

Fig. 5 Evolution of Photosynthesis (μmol CO_2 m^{-2} s^{-1}) as a consequence of decrease of Soil Water Content (SWC) of Wild Type (◇), B5H (■) and B1F (▲) plants. Trend lines were established for WT (▬), B5H (—) and B1F (▬) lines

stabilization after the initial decrease is situated before the one verified for WT plants and at higher levels of Φ_{PSII}; and the final decrease trendline displays a slope less pronounced than the one verified for the other two lines in study.

The qP (coefficient of photochemical quenching) results are presented in Figs. 12, 13 and 14.

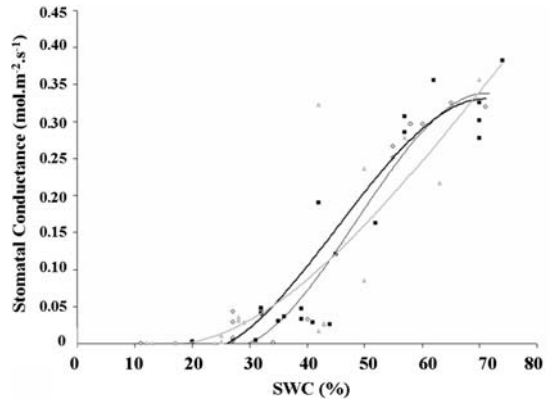

Fig. 6 Evolution of Stomatal Conductance (mol H_2O m^{-2} s^{-1}) as a consequence of decrease of Soil Water Content (SWC) of Wild Type (◇), B5H (■) and B1F (▲) plants. Trend lines were established for WT (▬), B5H (—) and B1F (▬) lines

They show that three plant lines have an initial decrease during the first phase of the experiment. After this phase, qP tends to stabilize until SWC of 12%, after which decreases markedly. Decreases for WT and B1F lines are more pronounced than those of the B5H line. Contrary to three previously mentioned parameters, the coefficient of non-photochemical quenching (qN) is stable throughout the assay and very similar for all plant lines (data not shown).

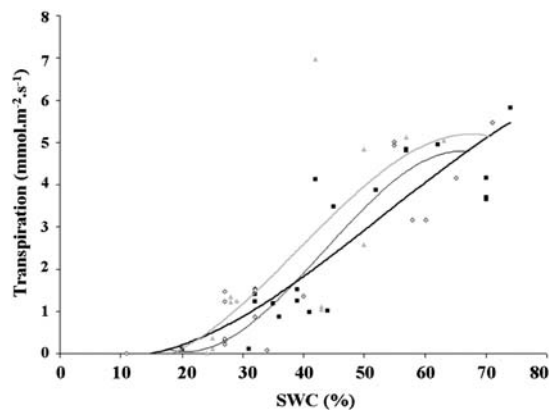

Fig. 7 Evolution of Transpiration (mmol $H_2O.m^{-2}$ s^{-1}) as a consequence of decrease of Soil Water Content (SWC) of Wild Type (◇), B5H (■) and B1F (▲) plants. Trend lines were established for WT (▬), B5H (—) and B1F (▬) lines

Table 1 Trendline equations and correlation coefficients (R^2) for the Gas exchange experiment

Parameter	WT	B5H	B1F
Photosynthesis	$y = -0.0002x^3 + 0.0267x^2 - 0.817x + 6.9399$ $R^2 = 0.9641$	$y = -0.0002x^3 + 0.0314x^2 - 1.125x + 12{,}527$ $R^2 = 0.8097$	$y = -0.0003x^3 + 0.0373x^2 - 1.168x + 9.7943$ $R^2 = 0.8206$
Stomatal conductance	$y = -5E^{-06}x^3 + 0.0007x^2 - 0.0221x + 0.1853$ $R^2 = 0.9694$	$y = -8E^{-06}x^3 + 0.0011x^2 - 0.0433x + 0.4943$ $R^2 = 0.8912$	$y = -1E^{-06}x^3 + 0.0002x^2 - 0.0057x + 0.0394$ $R^2 = 0.6582$
Transpiration	$y = -2E^{-05}x^3 + 0.0031x^2 - 0.0508x + 0.1358$ $R^2 = 0.8603$	$y = -0.0001x^3 + 0.0134x^2 - 0.4211x + 3.9457$ $R^2 = 0.7755$	$y = -6E^{-05}x^3 + 0.0072x^2 - 0.1377x + 0.4721$ $R^2 = 0.6264$

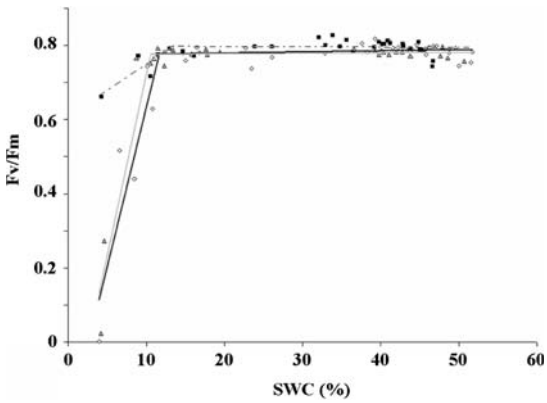

Fig. 8 Quantum yields of a leaf adapted to darkness (Fv/Fm) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC). Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

Trendline equations and regression coefficients at maximal stress for F_v/F_m, Φ_{PSII} and qP are presented in Table 2.

Discussion

We demonstrated to obtain transgenic tobacco plants expressing the trehalose-6-phosphate synthase gene from *Arabidopsis thaliana* as could be asserted by northern and western blot analysis (Almeida et al. 2005). Such plants were able to withstand several abiotic stresses at the germination level. In this paper, we mainly aim to study differences at the photosynthetic level and level of accumulation of trehalose-6-phosphate and sugar profiling between transgenic lines showing high (B5H) and low (B1F) patterns of expression of the transgene and non-transformed wild type plants.

The analysis of accumulation of T6P described in this work is in accordance with the results of expression levels found previously (Almeida et al. 2005). In fact, we have registered a residual level of accumulation of T6P in the WT line (3.20 ± 0.9 μmol m^{-2}), whilst in the transgenic

Table 2 Trendline equations and correlation coefficients (R^2) for the Chlorophyll *a* fluorescence assay at maximal stress

Parameter	WT	B5H	B1F
F_v/F_m	$y = 0.086x–0.2306$ $R^2 = 0.837$	$y = 0.014x + 0.6078$ $R^2 = 0.7482$	$y = 0.0977x–0.2589$ $R^2 = 0.8928$
Φ PSII (low actinic light)	$y = 0.0404x–0.119$ $R^2 = 0.7768$	$y = 0.0172x + 0.2941$ $R^2 = 0.8656$	$y = 0.0484x–0.1067$ $R^2 = 0.8238$
Φ PSII (medium actinic light)	$y = 0.0241x–0.0961$ $R^2 = 0.836$	$y = 0.0117x + 0.0869$ $R^2 = 0.6793$	$y = 0.0277x–0.0951$ $R^2 = 0.817$
Φ PSII (high actinic light)	$y = 0.0063x–0.0273$ $R^2 = 0.8133$	$y = 0.004x + 0.0138$ $R^2 = 0.8662$	$y = 0.0073x–0.025$ $R^2 = 08483$
qP (low actinic light)	$y = 0.0542x–0.0149$ $R^2 = 0.7199$	$y = 0.0136x + 0.5472$ $R^2 = 0.6879$	$y = 0.0598x–0.0475$ $R^2 = 0.7958$
qP (medium actinic light)	$y = 0.0319x–0.092$ $R^2 = 0.7154$	$y = 0.0219x + 0.0743$ $R^2 = 0.7838$	$y = 0.0286x–0.0212$ $R^2 = 0.6994$
qP (high actinic light)	$y = 0.0042x + 0.0319$ $R^2 = 0.6315$	$y = 0.0084x + 0.0039$ $R^2 = 0.5601$	$y = 0.0123x–0.0377$ $R^2 = 0.7667$

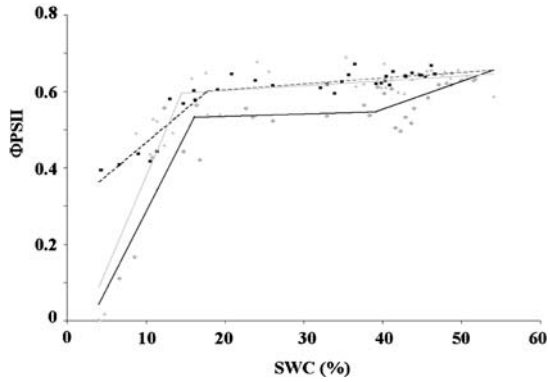

Fig. 9 Effective Quantum Yield of Photosystem II (Φ_{PSII}) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC) under an actinic light of 110 μmol photons m^{-2} s^{-1} Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

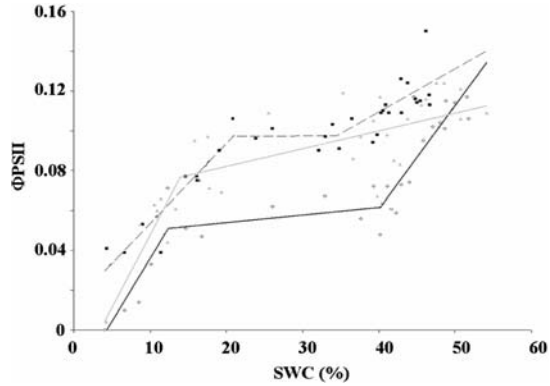

Fig. 11 Effective Quantum Yield of Photosystem II (Φ_{PSII}) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC) under an actinic light of 1850 μmol photons m^{-2} s^{-1}. Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

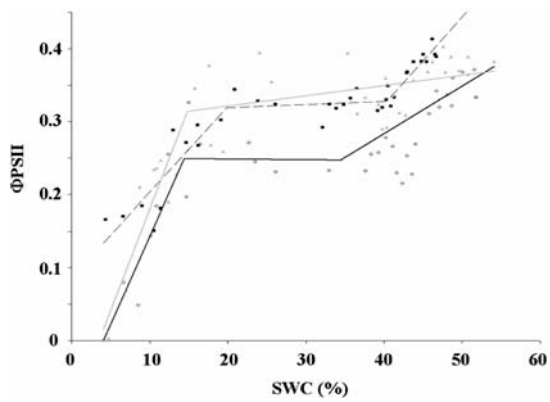

Fig. 10 Effective Quantum Yield of Photosystem II (Φ_{PSII}) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC) under an actinic light 590 μmol photons m^{-2} s^{-1}. Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

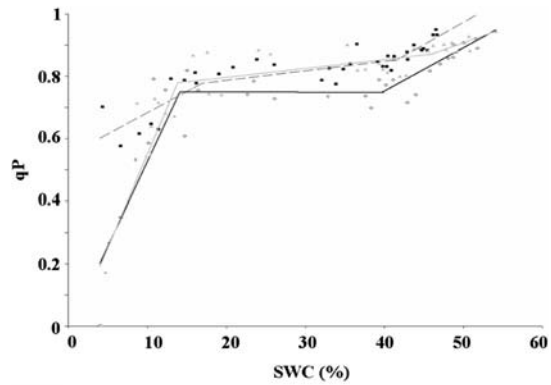

Fig. 12 Coefficient of Photochemical Quenching (qP) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC) under an actinic light of 110 μmol photons m^{-2} s^{-1} Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

lines T6P accumulation increases to levels of 5.12 ± 0.56 and 10.02 ± 1.89 μmol m^{-2} respectively in the B1F and B5H lines. There results are slightly higher than those reported by Pellny et al. (2004) using transgenic tobacco transformed with TPS gene of *E. coli* origin. Such data seem to confirm the accumulation of T6P suggested by the preliminary results (Almeida et al. 2005) hence suggesting that transgenic plants would accumulate trehalose similarly to results reported previously (Romero et al. 1997; Pellny et al. 2004). They also suggest that, due to the accumulation of T6P the obtained transgenic plants would show higher tolerance to water deficit than WT plants, higher in B5H and intermediate in B1F.

Similar concentrations of reducing sugars were quantified among the three lines studied under regular watering conditions. Such results do not agree with results of Romero et al. (1997) and Pilon-Smits et al. (1998) where differences were found for fructose and glucose. In contrast, a significant higher concentration of sucrose was

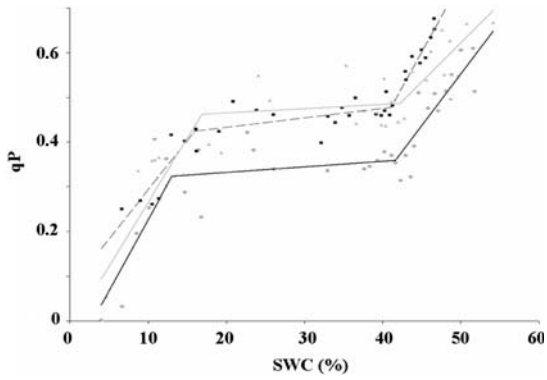

Fig. 13 Coefficient of Photochemical Quenching (qP) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC) under an actinic light 590 μmol photons m^{-2} s^{-1}. Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

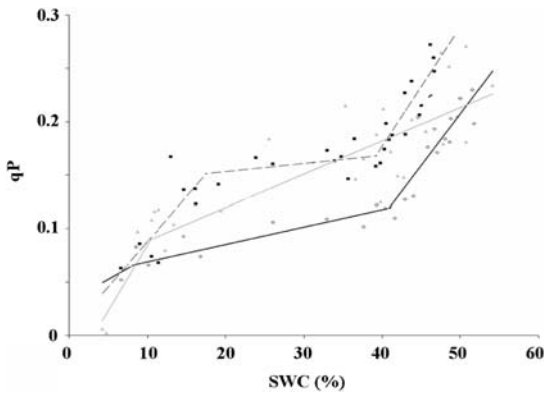

Fig. 14 Coefficient of Photochemical Quenching (qP) of Wild Type (◇), B5H (■) and B1F (▲) plants as a consequence of changes in soil water content (SWC) under an actinic light of 1850 μmol photons m^{-2} s^{-1}. Polyphasic trend lines were established for WT (black line), B5H (dashed line) and B1F (gray line) plant lines

found in the two transgenic lines. Such characteristic has been described for tobacco plants transformed with genes of *E. coli* origin involved in trehalose biosynthesis and accumulating trehalose (Pilon-Smits et al. 1998) and in plants accumulating the osmoprotectant fructan (Pilon-Smits et al. 1995). Although the mechanisms by which the insertion of trehalose-6-phosphate synthase gene affects the sugar metabolism are not fully understood, it could be suggested that the expression of certain genes or enzyme activities are affected, hence contributing to the alteration in sucrose profiles (Pilon-Smits et al. 1998). Such an increase in sucrose concentration could possibly be related to a higher capacity of osmotic adjustment of transgenic plants hence justifying the results of osmotic stress tolerance presented by these plants at the germination level (Almeida et al. 2005). In fact, sucrose accumulation, altered sugar metabolism and osmotic stress tolerance are three features often observed in plants accumulating T6P, even at minimal amounts (Romero et al. 1997).

Non-stressed wild type and transgenic plants had similar Relative Water Contents (RWC). After 21 days of stress, RWC is significantly higher in the transgenic line B5H when compared to wild type and transgenic B1F plants that show intermediate levels. These results indicate that a higher capacity to withstand water withdrawal of transgenic lines is linked both with the level of AtTPS1 expression and T6P accumulation. Such feature necessarily leads to alterations in the photosynthetic apparatus (Wingler 2002; Paul et al. 2001) that can be assessed by the use of methods such as Gas Exchange Analysis and Modulated Chlorophyll *a* fluorescence.

Data obtained in this gas exchange analysis assay points out to a very similar behavior of the three lines in study, with the exception of the B1F line initial photosynthesis rate. Pellny et al. (2004) studied CO_2 assimilation under growth and saturation lights. In both cases, transgenic plants accumulating trehalose, showed significantly higher CO_2 assimilation when compared to wild type. These results refer to hydrated plants and seem to be in accordance with the ones we have obtained in the gas exchange experiment. In fact, at a SWC of 70%, B1F plants have a photosynthesis rate of nearly 15 μmol m^{-2} s^{-1}, three units higher than those of WT or B5H plants, a difference similar others previously reported (Pellny et al. 2004).

When we look at results obtained at SWC smaller than 50%, analyzed parameters are very similar between the plant lines. These results are contrary to those observed in other plants accumulating osmoprotectant such as D-ononitol (Sheveleva et al. 1997) or GF14λ (Yan et al. 2004). Such results however imply a more rapid

stress induction than the one used in our assays. It is expectable that under the drought conditions of this assay, long period imposing stress would lead to a continuous adaptability to drought of WT plants than the one observed in assays previously reported.

Results from modulated chlorophyll *a* fluorescence experiment show that at a SWC below 12% and 15% the Fv/Fm values for B5H plants decrease less than those observed in WT or B1F plants, similarly to results obtained in other transformation events (Pilon-Smits et al. 1998; Jang et al. 2003). Quantum yields of leaves adapted to darkness (F_v/F_m) reflect the potential quantum efficiency of PSII and are sensitive indicators of the plant photosynthetic performances (Maxwell & Johnson, 2000). Our results seem therefore to indicate that, when subjected to advanced drought stress, B5H plants have better levels of photosynthetic performance potential when soil water content is less than 20% than WT or B1F plants.

Under an actinic light of 110 μmol photons $m^{-2}s^{-1}$, the effective quantum yield of PSII (Φ_{PSII}) is higher for the transgenic lines that, contrary to WT plants, do not seem to show an initial decrease in the values of the parameter immediately after stress is initiated. Under higher levels of actinic light, transgenic plants have higher Φ_{PSII} than WT plants. Such results are in accordance with those reported by Garg et al. (2002) where decrease of Φ_{PSII} is lower to those verified for WT plants. Φ_{PSII} measures the effective efficiency of PSII photochemistry (Maxwell and Johnson, 2000). We can therefore conclude that the efficiency of PSII photochemistry is higher in the transgenic lines (with special reference to the B5H line that has higher levels of expression of the gene and T6P accumulation) than in wild type plants, particularly at low SWC.

The pattern of results of Φ_{PSII} is again observed for the coefficient of photochemical quenching (qP). Higher qP was previously reported in plants engineered towards trehalose accumulation (Pilon-Smits et al. 1998), accordingly to our results. This parameter is an indication of the proportion of PSII reaction centers that are open, and decreases in the coefficient values are a consequence of reaction center closure (Maxwell and Johnson 2000). Our results indicate that under an actinic light of 110 μmol photons $m^{-2}s^{-1}$ and at SWC less than 15%, transgenic B5H plants are capable of maintaining more reaction centers opened, while under higher levels of actinic light, both transgenic lines have higher qP and hence more reaction centers opened than wild type plants.

The fact that under actinic lights of 590 and 1,850 μmol photons $m^{-2}s^{-1}$, transgenic lines show higher values of qP and Φ_{PSII} than WT plants could be an indication of the ability of these plants to withstand higher levels of saturating light. Garg et al. (2002) previously reported such trait. At SWCs under 10–15%, both qP and Φ_{PSII} data show that the photosynthetic efficiency of B5H plants is higher than the one of WT plants. Such characteristic is of extreme importance concerning the ability to withstand a certain level of drought stress and being able to recover during subsequent rehydration. In fact, it could be inferred that, if rehydration occurred after SWCs of 10–12%; transgenic B5H plants would have better chances of recovery than WT plants. It could hence be interesting to conduct research regarding the physiology of B5H upon post-stress rehydration.

The coefficient of non-photochemical quenching (qN) is a measure of changes in the efficiency of heat dissipation relatively to the dark-adapted state (Holt et al. 2004). As this parameter was not altered throughout the assay, it can be assumed that there are no differences regarding this point.

The transgenic plants obtained accumulate T6P and demonstrate a higher tolerance to water deficit stress, noticeable by the increase in photosynthetic parameters. Such increase seems to be higher for the B5H line than for the B1F line. Paul et al. (2001) propose a model by which T6P interact with plant hexokinase as in yeast. According to the theory, plants have several forms of hexokinase and T6P might interact with one of these, modifying the perception of glucose content, sensing a Carbon deficit that would result in changes in photosynthetic capacity (Paul et al. 2001). Such theory possibly explains the higher photosynthetic parameters, namely Φ_{PSII} and qP observed in the transgenic lines at the onset of water deficit stress. Such difference is more

noticeable as stress progresses, possibly explaining the results obtained in the Modulated Chlorophyll *a* experiment. However other theories could be forwarded to explain the differences in photosynthetic parameters found in these assays, namely alterations in sugar metabolism with its consequences in osmotic adjustment through the maintenance of osmotic potential, or the protective role of trehalose by oxidative detoxification (Almeida et al. 2005). Morphology changes described earlier probably also concur to the observed differences in stress tolerance.

The results obtained in this assays contribute to understand the physiological parameters involved in the higher resistance to osmotic and temperature stresses previously verified (Almeida et al. 2005). These data are of particular importance in what concerns the plant lines reaction to slowly imposed water stress, a feature associated to field conditions. However, rapidly imposed stress could equally be important in the field and plants show distinct reactions to it (Marques da Silva and Arrabaça 2004). Therefore, it seems important to conduct future research studying the reaction of plants to rapidly imposed stress.

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