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ORIGINAL ARTICLE

Effects of drought on water content and photosynthetic parameters in potato plants expressing the trehalose-6-phosphate synthase gene of *Saccharomyces cerevisiae*

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Abstract Two transgenic potato lines, T1 and T2, expressing the trehalose-6-phosphate synthase (*TPS1*) gene of yeast were isolated. In our experimental approach, we applied two novelties, namely the fusion of the drought-inducible promoter *StDS2* to *TPS1* and a marker-free transformation method. In contrast to the expected drought-induced expression, only a very low constitutive *TPS1* expression was detected in the transgenic lines, probably due to chromosomal position effects. The observed expression pattern, however, was sufficient to alter the drought response of plants. Detached leaves of T1 and T2 showed an 8 h delay in wilting compared to the non-transformed control. Potted plants of T1 and T2 kept water 6 days longer than control plants and maintained high stomatal conductance and a satisfactory rate of net photosynthesis. During drought treatment, CO_2 assimilation rate measured at saturating CO_2 level was maintained at maximum level for 6–9 days in transgenic plants while it decreased rapidly after 3 days in the wild type plants. Under optimal growth conditions, lower CO_2 fixation was detected in the transgenic than in the control plants. Stomatal densities of T1 and T2 leaves were reduced by 30–40%. This may have contributed to the lower CO_2 fixation rate and altered drought response.

Ibolya Stiller, and Sándor Dulai contributed equally to this work.

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Abbreviations

A	Net photosynthetic CO_2 assimilation rate
g_s	Stomatal conductance
RWC	Relative water content
TPS1	Trehalose-6-phosphate synthase 1

Introduction

Drought is a critical environmental factor that limits agricultural yield worldwide. Water shortage substantially decreases the relative water content (RWC), the water potential (ψ), and the net photosynthetic CO_2 assimilation rate (A) of leaves (Bajjii et al. 2001; Molnár et al. 2004). The closure of stomata due to water deficit is in part responsible for the reduction of A, as decrease of stomatal conductance (g_s) is the most efficient way to reduce water loss. Stomatal closure restricts the diffusion of CO_2 into the leaves, and results in a lower intercellular CO_2 concentration (C_i) (Cornic 2000). The limitation of CO_2 fixation during drought is also influenced by physical processes such as the diffusion of CO_2 from the intercellular spaces to chloroplasts (Delfine et al. 1999; Loreto et al. 2003), and metabolic processes such as changes in the capacity of ribulose-1,5-bisphosphate-carboxylase-oxygenase (Rubisco) and the perturbed regeneration of ribulose-1,5-bisphosphate (Medrano et al. 1997; Maroco et al. 2002; Centritto et al. 2003; Chaves et al. 2003).

The development of drought tolerant crops bears significant economical importance, as water shortage decreases the growth, dry matter content and yield of cultivated plants (Blum et al. 1997; Frensch 1997; Araus et al. 2002; Munns 2002). To date, various approaches have been used to produce drought tolerant plants. One of the approaches is the engineering of transgenic cultivars expressing osmolyte biosynthesis genes involved in the biosynthesis of glycinebetaine, proline, polyamine, mannitol or trehalose (for a review, see Cherian et al. 2006).

Trehalose (α,α-1,1-diglucose) is synthesised from UDP-glucose and glucose-6-phosphate. In yeast (*Saccharomyces cerevisiae*), a complex containing trehalose-6-phosphate synthase (TPS, encoded by *TPS1*) and trehalose-6-phosphate phosphatase (TPP, encoded by *TPS2*) is involved in the synthesis of trehalose. In *Escherichia coli*, TPS and TPP are encoded by the *otsA* and *otsB* genes, respectively. The first report on improving drought tolerance by introducing *TPS1* of yeast fused to the Rubisco small subunit promoter into tobacco was by Holmström et al. (1996). Although transgenic plants contained more trehalose than the non-transformed controls, trehalose-6-phosphate (T-6-P) was not found in leaves. This suggests that an endogenous phosphatase might be involved in the dephosphorylation of T-6-P. Trehalose accumulation caused no obvious morphological changes, but decreased the growth rate of transgenic tobacco plants by 30–50% under conditions which were optimal for the control plants. High level of expression of *TPS1* from the constitutive *CaMV35S* promoter also resulted in drought tolerant tobacco plants. These plants, however, exhibited multiple phenotypic alterations, including stunted growth and lancet-shaped leaves (Romero et al. 1997). Goddijn et al. (1997) generated high trehalose sythesising tobacco and potato by introducing both the *otsA* and *otsB* genes of *E. coli* under the control of either the constitutive *CaMV35S* promoter, or the tuber-specific patatin promoter. In the leaves of the obtained transgenic tobacco plants, very low levels of trehalose accumulation were observed, whereas transgenic potato tubers showed no trehalose accumulation at all. Endogenous trehalase activity was shown to affect the accumulation of trehalose in these plants.

The concentration of trehalose in transgenic tobacco plants seems insufficient for osmotic adjustment. Therefore the mechanism underlying the enhanced desiccation tolerance of *TPS* overexpressing plants is still unknown. Experiments with the model plant *Arabidopsis thaliana* indicate that the accumulation of T-6-P as opposed to trehalose determines the phenotypes of *TPS* transgenic plants (Schluepmann et al. 2003, 2004). Interestingly, constitutive overexpression of one of the endogenous *A. thaliana* TPS-coding gene, *AtTPS1*, results in a phenotype tolerant of dehydration without showing morphological alterations other than flowering delay (Avonce et al. 2004). Rice (*Oryza sativa*) appears to be more tolerant to trehalose than dicot plants. Overproduction of trehalose in *O. sativa* by a bifunctional fusion enzyme (TPSP) derived from the *otsA* and *otsB* genes produced trehalose of up to 0.1% of the fresh weight without any detrimental effect on plant growth and grain yield, but resulted in high tolerance against different abiotic stresses (Garg et al. 2002; Jang et al. 2003).

Compared to other crops, potato (*Solanum tuberosum* L.) is considered drought sensitive and even short periods of drought stress can cause significant reduction in tuber yield (van Loon 1981). To improve the drought tolerance of potato, Yeo et al. (2000) introduced the yeast (*S. cerevisiae*) *TPS1* gene under the control of *CaMV35S* promoter into the cv. Dejima. The obtained *TPS1* transgenic plants exhibited various morphological changes in phenotype in culture tubes, but these negative changes disappeared when the plants were grown in pots. In contrast to these findings, our preliminary experiments in the potato cvs. Désirée and White Lady have shown that expression of the *TPS1* gene under the regulation of the *CaMV35S* promoter inhibits growth in both tissue culture and soil.

Regulation of transgenes by stress-inducible promoters can protect transgenic plants from growth abnormalities (Kasuga et al. 1999, 2004). It has been shown that expression of the *DS2* gene of potato is highly desiccation-specific (Silhavy et al. 1995; Dóczi et al. 2002, 2005). The promoter region of the *Solanum tuberosum DS2* gene (*StDS2*) was isolated from the cv. White Lady and was proven to be drought-activated (Dóczi et al. 2002).

We report the generation of two *StDS2* promoter-*TPS1* transgenic lines of potato cv. White Lady by marker-free transformation. Although the expression of *TPS1* is not inducible in these transgenic lines, the very low level of constitutive *TPS1* expression detected in the leaves is sufficient to alter their response to drought.

Materials and methods

Plant material, growth conditions and transformation

The Hungarian commercial potato (*Solanum tuberosum* L.) cv. White Lady was vegetatively propagated from cuttings on MS or RM medium (Murashige and Skoog 1962) containing 2% (w/v) sucrose at 24°C under a light regime of 16 h light at 5,000 lux intensity and 8 h of dark.

Transgenic lines were obtained by marker-free tuber disc transformation according to Bánfalvi et al. (2000) using the *Agrobacterium tumefaciens* strain C58C1 containing pGV2260 (Deblaere et al. 1985). Tuber slices from 4-month-old in vitro cultures of *S. tuberosum* were

co-cultivated with *A. tumefaciens* on RM medium solidified with 0.5% of agar. After 2 days of co-cultivation, the tuber slices were washed with 500 mg l^{-1} cefotaxime and placed on MS medium containing 2% sucrose (w/v), 20 μg l^{-1} naphtalene acetic acid, 20 μg l^{-1} gibberellic acid, 2 μg l^{-1} trans-zeatin riboside, 500 mg l^{-1} cefotaxime and 0.8% agar. Regenerated shoots were excised and placed into RM medium containing cefotaxime for rooting.

Six-week-old control plants obtained by tissue culture and their transgenic derivatives were transferred into pots containing A200 sterile soil (500 g/pot) and grown further under greenhouse conditions at 18–28°C. Optimal growth conditions were provided by regular watering (70–80% soil humidity) and supplemental lighting from November to March using sodium lamps.

To measure diurnal changes of in situ stomatal conductance, 1-month-old plants grown in pots under optimal greenhouse conditions were acclimated to field conditions (fully open area, 1,700–2,000 μmol photo nm^{-2} s^{-1} PPFD, maximum air temperature of 26–32°C) for 3 weeks in June. Before starting stomatal conductance measurements, plants were watered nightly with 400 ml of water for 1 week.

Construction of the *StDS2* promoter-*TPS1*-*nosT* fusion and cloning into the binary vector pAB10

The *TPS1* gene of *Saccharomyces cerevisiae* was isolated by PCR and cloned into pBluescript II SK (Stratagene). Its functionality was tested in *E. coli*, and by producing desiccation tolerant transgenic nematodes (Vellai et al. 1999). A vector carrying the *TPS1* gene for plant transformation was constructed as follows. First, the 1,140-bp *StDS2* promoter (Dóczi et al. 2002) and the *nostT* terminator of plasmid pDV411 (kindly provided by G. Dallmann, Agricultural Biotechnology Center, Gödöllő, Hungary) were inserted in the appropriate order into the *Eco*RI and *Sac*I sites of pBluescript II KS. *TPS1* was subsequently isolated as a *Hind*III-*Sma*I fragment from the cloning vector, and the *Hind*III site was filled with Klenow polymerase. *TPS1* was inserted between the promoter and terminator sequences into the *Sma*I site of pBluescript II KS. Orientation of the *TPS1* gene was determined by *Eco*RI digestion (results not shown).

A binary vector for marker-free plant transformation was constructed from pGSGLUC1 (Plant Genetic Systems). The *nptII* marker gene and the β-glucuronidase gene of pGSGLUC1 was removed by *Bgl*II and *Sal*I digestions and replaced with the multicloning site of pBluescript II KS. The resulting vector was designated pAB10.

The *StDS2* promoter-*TPS1*-*nosT* fusion was cloned into the *Eco*RV site of pAB10 as a *Pvu*II fragment.

Nucleic acid isolation and analysis

To screen regenerated plants for the presence of the *TPS1* gene, genomic DNA was isolated from plant leaves as described by Shure et al. (1983), and PCR reactions were performed using primers specific to the *StDS2* promoter (5′-ttg atc ccg gtg aaa gaa ctc c-3′) and the *TPS1* gene (5′-cac cct tca aaa tct ctt gtc tg-3′).

Total RNA was extracted from leaves using the method of Stiekema et al. (1988). Northern blotting and hybridisation using a radioactive DNA probe was carried out under stringent conditions as described by Dóczi et al. (2002). The *StDS2* gene specific probe was obtained by PCR using the primers 5′-ttg atc ccg gtg aaa gaa ctc c-3′ and 5′-cag cac aca aca aag aga ggt a-3′ for ^{32}P-labelling.

Promega's Access RT-PCR Introductory System Kit was used for reverse transcription and PCR amplification according to the manufacturer's instructions, using one microgram of total RNA and the *TPS1* specific primers 5′-att ctg gat gct cgt tca-3′ and 5′-gat gaa atc gca gac tta ca-3′.

Drought stress, measurement of water content, stomatal conductance, and photosynthetic parameters

All experiments were performed on the fully expanded intact leaves of potato cv. White Lady. Before treatment, the height and leaf area of wild and mutant plants was estimated. Treatments and measurements were carried out on leaves considered uniform.

Water deficit was induced by wilting detached leaves at room temperature or by withholding the water supply in the soil. The water status of plants was traced by determining the relative water content (RWC) according to the following equation:

$$\mathrm{RWC} = (\mathrm{FW} - \mathrm{DW}) \times 100/(\mathrm{SW} - \mathrm{DW}),$$

where FW is the fresh weight, SW the water saturated weight and DW the dry weight after drying for 12 h at 105°C.

The response of the in vivo chlorophyll *a* fluorescence in leaves of wild, T1 and T2 potato lines to water deficit was measured in dark-adapted intact leaves using a pulse amplitude modulation fluorometer (PAM 101-103, Walz, Effeltrich, Germany) as described by Dulai et al. (1998) and recorded with a potentiometric chart recorder (NE-244, EMG, Budapest, Hungary) and a computer. The initial level (F_0) of fluorescence was excited by a weak 650-nm light beam modulated at 1.6 kHz. The fluorescence was detected by a PIN S1723 photodiode. The maximal fluorescence level (F_m) of the dark-adapted leaves was induced by a white saturating flash (4,000 μmol m^{-2} s^{-1}) of 0.8-s

duration, using a Schott KL-1500 light source (Schott, Essex, UK). Photosynthesis was induced for 15 min by continuous actinic light of 1,000 μmol m^{-1} s^{-1}. The variables and equations for quenching analysis were determined according to van Kooten and Snel (1990). The quantum efficiency of photochemistry was calculated as $\Delta F/F_m'$, as described by Genty et al. (1989).

CO_2 assimilation of intact leaves was measured with an infrared gas analyser (LCA-2, Analytical Development Co. Ltd., Hoddesdon, UK). The white light for excitation of photosynthesis was provided by a Schott KL-1500 light source through a fiber-optic cable. The net CO_2 assimilation rate (A) and stomatal conductance (g_s) were calculated in the light saturated state of photosynthesis by using the equations of von Caemmerer and Farquhar (1981). The light response curve of A was determined in the range of 100–1,200 μmol m^{-2} s^{-1}.

The responses of A to changing in ambient CO_2 concentration was measured at 345 and 1,200 ppm CO_2 at 1,000 μmol m^{-2} s^{-1} light intensity using a gas diluter (Analytical Development Co. Ltd., Hoddesdon, UK).

Diurnal change of in situ stomatal conductance in response to drought was measured in intact leaves using a Delta-T AP4 porometer (Delta-T devices, Cambridge, UK). Measurements were made every second hour between 8 and 20 at field conditions during the drought period.

Determination of stomatal density

We used a nail polish replica to determine the number of stomata under the light microscope at 400× magnification. The measuring and counting was carried out using the Soft Imaging System (Münster, Germany) software package.

Results

Isolation of *TPS1* transgenic White Lady plants by marker-free transformation

To develop an acceptable and safe potato cultivar for potential market application, the *TPS1* gene of yeast was fused to the drought-inducible *StDS2* promoter and cloned into the binary vector pAB10 developed for marker-free transformation (see "Materials and methods"). The recombinant binary vector was introduced into *A. tumefaciens*. Forty sterile microtuber slices of potato cv. White Lady were infected with *Agrobacterium*, co-cultivated for 2 days, and placed on selection-free regeneration medium in Erlenmeyer flasks. After 4 weeks approximately 50 shoots emerged from the tuber discs. The number of shoots increased to 250 after 6 weeks, and measured between 3 and 5 cm in length. Ninety shoots were excised and placed on rooting medium.

To isolate transgenic plants, leaf samples were taken from the 90 plants in tissue culture. The samples were analysed by PCR using a transgene-specific primer pair. Seven putative transgenic lines were identified. These were propagated further in the presence of cefotaxime on a culture medium to select against *Agrobacterium*. After 2 months in tissue culture the plants were potted into soil and grown in greenhouse. PCR analysis with the transgene-specific primers was repeated. At this time, however, only two PCR positive lines could be identified (Fig. 1). This indicates that in some of the lines, agrobacteria were able to survive at least until the time of the first screen. The two plants carrying the transgenes, designated T1 and T2, were derived from two different tuber slices and thus could be regarded as independent transformants.

Expression of the *TPS1* gene in the leaves of transgenic plants

Expression of *TPS1* in the T1 and T2 lines was tested in leaves collected from 6-week-old plants grown under optimal growth conditions in a greenhouse. To induce expression of the transgene, detached leaves were wilted at room temperature and samples were taken at every 8 h. Figure 2a shows that the non-transformed leaves lost their water content significantly faster than the leaves of transgenic lines. RNA was isolated from the leaves and tested for *StDS2* and *TPS1* expression by northern hybridisation. Severity of water loss after 16 h was strong enough to detect expression of *StDS2* not only in non-transformed, but also in transgenic lines (Fig. 2b). This experiment, however, did not result in the detection of *TPS1* mRNA, suggesting a very low level of *TPS1* expression in transgenic lines. RT-PCR was therefore used to detect the expression of *TPS1* in the leaves, as the level of sensitivity it provides increases the chances of finding low copy number transcripts. Figure 2c shows that even using this method, only a basal level of *TPS1* expression was found, and no elevation of *TPS1* mRNA levels was detected in wilted leaves. The failure of stress-inducible promoters to

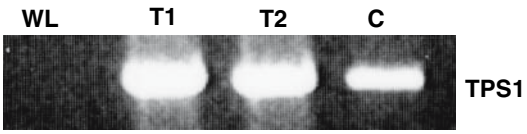

Fig. 1 PCR analysis of marker-free *TPS1* transgenic White Lady plants, T1 and T2. PCR analysis was carried out with primers specific for the transgene. pAB10 carrying the *TPS1* gene was used as a positive (C) and non-transformed plant DNA as a negative control (WL)

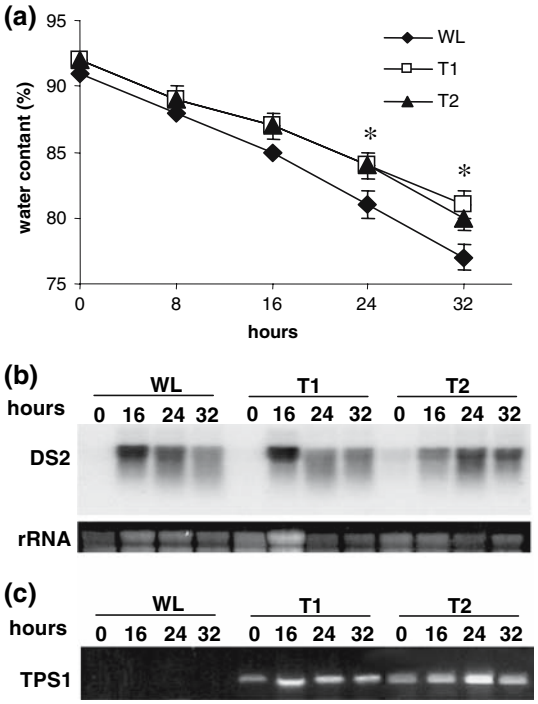

Fig. 2 Experiments with detached leaves. **a** Decrease in water content of non-transformed (WL) and *TPS1* transgenic leaves, T1 and T2, wilted at room temperature. Samples were taken from 10 plants, 3 leaves from each. Data are the mean ± SD. *Asterisks* depict differences significant at $P = 0.01$ (t probe) for both transgenic lines compared to the non-transformed control. **b** Expression of *StDS2* in wilted leaves. 0, 1, 2, 3 indicate 0, 16, 24 and 32 h time points, respectively. Hybridisation was carried out under stringent conditions with a ^{32}P-labeled *StDS2*-specific probe. Ethidium bromide-stained rRNA bands are shown as loading controls. **c** RT-PCR analysis to detect *TPS1* expression. The same set of RNA isolates was used for RNA gel blot as for RT-PCR

function in transgenic lines is not unprecedented and might be explained by the chromatin structure of integration site (Busk and Pages 1998; Dóczi et al. 2002).

Effect of drought on water content, stomatal conductance and photosynthetic parameters

TPS1 transgenic and control plants were potted and grown in a greenhouse for 6 weeks under optimal conditions. At 6 weeks, irrigation was stopped and the response of the plants was studied over time. In both T1 and T2 plants, water loss was slower than in the control plants. A significant decrease of RWC was only observed after 9–12 days. Furthermore, the water content of the T1 and T2 plants was significantly higher than that of the control during the dry period (Fig. 3a).

In the wild type, the initial high stomatal conductance (g_s) decreased in parallel with the water loss from day three of the desiccation period. The decline in g_s of T1 and T2 was slower than in the control (Fig. 3b).

CO_2 gas analysis revealed a lower CO_2 fixation rate in irrigated transgenic plants compared to the non-transformed control (Fig. 3c). The A value, similarly to g_s sharply dropped in the wild type plants after 3 days of dehydration, while in the transgenic plants it remained at the original level for 6–9 days.

Drought-induced reduction of CO_2 assimilation is influenced by stomatal and non-stomatal factors. When stomatal limitation occurs, then A_{max} can be restored by saturating CO_2 levels (Lawlor 2002). In well-watered leaves, the difference between the A levels of the wild type and transgenic plants remained significant even by raising the ambient CO_2 concentration (Fig. 3d). During drought treatment the A values measured at saturating CO_2 level decreased continuously and intensively in the wild type, while it was maintained at maximum level for 6–9 days in the transgenic plants.

Up to day twelve of the dehydration period, no significant difference was found in the optimal quantum yield (F_v/F_m). This reflects the capacity of charge separation and relative number of active PS II reaction centres. No difference in the effective quantum yield of PS II photochemistry ($\Delta F/F_m'$) was detected between the wild type and transgenic plants at 1,000 μmol m^{-2} s^{-1} light intensity. These parameters decreased significantly only at severe water stress beyond day 12 and 16 (Fig. 4a, b).

Responses of diurnal changes of in situ stomatal conductance to drought were also measured (Fig. 5). For this purpose 1-month-old plants in pots were transferred from the greenhouse to field conditions and acclimated for 3 weeks. After 3 weeks, the irrigation was stopped. At the beginning of the desiccation period, g_s was lower in the leaves of T1 and T2 than in the wild type, especially from 2–4 p.m. until the end of photoperiod. Nevertheless, g_s was only moderately decreased to further drought treatment in transgenic plants. It remained relatively high even at the end of the treatment, while it was only a small fraction of the original value in the non-transformed control.

Stomatal densities of *TPS1* transgenic plants

Leaf anatomical features contribute to transpiration efficiency and water loss under drought conditions. Epidermal phenotype of *TPS1* transgenic lines grown in pots in greenhouse under optimal conditions was investigated using a light microscope. This experiment revealed that the stomatal densities of T1 and T2 lines are reduced by 30–40% compared to the non-transformed control (Fig. 6).

Discussion

Effects of drought stress on the water content of leaves

In this study, two transgenic potato lines expressing the *TPS1* gene of yeast at a very low constitutive level were isolated (T1 and T2). Detached leaves of non-transformed control plants lost their water content significantly faster than the leaves of *TPS1* transgenic lines. After withholding the water supply in pots, water loss was also faster in non-transformed plants than in transgenic lines. A significant decrease in RWC was detectable in the non-transformed plants after the day 3, and their water content was significantly lower than that of T1 and T2 by the end of the dehydration period. It was shown that T1 and T2 potato plants possess 30–40% less stomata than the non-transformed control plants, which may contribute to their slower rate of water loss. However, the g_s value of transgenic plants under irrigated conditions was only slightly lower than that of the wild type plants. Decrease in g_s value was shown as the most efficient way of reducing transpirational water loss (Cornic 2000). In spite of the rapid drop of the g_s value, a continuous reduction was observed in the RWC values of the wild type plants. It is interesting to note that while efficiently keeping water, the decline in g_s value of T1 and T2 plants was slower than in the wild type plants. This might be explained by metabolic alterations and improved osmoregulation in transgenic plants.

Effects of drought stress on the gas exchange and fluorescence induction parameters

It has been reported that during drought stomata play an important role not only in the regulation of transpirational water loss but also in the inhibition of photosynthetic CO_2 fixation partly by stomatal closure (Sharkey 1990; Chaves 1991; Cornic 1994; Molnár et al. 2004). The g_s value, though not to the same degree, decreased in all examined lines during the desiccation period, and correlated with RWC decrease. The strongest stomatal closure was detected in wild type plants. Contrary to this, stomata in the T1 and T2 lines remained more open for a longer time during the desiccation period than in the wild type. When mesophyll conductance and metabolic factors do not limit the carboxylation processes, this decrease of g_s, which may restrict the diffusion of CO_2 into the leaves, has been reported to lead to a decrease in photosynthetic CO_2 fixation (Flexas and Medrano 2002). *A* was strongly restricted as g_s rapidly fell in the case of the wild type, while T1 and T2 were capable of maintaining a satisfactory rate of net photosynthesis for longer time in parallel with a moderate decrease of stomatal conductance. In situ measurements of

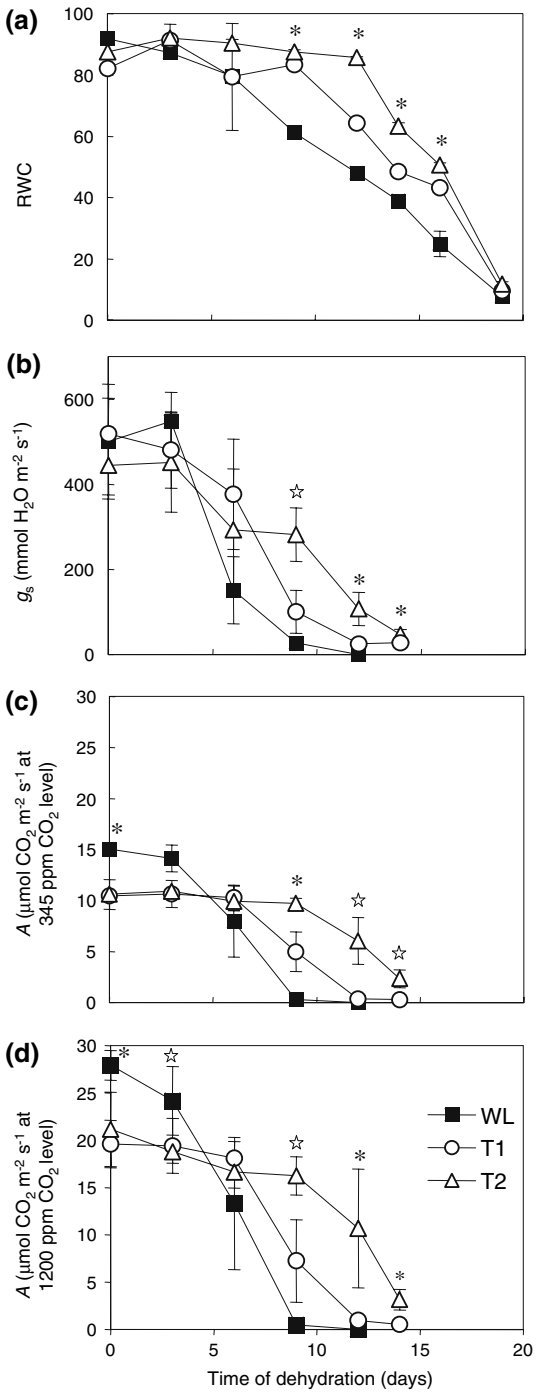

Fig. 3 **a** Effects of drought stress on relative water content (*RWC*), **b** stomatal conductance (g_s) and net assimilation rates (*A*) at **c** 345 and **d** 1,200 ppm CO_2 level of non-transformed (WL) and *TPS1* transgenic, T1 and T2 potato plants. The results are means ± SD of data of five independent measurements on different leaves from different plants. *Asterisks* depict differences significant at $P = 0.01$ (*t* probe) for both transgenic lines compared to the non-transformed control, while the differences significant at the same level only for T2 are labelled with *stars*

g_s also suggest that stomatal conductance in T1 and T2 lines is less susceptible to drought than in wild type. These results indicate that the transgenic lines could retain their

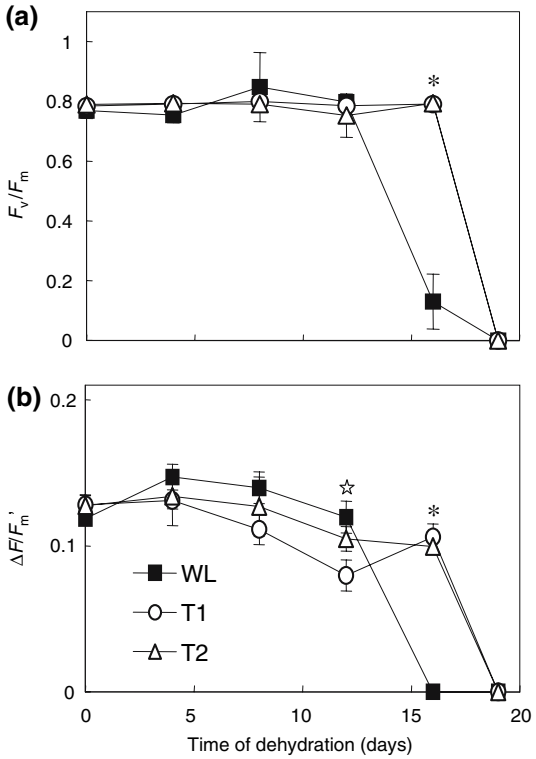

Fig. 4 Changes in **a** optimal (F_v/F_m) and **b** effective ($\Delta F/F_m'$) quantum yield of photosystem II in non-transformed (WL) and *TPS1* transgenic, T1 and T2, potato plants during the desiccation period. The results are means ± SD of data of three independent measurements on different leaves from different plants. *Asterisks* depict differences significant at $P = 0.01$ (t probe) for both transgenic lines compared to the non-transformed control, while a difference significant at the same level only for T1 is labelled with a *star*

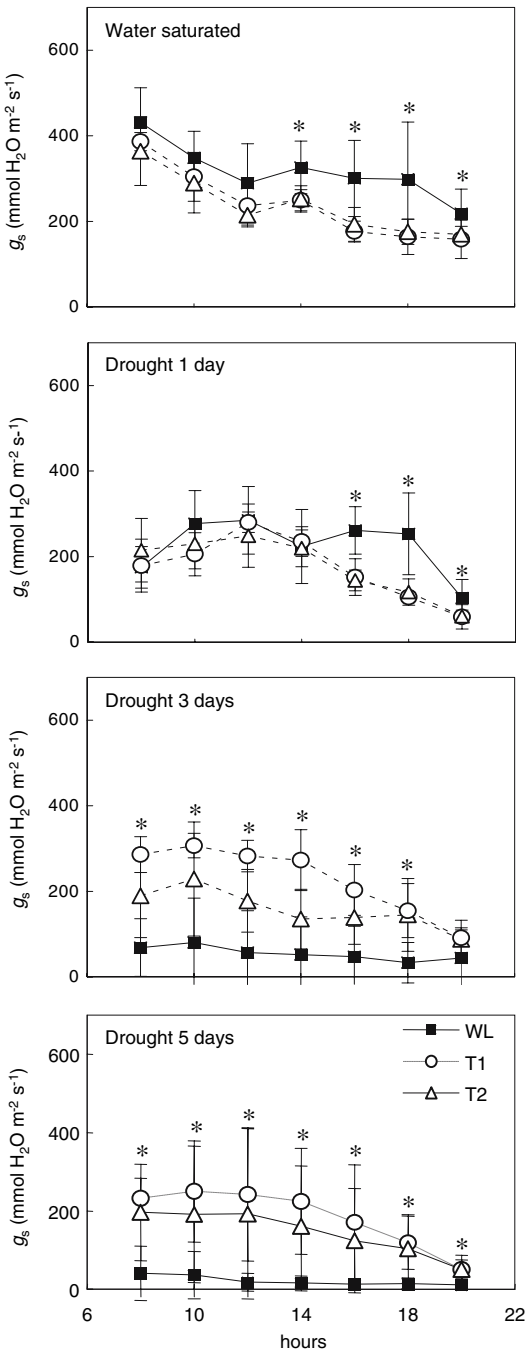

Fig. 5 Effects of drought stress on the diurnal changes of stomatal conductance (g_s) for non-transformed (WL) and for *TPS1* transgenic, T1 and T2, potato plants measured during daylight from 8 to 20 h. *Asterisks* depict differences significant at $P = 0.01$ (t probe) for both transgenic lines compared to the non-transformed control

CO_2 fixation rate for a longer period under drought stress. However, CO_2 gas analysis revealed a lower rate of CO_2 fixation in transgenic lines as compared with the wild type under optimal growth conditions. This raises the question whether there is a correlation between the lower stomatal density and CO_2 fixation rate detected in transgenic plants compared to the wild type, or if both are the consequences of metabolic processes. In spite of the extremely low level of *TPS1* expression (detectable only by RT-PCR) a slight decrease in the growth rates of transgenic plants was observed (data not shown). The lower CO_2 fixation in transgenic lines compared to the wild type under optimal growth conditions may explain the slower growth rate of *TPS1*-expressing transgenic plants. The smaller size of the plants, however, could not be the only explanation for maintaining the leaf RWC at high level when watering is withheld, since it had been shown that detached non-transformed leaves also lost their water content significantly faster than the leaves of transgenic lines.

In water-saturated C_3 plants kept at ambient CO_2 concentration, the A value does not reach that maximum level which otherwise would be measurable at saturating CO_2 concentration (A_{max}). As suggested previously, stomatal closure is the main limitation to CO_2 fixation during drought since A_{max} can be restored by a high CO_2 level (Cornic 2000; Cornic and Fresneau 2002). Several studies, however, have also reported that the maximal rate of net photosynthesis is not fully restored by increased CO_2

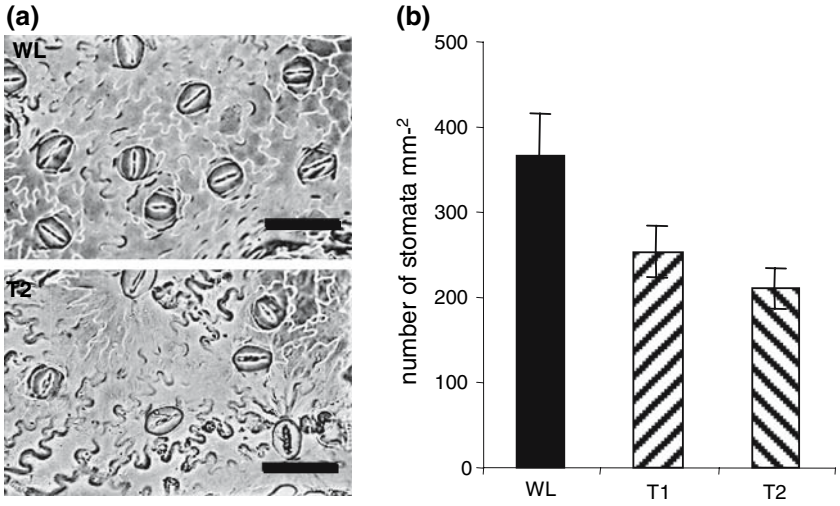

Fig. 6 Stomatal densities. **a** Microscopic image derived from the epidermis of non-transformed (WL) and *TPS1* transgenic T1 leaves. **b** Effects of *TPS1* expression on stomatal densities of *TPS1* transgenic lines T1 and T2. The data were obtained from three plants per line, three leaves from each with the same size and by analysing 30 fields of vision per leaf with the Soft Imaging System (Analysis 2.11 software). Data are the mean ± SD. Scale bar, 50 μm

concentration. Decrease of the *A* value can also result from reduced mesophyll conductance (Delfine et al. 1999; Flexas et al. 2002; Loreto et al. 2003), or other important metabolic factors (Tezara et al. 1999; Delfine et al. 2001; Lawlor and Cornic 2002; Centritto et al. 2003; Chaves et al. 2003). In well-watered leaves the net assimilation rate at normal ambient CO_2 concentration was significantly lower in transformed than in wild type plants, and this difference was also detectable at saturating levels of CO_2 (1,200 ppm), suggesting differences in the mesophyll conductance and/or metabolism between transgenic and wild type plants.

During drought treatment, the *A* values measured at saturating CO_2 level remained virtually unchanged in the T1 and T2 lines up to day 6, indicating the relative importance of stomatal factors in the limitation of *A* in the transformed plants. When non-stomatal limitation occurs, the CO_2 assimilation does not reach the maximal rate measured at normal water conditions (Lawlor 2002). In wild type plants, the maximal CO_2 fixation capacity decreased intensively and continuously. These results indicate that biochemical changes rapidly occur after withholding the water supply in the leaves of wild type plants due to the fast water loss. In contrast, the non-stomatal limitation is not significant in the T1 and T2 plants in this period of drought stress as a consequence of the practically unchanged water content.

Evidence suggests that one of the metabolic factors affecting CO_2 assimilation during water stress is the inhibition of photochemical and electron transport processes (Keck and Boyer 1974; Giardi et al. 1996). However, in the present study, the optimal quantum yield (F_v/F_m) was completely unaffected by the withholding of the water supply for up to 12 days of the drought treatment in the wild type, and 16 days in transgenic lines. Consequently, a considerable decrease in F_v/F_m was noticeable only at a very severe RWC decrease in all examined lines. Therefore, our results suggest that moderate drought has a marginal effect on the capacity of the primary charge separation as has also been reported in other studies (Ben et al. 1987; Grieu et al. 1995). Nevertheless, the photosynthetic electron transport processes were not significantly down-regulated either in wild type or in T1 and T2 plants at moderate or medium water deficit as reflected in the changes of effective quantum yield of PS II photochemistry. Consequently, we expect the presumed differences in structural and functional damage of PS II between wild and transgenic lines to originate only from the altered velocity of water loss.

In conclusion, our results suggest that the two independent *TPS1*-expressing transgenic potato lines, which efficiently keep water during drought treatment, are able to maintain an acceptable level of activity of their photosynthetic processes for a longer time than wild type. Under optimal growth conditions, the CO_2 fixation rate of transgenic plants is lower than that of the non-transformed control plants. Stomatal densities of T1 and T2 plants are also reduced, and this may contribute to the lower CO_2 fixation rate and the altered drought response.

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