Low temperature tolerance of tobacco plants transformed to accumulate proline, fructans, or glycine betaine. Variable chlorophyll fluorescence evidence

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Abstract

Tobacco (*Nicotiana tabacum* L.) has been transformed to accumulate different compatible solutes (proline, fructans, or glycine betaine) in order to improve its tolerance to abiotic stress. Photosynthetic activity of wild type (wt) and transformed tobacco plants before and after freezing stress was studied by measuring chlorophyll (Chl) fluorescence. The JIP test of Chl fluorescence induction was used to analyze in details the functional activity of photosystem 2. No significant differences were found among wild type and transgenic plants after 12 h of freezing. Both plant types maintained the same values of the measured parameters [F_V/F_M, PI(CS_M), ABS/RC, TR_0/RC, ET/RC] after recovery of stress. The studied Chl fluorescence parameters decreased only for the wild type plants, stressed for 24 h at –2 ºC. The strong inhibition of photosynthetic reactions in the wt plant after 24 h of freezing could not be restored. The evaluated parameters of transgenic plants did not change significantly after 24 h at –2 ºC and successfully survived freezing stress.

Additional key words: freezing stress; JIP test; *Nicotiana tabacum*; transgenic plants.

Introduction

During their development plants are subjected to various environmental stresses (Lichtentaler 1996). Drought, salinity, or low and high temperatures determine to a great extent the geographical distribution and growth of crop plants. All these stresses, generally accepted to be osmotic by nature, sharply decrease agricultural production worldwide (Levitt 1980, Boyer 1982). In addition to the changes at structural, molecular, and biochemical levels, plants accumulate various low mass organic metabolites. These are polyhydrated sugar alcohols, amino acids and their derivatives, or tertiary sulphonium and quaternary ammonium compounds that allow plants to withstand the unfavourable conditions (Rhodes and Hanson 1993, Kishitany *et al*. 1994, Bohnert and Jernsen 1996, Rajasheker 2000). These compatible solutes serve as organic osmolytes, increasing the ability of cells to retain water but not disturbing normal cellular functions (Yancey *et al.* 1982). Other mechanisms by which these substances might increase stress tolerance are far from being completely understood. They may serve also as scavengers of hydroxyl radicals or their protective function might be due to maintaining the hydration sphere of proteins under dehydration (Sheveleva *et al*. 1997).

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Abbreviations: ABS/RC – the flux of photons absorbed by the antenna chlorophylls (ABS) per reaction centre (RC); Chl – chlorophyll; ET_0/RC – the initial rate of the reopening of RCs by re-oxidation of Q_A^- (ET_0) per RC; F_0 – initial Chl fluorescence; F_M – maximum Chl fluorescence; F_V – variable Chl fluorescence; Fru – fructans; GB – glycine betaine; PI(CS_M) – performance index defined on cross section (CS) basis at the moment of maximal fluorescence intensity; M_0 – normalised value of the initial slope of Chl fluorescence; Pro – proline; PS – photosystem; Q_A – the primary quinone electron acceptor of PS2; RC – reaction centre; TR_0/RC – the initial rate of conversion of the excitation energy to redox energy by reducing Q_A to Q_A^- (TR_0) per RC; V_J – the relative variable Chl fluorescence at the J point (F_J) of the induction transient $(F_J - F_0)/(F_M - F_0)$.
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Classical breeding for manipulation of osmoprotectant accumulation in plants could be a very difficult and time-consuming process. Recently genetic engineering of different crops gained significant importance and was promising for agriculture. Transgenic plants with increased contents of compatible solutes show a strong potential for improving their stress tolerance (Bajaj *et al*. 1999). As far as tobacco does not naturally accumulate fructans (Fru) and glycine betaine (GB), genetic manipulation of this economically important crop to synthesize these osmolytes could be of particular interest.

Low temperature stress is often studied in a combination with high photon flux density. Both these factors lead to photoinhibition, as the larger portion of the absorbed photon energy becomes excessive, due to the reduced photosynthetic capacity at low temperature, accompanied with a degradation of the D1 protein of the photosystem 2 (PS2) reaction centre (RC) (Huner *et al*. 1993, Krause 1994).

In vivo chlorophyll (Chl) fluorescence measurements became a valuable tool to evaluate plant viability and to document the effects of freezing stress on the photosynthetic apparatus (Klosson and Krause 1981, Strand and Öquist 1988). The ratio of variable to maximal Chl fluorescence (F_V/F_M) is often used as a reliable quantitative indicator of the potential quantum yield of photochemical capacity of PS2 and for assessment of stress damage in plants in a non-destructive manner (Björkman and Demmig 1987). Recent advances in Chl fluorescence measuring technique allowed the development of the JIP-test as a tool for rapid screening of many samples *in vivo*, providing adequate and detailed information about the structure, conformation, and function of the photosynthetic apparatus (Strasser and Strasser 1995).

In previous experiments we have transformed commercial oriental type tobacco to accumulate proline (Pro), Fru, or GB (Konstantinova *et al*. 2002). Stable transgenic lines have been selected using response to sub-zero temperatures and osmotic stress for screening. Several subsequent progenies survived at controlled and field conditions.

Our aim in this study was to examine the functioning of PS2 of these transgenic tobacco lines expressing genes for accumulation of the above mentioned osmoprotectants when subjected to sub-zero temperature.

Materials and methods

Plant material: We used oriental type tobacco plants (*Nicotiana tabacum* cv. Nevrokop 1146) transformed to accumulate Pro, Fru, or GB (Konstantinova *et al*. 2002). Overproduction of Pro was achieved by transformation with genes from two higher plants, *Arabidopsis thaliana* (AtP5Cs) (Yoshiba *et al.* 1995) or *Vigna acconitifolia* (VacP5Cs) (Hu *et al.* 1992). The gene for synthesis of Fru (SacB) was isolated from *Bacillus subtilis* (Steinmetz *et al.* 1985). The gene for GB accumulation (codA) was isoated from *Arthrobacter globiformis* (Deshnium *et al*. 1995). All genes were introduced separately into the tobacco genome and effectively expressed (Konstantinova *et al*. 2002). Several T1 lines were obtained that showed 75 % resistance to antibiotics (100 g m^{-3} Km and 7 g m^{-3} Hyg). The presence of the introduced genes was confirmed by PCR.

Among the T1 lines selected, we chose one per every respective osmoprotectant: AtPro26 (with P5Cs gene from *Arabidopsis thalina* for Pro accumulation), VacPro29 (with P5Cs gene from *Vigna aconitifolia* for Pro accumulation), Fru52 (with SacB gene for Fru accumulation), and GB9 (with codA gene for GB accumulation). Untransformed plants were designated as wild-type (wt).

In vitro cloned transformed and wild type plants were grown on a MS basic medium (Murashige and Skoog 1963). After 30 d they were potted and grown at 22 ºC under 16 h of light at 30 μmol m^{-2} s^{-1} for 14 d. Potted plants at a stage of 4–5 leaves were used for low temperature treatments.

Freezing procedure: Stress conditions were applied as described in Konstantinova *et al.* (2002). Before subjected to –2ºC the pot plants were acclimated for 5 d at 2 °C (16 h at 5 μmol m^{-2} s^{-1} and 8 h dark). The low irradiance was used to prevent photoinhibition. The plants were then moved in a growth chamber and kept for 12 or 24 h at –2 ºC under continuous irradiation (5 μmol m^{-2} s^{-1}). After the respective period of freezing, wt plants and transformants were allowed to recover at room temperature for 24 h. Light/dark conditions were 16 h at 30 μmol m^{-2} s^{-1} and 8 h dark. Measurements were performed on whole leaves before acclimation, before transfer to –2 ºC, after 12 or 24 h at –2 ºC, and after 24 h of recovery at 22 ºC. At every step, control measurements were taken on plants of the same age grown at room temperature.

Chl fluorescence parameters: Chl fluorescence induction curves were registered by using a Handy PEA fluorimeter (*Hansatech Instruments*, UK) at room temperature, the analyzed leaf area being dark-adapted for 3 min. Induction kinetic curves were recorded for a period of 1 s at actinic irradiance of 3 000 μmol m^{-2} s^{-1}. Data obtained were processed using the *Biolyzer 2.5* software (Maldonado-Rodriguez 2002). Each experimental point is a mean of 6–8 repetitions on 2 different plants (n = 12–15).

Photosynthetic activity was monitored by Chl fluorescence parameters provided by the JIP test (Fig. 1) (Strasser and Strasser 1995, Strasser *et al*. 2000). The following key parameters were calculated and analyzed in parallel: F_V/F_M is the maximal quantum yield of PS2

photochemistry (Kitajama and Butler 1975). F_0 is the normalized value of the initial slope of Chl fluorescence and indicates the net rate of closure of the RCs. Reducing Q_A to Q_A^- increases the number of closed RCs and electron transport decreases it: $M_0 = TR_0/RC - ET_0/RC$. The performance index $I(CS_M)$ is defined on cross section (CS) basis at the moment of maximal level of Chl fluorescence intensity. This index combines structural and functional criteria of PS2. It represents the effectiveness (or probabilities) of photon energy transformation reactions in PS2 that occur simultaneously. In terms of experimental procedure PI is defined as:

$$PI_{CS_M} = F_M \cdot \frac{1-(F_0/F_M)}{M_0/V_J} \cdot \frac{F_M - F_0}{F_0} \cdot \frac{1-V_J}{V_J}$$

ABS/RC refers to the flux of photons absorbed by the antenna Chls per RC. It is calculated as $ABS/RC = (M_0/V_J)/(F_V/F_M)$. ABS/RC depends on the amount of light-harvesting Chls and the efficiency of excitation energy migration toward the PS2 RC, *i.e.* the volume and the integrity of the PS2 antennae. Stress-induced damage or migration of some PS2 antenna complexes to photosystem 1 is reflected in change of this parameter.

TR_0/RC is the initial rate of conversion of the excitation energy to redox energy by reducing Q_A to Q_A^-. From experimental data (Fig. 1) TR_0/RC is calculated as: $TR_0/RC = M_0/V_J$. This parameter expresses the initial rate of the closure of RCs as a fractional expression over the total number of RCs that can be closed. Under stress some RCs are inactivated in the sense of being transformed to quenching sinks (Krause and Weis 1991) without reducing Q_A to Q_A^-. In such a case, TR_0/RC still refers only to the active (Q_A to Q_A^- reducing) centres. The same is valid for the other two fluxes (ABS/RC and ET_0/RC), since their derivation is based on TR_0/RC (Strasser *et al.* 2000).

ET_0/RC expresses the initial rate of the reopening of RCs by re-oxidation of Q_A^-: $ET_0/RC = (M_0/V_J)(1 - V_J)$. ET_0/RC is a measure of the rate of electron transport after Q_A. A change in this parameter could indicate stress-induced conformational changes in the Q_B-binding site of the D1 protein.

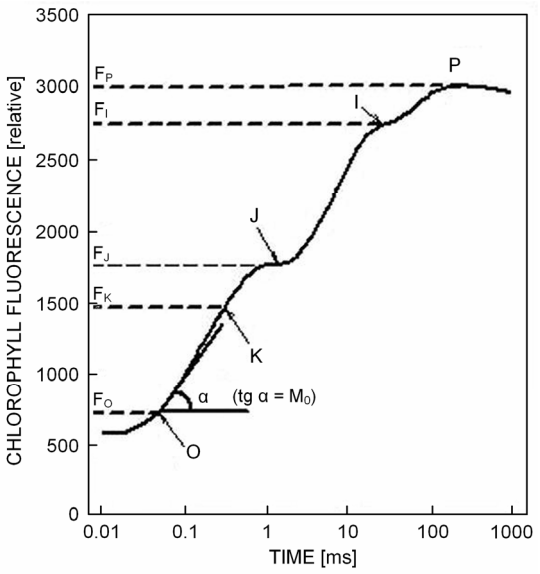

Fig. 1. Chlorophyll (Chl) fluorescence transients exhibited upon irradiation of leaves from tobacco wild type plants after 3-min dark adaptation. The transients are presented in log time scale. Letters O, J, I, and P show the characteristic points (Papageorgiou 1975, Strasser *et al.* 1995). F_0, F_K, F_J, F_I, and F_P indicate Chl fluorescence values at the corresponding points. M_0 characterizes the initial slope of photoinduced Chl fluorescence increase. F_0 is measured 50 μs after beginning of irradiation (F_{50}), $F_K = F_{300}$, $F_J = F_{2\ ms}$. M_0 is calculated as the ratio $(F_{150} - F_{50})/0.1$ ms. F_P is determined as the maximum of the induction transient. At the used actinic irradiance (3 000 μmol m^{-2} s^{-1}) F_P is approximately equal to maximal Chl fluorescence intensity F_M ($F_P \approx F_M$).

Results

Acclimation of wt and transgenic tobacco plants for 5 d at low positive temperature (2 ºC) did not lead to any significant changes in their morphology. When transferred to –2 ºC for 12 h no visible injuries were found and plants were able to recover very fast. When the freezing stress period was extended to 24 h, the wt plants were severely damaged (the leaves became fragile and water soaked). At this temperature the soil in the pots was completely frozen. During the recovery period, as the leaves thawed, they remained wilted and water soaked. The transgenic plants were green, without visible injuries and showed 100 % survival (Fig. 2). They recovered at normal temperatures without damages.

The effect of freezing on the PS2 activity of mature leaves was examined by monitoring the Chl *a* fluorescence. The F_v/F_M values were measured on acclimated tobacco plants (0 h) and after 12 or 24 h at –2 °C (Table 1). This ratio is widely used for determining the physiological state of PS2 and for all groups of plants it had similar values. The only significant decline (17 %) was found for the wt plants, stressed for 24 h. As the F_v/F_M ratio characterizes only the PS2 primary photochemical activity, we used the JIP-test for further analyzing the effect of freezing. This test links the shape of the Chl fluorescence induction curves with energy fluxes through PS2.

Minor alterations occurred in the photosynthetic parameters, evaluated by the JIP-test, *i.e.* $PI(CS_M)$, ABS/RC, TR_o/RC, and ET_0/RC, of wt and transformed tobacco plants at acclimation (5 d at 2 ºC) (Fig. 3). The values of $PI(CS_M)$ for wt and the AtPro26 transformant insignificantly decreased in comparison with their respective control grown at 22 ºC. For all other transformants the values remained unchanged before and after acclimation (Fig. 3*A*). As for the rest of the studied parameters, no

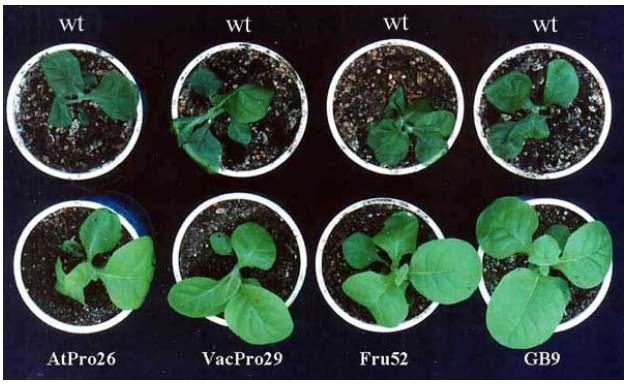

Fig. 2. Freezing stress for 24 h of wild type (wt) and transgenic plants (AtPro26, VacPro29, Fru52, and GB9).

Table 1. F_v/F_M ratio of wild type (wt) and transgenic tobacco plants after 12 and 24 h at –2 °C; 0 h – plants acclimated at 2 ºC for 5 d.

Time [h]	Genotypes				
	wt	AtPro26	VacPro29	GB9	Fru52
0	0.75	0.75	0.75	0.76	0.76
12	0.76	0.75	0.75	0.76	0.76
24	0.62	0.74	0.73	0.75	0.75

significant changes were observed between the wt and transformants acclimated for 5 d at 2 ºC in comparison with the respective controls (Fig. 3*B,C*). The values for the electron transport (Fig. 3*D*) increased after acclimation, especially for VacPro29 and for Fru52.

After 5 d of acclimation at low positive temperature (2 ºC), the plants were grown for 12 or 24 h at –2 ºC. After the respective period of stress the photosynthetic parameters were evaluated by the JIP-test (Fig. 4). The values of $PI(CS_M)$ after 12 h of freezing were practically the same as of the non stressed (control) plants (Fig. 4*A*). Only the Fru-accumulating transformant (Fru52) showed a slight decrease. When the period of freezing was extended to 24 h, the wt plants showed 4-fold decrease of $PI(CS_M)$ in comparison with the control ones. A very slight decrease was observed also for plants transformed with the gene for Pro over-accumulation from *Vigna* (VacPro29). For the rest of the transformants (AtPro26, Fru52, GB9) no alterations were found. For the wt plants grown at -2^0C for 24 h, the parameter ABS/RC (Fig. 4*B*) decreased by 20 %. No significant changes were observed for the transgenic and wt plants at –2 °C for 12 h. The Pro over-producers had higher values of this parameter after 24 h at –2 ºC. The course of alteration of the photosynthetic parameters, characterizing the functional activity of the RCs of PS2 (TR_0/RC and ET_0/RC), followed the same tendency as for ABS/RC (Fig. 4*C,D*). However, the decline for the wt plants after 24 h of freezing was more pronounced, *i.e.* 34 % for TR_0/RC and 47 % for ET_0/RC in comparison to the non-treated plants. An increase of the two parameters after 24 h at –2 ºC was observed for AtPro26. No significant changes were found for the other transformed plants, *i.e.* VacPro29, Fru52, and GB9 (Fig. 4*C,D*).

After the respective freezing period (12 or 24 h), the plants were allowed to recover for 24 h at room temperature. Wt plants did not survive after 24 h at –2 ºC. Therefore the values of recovery after 24 h are not presented. The performance index $PI(CS_M)$ at recovery showed activation of the photosynthetic reactions (Fig. 5). Significant increase of $PI(CS_M)$ was observed in wt and all

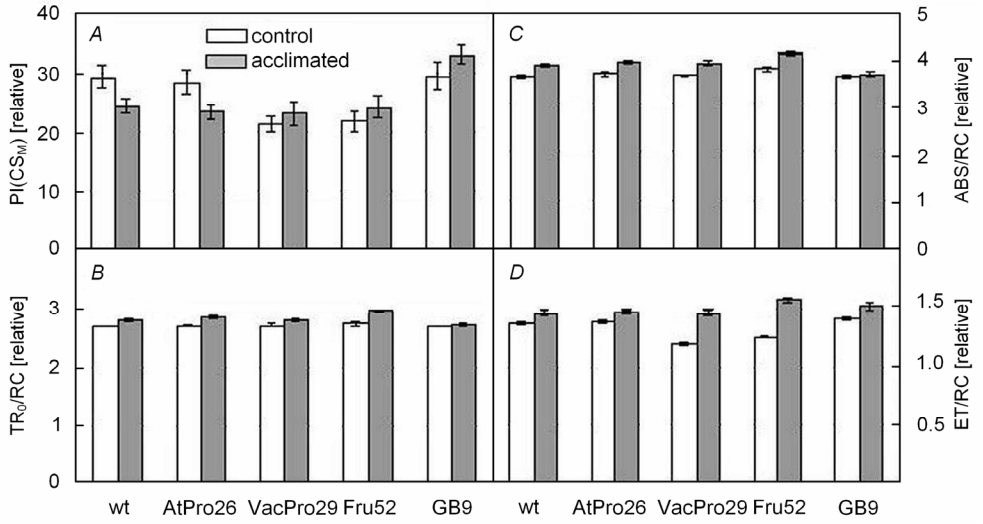

Fig. 3. Photosynthetic parameters evaluated by the JIP-test of wt and transgenic tobacco plants (AtPro26, VacPro29, Fru52, and GB9) after 5-d acclimation at 2 ºC. Control plants of the same age as the acclimated ones were grown at 22 ºC. $PI(CS_M)$ – performance index on cross section basis at the moment of maximal fluorescence intensity, ABS/RC – the flux of photons absorbed by the antenna chlorophylls per RC, TR_0/RC – the initial rate of conversion of the excitation energy to redox energy by reducing Q_A to Q_A^- per RC, ET_0/RC – the initial rate of the reopening of RCs by re-oxidation of Q_A^- per RC.

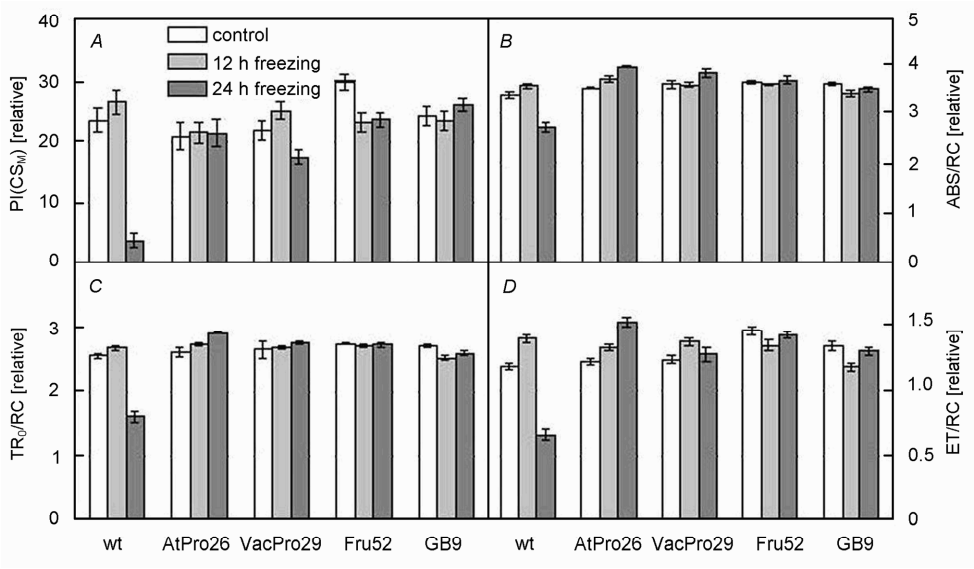

Fig. 4. Photosynthetic parameters evaluated by the JIP-test after freezing for 12 and 24 h. Plants, wt and transformants, were transferred to –2 ºC after 5 d of acclimation at 2 ºC. Control plants of the same age as the acclimated ones were grown at 22 ºC. JIP-test parameters are as in Fig. 3.

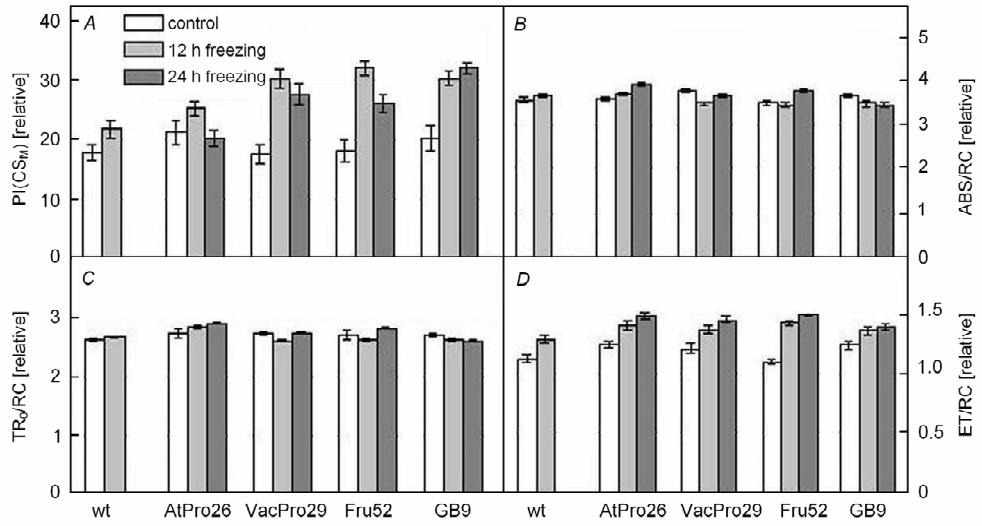

Fig. 5. Photosynthetic parameters evaluated by the JIP-test of wt and transgenic tobacco plants recovering for 24 h at 22 ºC after 12 and 24 h of freezing (-2 ºC). Control plants of the same age as the acclimated ones were grown at 22 ºC. JIP-test parameters are as in Fig. 3.

transformed plants after 12 h of freezing, especially for VacPro29, Fru52, and GB9. The tendency was the same for the transgenic plants after 24 h at –2 ºC. Only in AtPro26 the PI(CS$_M$) was equal to that of the control. The values of ABS/RC and TR$_0$/RC did not change significantly after the recovery. The last parameter, ET$_0$/RC, showed a gradually accelerating electron transport (Fig. 5*D*).

Discussion

Recently much attention is paid to genetic modification for increased accumulation of various osmolytes (Konstantinova *et al.* 2002). Understanding the regulatory mechanisms by which osmoprotectants increase freezing tolerance might be essential for development of crops, tolerant to various environmental stress conditions.

Freezing for 12 h did not lead to any significant alterations of wt tobacco plant morphology while a period of 24 h caused lethal changes. Plants died very soon after return to normal (22 ºC) temperature. In contrast, all our

transformants accumulating Pro, Fru, or GB survived successfully the freezing stress.

Because the multi-protein complex of PS2 is the most stress-sensitive part of the photosynthetic apparatus, we used Chl fluorescence for detailed screening of freezing-caused alterations. The most commonly used Chl fluorescence ratio F_V/F_M depends strongly on the plant species and type of stress. We observed changes only in the wt plants frozen for 24 h. In order to evaluate the exact site of freezing damage on PS2 *in vivo* we analyzed the more precise JIP parameters. The most sensitive part of the electron transport chain to high and freezing temperatures is the donor side of PS2, the oxygen-evolving complex (Popova 1993, Enami *et al.* 1994). In the Chl fluorescence induction curves, the appearance of a K-peak is attributed to inactivation of the oxygen-splitting complex (Srivastava and Strasser 1995, Srivastava *et al.* 1995). No K-peak was observed in the induction curves during our experiments (values not shown) indicating that under these experimental conditions the donor side of PS2 remained intact.

PI(CSM) gives complex information about absorption and transfer of excitation energy toward the reaction centres (ABS/RC), photochemical charge separation (TR_0/RC), and intersystem electron transport (ET_0/RC). The acclimation of the non-transformants led to a decrease of PI(CSM) that became drastic after 24 h of freezing. Under acclimation as well as after the 12 h period at –2 °C and subsequent recovery, the degree of absorbed photon energy and the process of charge separation did not show significant alterations. Only after 24 h of freezing a decline in two parameters (ABS/RC and ET_0/RC) was observed, but to a lesser extent than in the case of PI(CSM).

For the transgenic plants, which effectively survived the sub-zero stress, the parameters ABS/RC and TR_0/RC were stable. As the ratio ABS/RC is determined by the amount of light-harvesting complexes and the efficiency of excitation energy migration to the PS2 RC was not changed after freezing, we conclude that in our case the size and integrity of the PS2 antennae were not altered. The same was true for the ratio TR_0/RC, hence the RCs were not inactivated during freezing. Only the degree of electron transport at the acceptor side of PS2 (after Q_A) showed statistically significant changes, *i.e.* conformational changes in the Q_B-binding site of the D1 protein. It could be speculated that the alterations in PI might be due to changes in the rate of the photosynthetic electron transport at the acceptor side of PS2, most probably the D1 protein of the RC.

The recovery at room temperature slightly activated the photosynthetic reactions in transgenic plants stressed for 24 h at –2 °C, which is evident from the performance index PI(CSM). Comparing the parameters of recovered transgenic plants with that of non-treated ones one could assume that the increase of PI(CSM) is primarily due to accelerated electron transport (ET_0/RC) and to a lesser extent due to changes in the antenna complexes (ABS/RC).

It is still not clear how the accumulation of osmolytes helps the plant cell to survive freezing stress. Increased accumulation of compounds like Pro or GB is considered to contribute for "osmotic adjustment" which results in increased water retention (Yancey *et al.* 1982). The "compatible solutes" are believed also to associate with membrane lipids and/or proteins thus preventing dissociation of protein complexes (Bohnert and Jernsen 1996). Pro, Fru, and GB tend to be localized at the head group region of the membrane (Goughlan and Heber 1982, Rudolph *et al.* 1989, Ozaki and Hayashi 1996, Hincha *et al.* 2000, Popova and Busheva 2001). In our case, the gene transfer led to Pro and Fru accumulation outside the chloroplasts while the GB synthesis was targeted to the chloroplasts (Konstantinova *et al.* 2002). Despite these differences in localisation, the three osmolytes have probably a similar protective effect. Residing in the surface exposed regions of the cell membranes they stabilize indirectly the photosynthetic complex and consequently the photosynthetic effectiveness.

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