Journal of Plant Physiology **162** (2005) 650–661

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JOURNAL OF PLANT PHYSIOLOGY

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Chlorophyll accumulation is enhanced by osmotic stress in graminaceous chlorophyllic cells

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Received 12 July 2004; accepted 8 September 2004

KEYWORDS
Water stress;
Osmotic stress;
Chlorophyll;
Chloroplast size;
Chloroplast number;
Photoautotrophic cells

Summary

We have developed a new chlorophyllic cell line ('TADH-XO') from the highly water stress tolerant grass *Bouteloua gracilis* (blue grama). When grown under normal (non-stress) conditions, this new cell line accumulates higher levels of chlorophyll (up to 368.1 μg total chlorophyll g^{-1} FW) than a previously obtained cell line ('TIANSJ98'). Both cell lines are capable of developing substantially higher amounts of chlorophyll when subjected to osmotic stress. In order to explain these changes in the chlorophyll kinetics of the chlorophyllic cells, we analyzed the following population variables in cells subjected to polyethylene glycol 8000-induced osmotic stress: growth, viability, chlorophyll (total, 'a' and 'b'), cell size, percentage of green cells and chloroplast (number and size). Although previous studies in some chlorophyllic cells of dicots have already reported that chlorophyll increases under saline stress, in this report we show that, at least in this graminaceous cell line, the increase in chlorophyll is an immediate and proportional response to the osmotic stress applied and not the result of a progressive adaptation process. Consistent with previous studies, the increase in chlorophyll accumulation could be the result of chloroplast development (increased thylakoid number per chloroplast). On the basis of our results, the increases in chlorophyll accumulation previously observed in salt-adapted dicot cells may be the result of the osmotic shock (water deficit), rather than the ionic effect of salt on the physiology of chlorophyllic cells of

Abbreviations: DW, dry weight; FW, fresh weight; HSP, heat shock protein; PEG, polyethylene glycol

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0176-1617/$ - see front matter
doi:10.1016/j.jplph.2004.09.015

dicots. Under the cell population experimental approach we followed, our study provides important insights related to the physiological behavior of chlorophyllic cells subjected to osmotic stress.

Introduction

Drought stress is the main constraint for crop production in the world (Boyer, 1982). Intensive research is currently being conducted on basic and applied issues, from molecular to ecological approaches, linked to the effects of water deficit in plants in light of critical global scenarios related to water availability for human consumption and crop production anticipated to arise in the near future.

Since the development of the first chlorophyllous (photoautotrophic) cell cultures in *Nicotiana tabacum* by Bergmann (1967) and successive cell systems obtained later (Widholm, 1992; Aguado-Santacruz et al., 2001), the potential of chlorophyllic (photoautotrophic) cell cultures as models for analyzing the effects of water stress on photosynthetic metabolism has been recognized (Widholm, 1992). On the basis of some specific traits, such as chlorophyll content and photosynthetic rate, the physiology of these cells has been equated to that of dividing leaf cells (Rogers et al., 1987).

Water stress tolerance mechanisms can be visualized as a function of the plant organization level under analysis: cell (osmotic adjustment), tissue (water storage tissues), organ (deep or fast growing roots) and whole plant (water conductance). Although this situation poses difficulties for transferring basic knowledge to applied context, some successful cases of manipulating cellular mechanisms for increasing water stress tolerance have been reported, in which transgenic plants were tested under greenhouse or laboratory conditions (Bohnert and Shen, 1999; Garg et al., 2002). If we are to properly manipulate the stress tolerance of plants, it is necessary to increase our knowledge of plant responses at each level of organization, analyzing, for example, the effect of water stress on the photosynthetic behavior of plants and the contribution of stomatal vs. metabolic effects to the reduction in photosynthesis rate and, consequently, in crop productivity (Chang et al., 1997).

An intriguing phenomenon observed in chlorophyllous systems is that cells respond to saline stress developing more chlorophyll when grown under both heterotrophic (Winicov and Button, 1991; Locy et al., 1996) and photoautotrophic conditions (Chang et al., 1997). This correlates, in turn, with increases in photosynthesis-related mRNAs and protein levels (nuclear and chloroplast encoded), such as Rubisco, Cyt b_{559}, psbD, psaB, atpB, pCab4, pCab1, among others (Winicov and Seemann, 1990; Winicov and Button, 1991; Locy et al., 1996), greater Rubisco enzymatic activity and higher photosynthetic rates (Dalton and Street, 1977; Locy et al., 1996). Although both saline and osmotic stress share the characteristic of creating a water potential differential between the cell and its environment, which is finally conducive to a water deficit in the cell, the latter avoids the toxic effect of salt (Zhang et al., 2000), showing solely the effect of water deficit within the cell. Indeed, particular and specific responses are expected when cells are subjected to saline vs. water stress (Critchley, 1982; Iraki et al., 1989; Sabbah and Tal, 1990; Leonardi et al., 1995), with salt stress being more detrimental than osmotic stress (Sabbah and Tal, 1990) to cell growth because of the additional ionic effect of the sodium chloride. Salt tolerant cell lines can be derived from osmotically adapted cells (Bressan et al., 1981; Harms and Oertli, 1985), demonstrating that part of the mechanisms associated with salt tolerance can be attributed to processes allowing cell growth under osmotic stress (Sabbah and Tal, 1990).

Explanation of an increased chlorophyll content in cells subjected to saline or saline stress is not easy, because plants experiencing severe saline or water stress in their native environments do not become greener (Bokhari 1976a, b; Kingsbury and Epstein, 1986; Antolín and Sánchez-Díaz, 1993; Streb and Feierabend, 1996). The growth of crop plants, however, can be enhanced by low levels of NaCl (Winicov and Button, 1991), and some cereals experiencing severe drought conditions retain their green appearance (Rosenow et al., 1983; Thomas and Smart, 1993). In addition, the chloroplasts of some halophytes require high chloride concentrations for photosynthetic electron transport around PSII (Critchley, 1982).

Therefore, the question of what specific events or processes favor the increase in chlorophyll development remains to be properly addressed (Winicov and Button, 1991).

We obtained the first highly chlorophyllous cell line in Poaceae from the grass *Bouteloua gracilis*

(blue grama). Blue grama is one of the most drought and grazing tolerant grasses of North America (Lauenroth et al., 1994; Aguiar and Lauenroth, 2001), and there is extensive ecological and physiological information available for supporting molecular studies in this species. Our chlorophyllic system could help revealing the cellular mechanisms underlying the high drought tolerance of *B. gracilis*, while analyzing the effects of water stress on the photosynthesis and related biochemical processes of Poaceae at the cellular level.

In this work, we study the response of chlorophyllous cells of *B. gracilis* to osmotic stress, demonstrate that gramineaceous chlorophyllous cells proportionally increase their chlorophyll content when exposed to different concentrations of PEG 8000, and analyze some of the events that we predicted could contribute to increased chlorophyll production under osmotic stress. Detailed kinetics of chlorophyll development in the blue grama cell populations grown under osmotic stress is also described.

Materials and methods

Shoot apices-derived green calli were generated and cultured in MPC medium as described previously (Aguado-Santacruz et al., 2001). Following this protocol, we obtained a new green cell line, named 'TADH-XO', which was subcultured every 20 days by transferring 1 mL of the cell suspension into 24 mL of fresh liquid MPC medium. The response of this cell line to osmotic stress was evaluated by growing the chlorophyllic cells in liquid MPC medium containing 0%, 10% or 20% PEG 8000 (Amersham, Cleveland, OH, USA). From these initial experiments, it was evident that the 'TADH-XO' chlorophyllic line responded to osmotic stress, as did the previous cell line 'TIANSJ98', increasing its chlorophyll content as osmotic stress increased. However, it was also noted that this reaction was more consistent when an osmotic treatment of low strength and short duration (2.5% PEG; 10–20 days) was applied to the cells prior to testing treatments of lower osmotic potentials. Accordingly, 'TADH-XO' cells were grown first in MPC medium containing 2.5% PEG for 20 days (2 subcultures of 10 days each) and then cultured into 0%, 7%, 14%, 21% and 28% PEG batch cultures. One hundred and twenty-five flasks (125 mL capacity; 25 flasks per treatment) were filled with 24 mL of the respective osmotic medium, inoculated with 1 mL of the cell suspension (0.5 g) and then agitated at 95 rpm on a gyratory shaker under continuous fluorescent light (60 μmol s^{-1} m^{-2}; 30 $\pm$ 1 °C). After inoculation, a Wescor model HR 33 T microvoltmeter with model C52 thermocouple psychrometer chambers (Wescor, Inc., Logan, Utah, USA) was used to measure the osmotic potential of the utilized culture media. The quantified osmotic potentials were as follows: 0% PEG, $\Psi\pi = -5.1$ bar; 2.5%, $\Psi\pi = -5.25$ bar; 7% PEG, $\Psi\pi = -6.2$ bar; 14% PEG, $\Psi\pi = -7.8$ bar; 21% PEG, $\Psi\pi = -14.4$ bar; 28% PEG, $\Psi\pi = -20.0$ bar. Fresh weight, viability, cell size, percentage of green cells and chloroplast size and number were determined every 3 days (five flasks per treatment) during a 15-day period. Chlorophyll was extracted from cells with aqueous acetone (80% v/v) and determined according to Arnon (1949). For determining the viability, cell size, percentage of green cells and chloroplast size and number, the cells were stained with basic fucsin 1% and 50 microscope fields were analyzed per sample. Using Evans blue dye for the viability analysis generated similar results to basic fucsin 1%, because cells appearing completely stained had lost their membrane integrity. Chloroplast and cell size were determined using the formula for resolving the area of an ellipse.

Statistical analysis

A completely randomized experimental design was used for analyzing differences among treatments and among sampling dates in the cellular variables. When significant *F*-values were found, Duncan's multiple range test was utilized to separate significant ($P < 0.01$) mean differences (Steel and Torrie, 1960).

Results

A new, highly chlorophyllous cell line was obtained from culturing shoot apices-derived green calli in liquid MPC medium as described previously (Aguado-Santacruz et al., 2001). As with the earlier obtained cell line ('TIANSJ8'), this new green suspension, named 'TADH-XO', proportionally increased its chlorophyll concentration when exposed to different concentrations of polyethylene glycol 8000 (PEG 8000); other osmotica, such as sucrose (6–12%) or mannitol (0.5–1 M), were also tested with similar results. In comparison, 'TADH-XO' was able to accumulate much higher concentrations of total chlorophyll (up to 368.1 μg g^{-1} FW cells) than the 'TIANSJ98' cell line (maximum = 121.6 μg g^{-1} FW cells; Aguado-Santacruz et al., 2001) under normal non-osmotic conditions.

In addition, as indicated by its doubling time (calculated on a fresh weight basis) of 1.3 days, this new cell line displayed a faster growth than the predecessor line 'TIANSJ98' (doubling time = 7.2 days).

In the course of the experiment two different cell responses within cell growth (Fig. 1) and chlorophyll kinetics (Fig. 2) were evident; one of them derived from the sucrose availability and consumption, the other from the effect of the osmotic (water) stress itself on chlorophyllic cells physiology.

First, the harmful effect of the osmotic stress induced by polyethylene glycol 8000 on the physiological performance of the chlorophyllic cells was evidenced by a proportional reduction of fresh weight gain as a function of the osmotic tensions applied (Fig. 1). Relative to cells grown in 7% and 14% PEG, an initial decrease in the fresh weight of control cells was observed at day 6, which could be the result of the pre-conditioning treatment in 2.5% PEG applied to the cells before the initiation of the experiment. However, after this first decline, the control cells recovered to attain the highest fresh weight at the end of the cell kinetics (Fig. 1). The osmotic stress level limitative for sustained growth of the chlorophyllic cells was around 28% PEG, because the weight of the initial inoculum was maintained throughout the experiment in this treatment (Fig. 1) with low death cell percentages (close to 11% by the end of the cell growth; Table 1).

As a result of sucrose availability in the medium, an initial decrease in chlorophyll accumulation ('a', 'b' and total) of the 'TADH-XO' cell line was observed independently of the osmotic tension of the growing media (Fig. 2). This initial reduction in the chlorophyll content bears a strong relationship to carbohydrate availability; sucrose (among other carbohydrates) has been shown to inhibit the chlorophyll accumulation both in chlorophyllic cell systems as in intact plants (Edelman and Hanson, 1971; Widholm, 1992; Pego et al., 2000).

Following this initial reduction, carbohydrate consumption by the cells subsequently permitted a maximum accumulation of chlorophyll ('a', 'b' and total) by day 6 (Fig. 2) independently of the osmotic stress applied and the growth kinetics developed (Fig. 1).

It is evident from Fig. 2 that osmotic stress stimulated the chlorophyll production ('a', 'b' and total) by the 'TADH-XO' cells, and that, for below the highest PEG concentration tested (28% PEG), this increase was proportional to the strength of the osmotic stress applied. Furthermore, it has been found that the chlorophyll content of most of the obtained chlorophyllic cell systems up to now is much lower than that of the whole plant counterparts (Widholm, 1992), but in our system, osmotic stress stimulated the cells to reach the chlorophyll

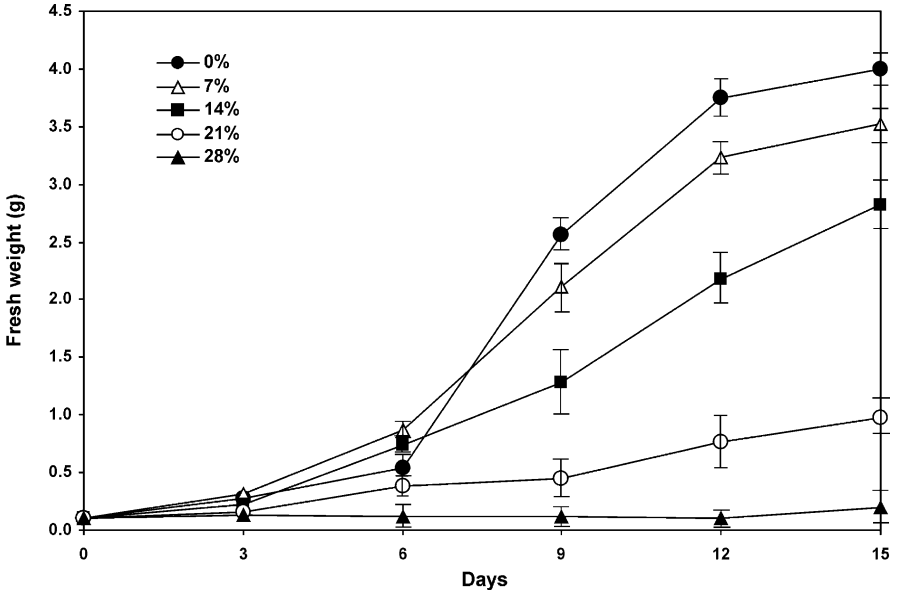

Figure 1. Effect of different osmotic stress strengths indiced by PEG 8000 on fresh weight gaining of 'TADH-XO' chlorophyllic cells of *Bouteloua gracilis* grown in batch cultures. Cells pre-adapted to grow in low osmotic strength medium (2.5% PEG) for 20 days were inoculated into 25 mL of the respective medium and harvested on the days indicated. Values are the mean of 10 replicates from two independent experiments. Bars represent ±SE. These samples were used for all analyses reported.

level determined in whole plants. Thus, as determined by the same method (Arnon, 1949) used for chlorophyll extraction from the 'TADH-XO' cells, blue grama plants regenerated from the chlorophyllic cells as described before (Aguado-Santacruz et al., 2001) contained practically the same chlorophyll content (918 $\mu g\, g^{-1}$ FW) developed by chlorophyllic cells grown in 21% PEG at day 6 (906.05 $\mu g\, g^{-1}$ FW; Fig. 2). As a reference, total chlorophyll values between 0.47–2.34 mg g DW^{-1} have been registered in field-grown blue grama plants depending on environmental factors (primarily water availability; Rauzi and Dobrenz, 1970). Under controlled conditions, these values are higher, ranging between 4 and 7 mg g DW^{-1} as a function of nutrient and water availability, as well as temperature regime (Bokhari, 1976a).

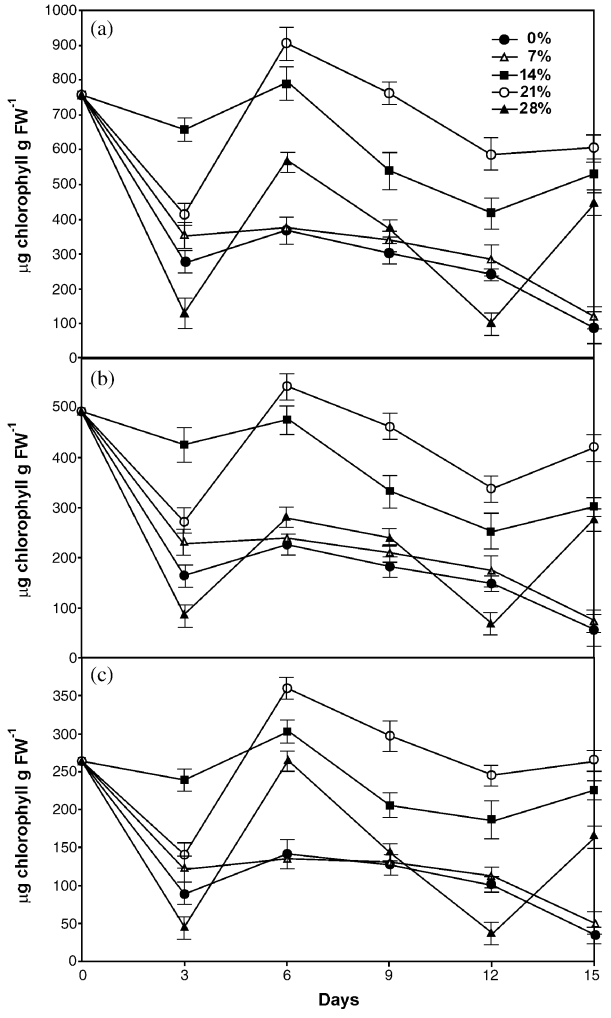

Figure 2. Chlorophyll kinetics in 'TADH-XO' chlorophyllic cells grown under different osmotic stress strengths induced by PEG 8000: (a) total chlorophyll, (b) 'a' chlorophyll and (c) 'b' chlorophyll. Values are the mean of 10 replicates from two independent experiments. Bars represent $\pm$SE.

The increased chlorophyll production observed at day 6, cannot be explained by a correlative increase in chloroplast number because these plastids were more abundant at day 9 for treatments 0%, 7%, 14% and 28% PEG, and at day 15 for treatment 21% PEG (Table 2). On the other hand, chloroplast size varied greatly among treatments (Fig. 3) and sampling dates, but the largest chloroplasts were observed at day 12 in all treatments (Table 3).

A further increase in chlorophyll content was also experienced by cells subjected to stress levels of 14%, 21% and 28% PEG from day 12 to day 15 (Fig. 2). Unlike the first registered chlorophyll increase, in which a relation to the chloroplast number was not clear, in this second chlorophyll increase a relation to augmented chloroplast number was detected (Table 2) as part of the normal cycles of division of these plastids.

This increased chloroplast number is congruent with a previous increase in chloroplast size during day 12 (Table 3) and a subsequent increase in cell size at day 15 (Table 4). Although increases in these variables were also observed in treatment 7% PEG, in that case the increases were not clearly related to chloroplast number (Table 2) or chlorophyll accumulation (Fig. 2) because a comparatively faster growth of these cells (Fig. 1) caused a more rapidly consumption of the required nutrients for chlorophyll synthesis in the batch cultures at day

Table 1. Cell death (%) in graminaceous chlorophyllic cells under five different osmotic treatments

	Days					
Treatments	0	3	6	9	12	15
0%	6.1 Ba*	6.7 Bb	0.0 Aa	4.8 Bb	25.9 Cb	29.2 Dc
7%	6.1 Ca	0.0 Aa	1.2 ABa	3.7 BCb	0.0 Aa	25.6 Dc
14%	6.1 Ca	0.0 Aa	1.7 ABa	3.5 Bb	0.0 Aa	0.0 Aa
21%	6.1 Ba	0.0 Aa	0.9 Aa	0.0 Aa	0.0 Aa	0.0 Aa
28%	6.1 Ba	0.0 Aa	1.3 Aa	9.1 Cc	0.0 Aa	10.9 Cb

*Means followed by different letters are significantly different among sampling dates (capital letters) or among treatments (small letters) using Duncan's multiple range test ($P<0.01$).

Table 2. Chloroplast number in graminaceous chlorophyllic cells under five different osmotic treatments

	Days					
Treatments	0	3	6	9	12	15
0%	27.2 Ca*	23.5 Bb	26.4 Cb	36.7 Dc	27.8 Cc	17.4 Aa
7%	27.2 BCa	25.4 Bb	29.5 BCb	36.1 Dc	30.2 Cc	21.2 Ab
14%	27.2 Ba	29.5 BCc	26.8 Bb	31.3 Cb	23.1 Ab	28.7 BCc
21%	27.2 Aa	25.6 Ab	33.2 Cc	35.6 Cc	29.7 Bc	42.0 Dd
28%	27.2 Da	16.4 ABa	15.4 Aa	28.4 Da	18.8 Ba	22.5 Cb

*Means followed by different letters are significantly different among sampling dates (capital letters) or among treatments (small letters) using Duncan's multiple range test ($P<0.01$).

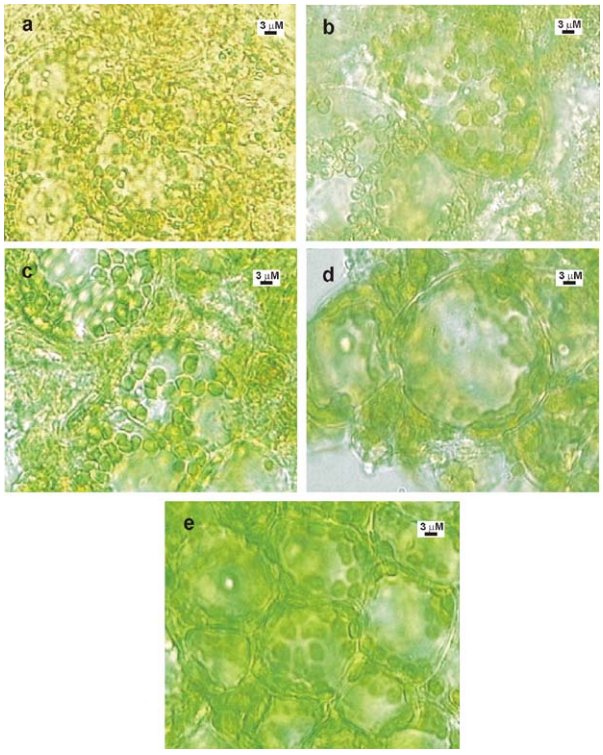

Figure 3. Microphotographs at day 10 of the blue grama chlorophyllic cells grown in the different osmotic treatments: (a) 0%, (b) 7%, (c) 14%, (d) 21% and (e) 28% PEG 8000.

15; doubling times for treatments 0%, 7%, 14% and 21% PEG were 1.3, 2.4, 3.8, 11.3 days, respectively.

The chlorophyll level determined for the initial inoculum was never reached by 0%, 7% or 28% PEG cells (Fig. 2), probably because the osmotic pre-adaptation period allowed development of relatively high amounts of chlorophyll in the inoculum cells. If this is the case, a gradual increase in chlorophyll development should be expected in cells continuously subcultured in osmotic stress media.

The early reduction in the fresh weight experienced by the cells grown in 0% PEG (Fig. 1) can be partially explained by the relatively higher cell death within the first 3 days observed in these cells as compared to those grown in the treatments containing PEG (Table 1), which probably also resulted from the osmotic pre-adaptation period. The percentage of dead cells during days 9 and 15 was around 10% in the 28% PEG-grown cells, whereas at the end of the experiment the maximum cell death percentages were reached in treatments 0% and 7% PEG (Table 1). This was attributed to the fact that the beginning of the cell growth plateau (Fig. 1) was reached in these latter treatments, as evidenced by the decreased chlorophyll levels (Fig. 2) and the relatively low percentage of green cells (Fig. 4) registered at this date. Green cells were, on the other hand, more abundant during day 12 in all treatments containing PEG. Interestingly, the osmotic stress apparently induced a physiological synchronization of the chlorophyllic cells, at least with relation to the development of thylakoids, because control cells always maintained a lower percentage of green cells throughout the experiment (Fig. 4).

The osmotic-induced increase in chlorophyll accumulation observed at day 6 in all treatments (Fig. 2) cannot be explained solely by the percentage of green cells (Fig. 4) nor by the chloroplast number (Table 2) or size (Table 3) because no treatment peaked in these variables at this date.

Discussion

In this study we have analyzed the effect of osmotic stress on the physiological performance of graminaceous chlorophyllic cells. After a short period of pre-adaptation in 2.5% PEG, the 'TADH-XO' cells were subcultured under four treatments

Table 3. Chloroplast size (μm^2) in graminaceous chlorophyllic cells under five different osmotic treatments

	Days					
Treatments	0	3	6	9	12	15
0%	9.2 Da*	4.9 Ba	3.7 Aa	7.6 Ca	10.3 Ea	3.8 Aa
7%	9.2 BCa	5.0 Aa	8.2 Bb	9.3 BCa	10.2 Ca	6.5 Ab
14%	9.2 Ba	8.3 ABb	13.2 Cc	13.6 Cb	15.3 Db	7.3 Ab
21%	9.2 Aa	15.9 Cc	13.2 Bc	9.1 Aa	23.2 Dc	15.4 BCc
28%	9.2 Aa	7.5 Ab	13.5 Bc	16.2 Cc	26.5 Ed	19.2 Dd

*Means followed by different letters are significantly different among sampling dates (capital letters) or among treatments (small letters) using Duncan's multiple range test ($P<0.01$).

Table 4. Cell size (μm^2) in graminaceous chlorophyllic cells under five different osmotic treatments

	Days					
Treatments	0	3	6	9	12	15
0%	712.0 Da*	588.3 Aa	655.6 Ca	870.4 Eb	867.7 Eb	617.4 Ba
7%	712.0 BCa	690.3 Bb	967.7 Dc	733.8 Ca	542.6 Aa	1112.2 Ed
14%	712.0 Ba	738.9 Cb	737.6 Cb	1122.7 Ec	547.9 Aa	916.0 Dc
21%	712.0 Aa	1148.2 Ed	753.0 Bb	844.6 Cb	1098.4 Dc	1918.3 Fe
28%	712.0 Ca	973.4 Fc	639.6 Ba	795.4 Eab	530.0 Aa	753.9 Db

*Means followed by different letters are significantly different among sampling dates (capital letters) or among treatments (small letters) using Duncan's multiple range test ($P<0.01$).

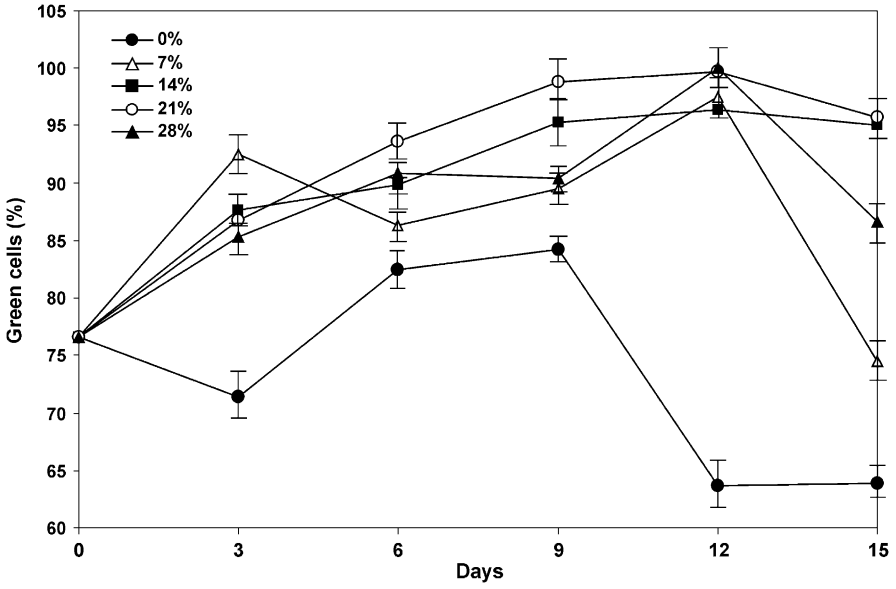

Figure 4. Percentage of green cells in TADH-XO' chlorophyllic cells grown under different osmotic stress strengths induced by PEG 8000. Values are the mean of 10 replicates from two independent experiments. Bars represent $\pm$SE.

of different osmotic potential. We were surprised by the rapidity with which the cells adapted to the pre-adaptation medium of low osmotic tension, which initially produced a decrease in cell fresh weight (Fig. 1) and a relatively higher cell death in the 0% as compared to the 7% and 14% PEG treatments (Table 1). By day 9, however, the cells recovered, and at the end of the experiment the greatest fresh weight gain was observed in cells grown in 0% PEG (Fig. 1). This also suggests a

remarkable physiological flexibility of the 'TADH-XO' cells for adapting to different osmotic tensions. This unexpected result of a process viewed initially as a cellular synchronizing treatment in relation to chlorophyll development (Fig. 4), raises the possibility that an adaptation, rather than a selection process, could be operating during the growth of chlorophyllic cells under osmotic stress. This was further supported by the low cell death percentages (<10%) registered in all treatments throughout the experiment up to day 9 (Table 1).

In our system, osmotic stress increased chlorophyll accumulation according to the strength of the osmotic stress applied. Although previous studies in chlorophyllic cells of dicots have documented increased chlorophyll production under saline stress (Winicov and Button, 1991; Locy et al., 1996; Chang et al., 1997), this is the first report showing (a proportionally) increased chlorophyll accumulation in response to osmotic stress in graminaceous chlorophyllic cells.

Although the increases in chlorophyll production under saline (and osmotic stress in this study) in chlorophyllic cell systems have been described, the causal factors have not been adequately addressed; neither has it been clarified if this increase in chlorophyll is incidental or an authentic cellular response to counteract the saline (osmotic) stress nor have the mechanistic links of acquired salt tolerance of plant cells with increased levels of transcripts for photosynthesis genes (Winicov and Button, 1991) been clearly established.

Because none of the variables analyzed peaked at the point of maximum chlorophyll accumulation (day 6), we assume that augmented chlorophyll production in response to osmotic stress could be related to chloroplast development, as it has been reported by other authors working with saline stress (Chang et al., 1997).

Although saline and osmotic stress share the characteristic of creating a water potential differential between the cell and its environment, which is ultimately conducive to water deficit in the cell, the latter avoids the toxic effect of salt (Zhang et al., 2000). Indeed, particular and specific responses are expected when cells are subjected to saline or water stress (Critchley, 1982; Iraki et al., 1989; Sabbah and Tal, 1990; Leonardi et al., 1995), with salt stress more detrimental than osmotic stress (Sabbah and Tal, 1990) to cell growth because of the additional ionic effect of sodium chloride. Conversely, Yamane et al. (2003) found PEG to have an apparently more detrimental effect than sodium chloride on chloroplast ultrastructure by causing destruction of the chloroplast envelope, in contrast to swelling of the chloroplast induced by NaCl.

Plastids are very plastic organelles (Mullet, 1988). Plastic responses in chloroplast osmotic adjustment and volume (Robinson, 1985), as well as in development (Chang et al., 1997), have been observed in response to saline or osmotic stress. But under prolonged or stronger stress conditions chloroplasts suffer from major alterations in their physiology or structure. These alterations include rupture of the chloroplast enveloped membrane, loss of its distinctiveness, and swelling of thylakoids which, in turn, results in an increase in intrathylakoid space, and formation of numerous vesicles. Some of these events manifested mainly in the chloroplasts of the 'TADH-XO' cells grown in 28% PEG.

It has been suggested that the increases in chlorophyll and related photosynthetic proteins observed in cultured cells play an important role in salt tolerance capability of salt-adapted alfalfa cells (Winicov and Seemann, 1990). But, is this increased chlorophyll (and increased photosynthesis rate) that permits the production of specific chloroplast compounds to overcome the salt stress? or is there an initial cellular mechanism that is turned on to protect the chloroplast that favors the increase in photosynthetic machinery components?

Because all reported increases in chlorophyll content in stressed chlorophyllic cells focused on responses to saline osmotica, ad hoc hypotheses have been postulated to explain this phenomenon: (a) salinity increases chlorophyll content by slowing cellular growth relative to chlorophyll synthesis and chloroplast biogenesis, (b) salt alters the adverse effect of sucrose on chlorophyll biosynthesis or chloroplast biogenesis, (c) saline adaptation interferes with the uptake of sucrose into the chloroplast compartment (Locy et al., 1996). Previous experiments with tobacco cells grown under saline stress have shown that this enhanced chlorophyll content is precisely due to increased thylakoid number (Chang et al., 1997).

Although our study was not conduced to address these questions, from the proportionally increased chlorophyll accumulation as a function of osmotic potential we can infer that osmotic stress seems to increase the potential of the chlorophyllic cells for thylakoid assemblage by an intriguing and unknown mechanism. In addition, our results suggest the possibility that increases in chlorophyll under saline (osmotic) stress reported by other authors are the result of osmotic stress on cell physiology rather than the effect of salt per se. Data newly collected by us (not reported in this paper) support this conclusion, and show a proportional increase in chlorophyll content as a function of salt concentration.

Chloroplasts play a central role in the plant's physiology and biochemistry. Besides photosynthesis, they are involved in the synthesis of amino acids, fatty acids, starch, and many secondary metabolism compounds, and play a pivotal role in the plant response to water stress. Therefore, an alternative explanation to increased chlorophyll production under saline or osmotic stress could be that protective mechanisms related to the chloroplasts would permit this increase in chlorophyll. Accordingly, in these plastids the first reaction involved in ABA biosynthesis takes place through epoxidation of zeaxanthin into antheraxanthin and violaxanthin (Marin et al., 1996). Chloroplasts from maize plants overproducing ABA seem to be more protected from the detrimental effects of drought stress than plants having low levels of ABA (Ristic and Cass, 1992). Although a role in stomata control by ABA is discarded in cultured plant cells, other effects mediated by ABA are important in the response of cells to water stress (Leonardi et al., 1995).

In higher plants, the pathways for synthesis of osmoprotectants, such as citrulline, glycinebetaine and proline, are localized in chloroplasts (Hanson et al., 1985; Weigel et al., 1988; Yokota et al., 2002). Compatible solutes play an important role in preventing thylakoid membrane damage during dehydration (Schwab and Heber, 1984). Heat shock proteins (HSPs) also appear to play an important role in protecting the stability of chloroplast membranes, since some HSPs and LEAs have been shown to be imported into the chloroplasts (Vierling et al., 1986; NDong et al., 2002), where they could assist in stabilization of proteins.

One of the most detrimental effects of biotic and abiotic stresses is related to the production of active oxygen species (AOS). Chloroplast protection has been associated with different antioxidant agents, such as Cu/Zn-SOD, Fe-SOD, glutathione, tocopherol, ascorbate, ascorbate peroxidase and carotenoids, which are synthesized by or imported into the chloroplast to accomplish this protective function. In particular, glutathione is an antioxidant which is concentrated mainly within the chloroplast, where it may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price et al., 1990). Carotenoids, on the other hand, can protect photosynthetic machinery by means of different mechanisms (Mathis and Kleo, 1973; Burton and Ingold, 1984).

Considering that chloroplasts are the centers for production of carbon skeletons and energy for cellular metabolism, allocation of great amount of energetic and metabolic resources by the cell to the chloroplast to protect photosynthetic machinery should not be surprising. Maintaining photosynthetic capacity is important for the cell to overcome the effects of water deficit. In this context, it has been shown that photosynthetic capability is obligatory for saline adaptation in alfalfa cells (Winicov and Seemann, 1990), but not in tobacco cells (Locy et al., 1996), while salinity increases the photosynthetic capacity of some green algae (Blumwald and Tel-Or, 1984; Takabe et al., 1988).

Whatever the mechanism or mechanisms responsible for this increase in chlorophyll content, an increased photosynthetic capacity will, at least in theory, ultimately be observed because chlorophyll levels correlate with the level of membrane-bound protein components of the photosynthetic apparatus (Evans, 1989; Winicov and Seemann, 1990). In fact, this seems to be the case in whole plants, as suggested by a tomato mutant (hp-1) which develops higher chlorophyll contents than the wild type and shows correlated increases in light absorption and net photosynthesis (Nieuwhof and van de Dijk, 1988; Peters et al., 1992).

Despite the observed increase in chlorophyll content in 'TADH-XO' osmotic stressed cells, an increase in fresh weight relative to control cells was not observed. Although a higher chlorophyll content may be correlated with increased photosynthetic activity, this may not ultimately be reflected in fresh weight gain in the cells because resources may be diverted to compounds (such as compatible solutes) produced to overcome the effects of osmotic stress (Takabe et al., 1988), or, in the case of salt stress, to compartmentalize salt in the vacuole against a large gradient (Winicov and Seemann, 1990; Locy et al., 1996).

While the first increase in chlorophyll accumulation was probably related to chloroplast development, the second peak, registered in treatments 14%, 21% and 28% PEG at the end of the cell kinetics, resulted from an increased chloroplast number linked to the normal cycles of growth and division of the cell. Increased chloroplast size (Table 3) at day 12 and augmented cell size (Table 4) during day 15 support this notion. Prior to division, chloroplasts have to attain a certain size (Ellis et al., 1983), and after chloroplast separation, cell size is a primary determinant of the chloroplast number (Dean and Leech, 1982). There seems to be a limit, imposed by cell size, to chloroplast biomass (size and number; Mullet, 1988). On the other hand, it was interesting to notice that 21% PEG-cells contained more chloroplasts than other treatments (Table 2), particularly at the end of the cell growth cycle, indicating that in addition to favoring chloroplast

development, osmotic stress can increase chloroplast number.

At this point of our discussion a question arises as to whether the increase in chlorophyll accumulation in response to osmotic stress is representative of what naturally occurs in whole plants. Plants suffering from drought stress reduce their chlorophyll content (Holaday et al., 1992; Parry et al., 2002) and this reduction is accompanied by an obvious reduction in photosynthesis machinery components and, finally, in photosynthesis and productivity (Bray, 1960; Mall et al., 1973; Bokhari, 1976b). However, some plants subjected to drought stress display a phenomenon, namely the stay-green trait, favoring the retention of chlorophyll after grain filling and the consequent maintenance of more photosynthetically active leaves (Rosenow et al., 1983) and increased yield (Borrell et al., 2000).

Thus, the question remains whether specific cellular physiological or biochemical responses to water stress are related to specific organizational levels (Willenbrink and Hüsemann, 1995).

Final remarks

From our results it is clear that a relation between osmotic potential of the growing media and chlorophyll accumulation exists after the initial decline in this pigment caused by the sucrose availability, while enhanced chlorophyll accumulation seems to be related to chloroplast development as shown by other authors. Our work opens lines of research related to the participation of chloroplasts in the response of graminaceous chlorophyllic cells to water deficit, as well as to definition of the specific mechanisms involved in the protection of photosynthetic machinery resulting in increased chlorophyll content (thylakoid development). First, studies directly focused on specific mechanisms, including the possibilities discussed above, that promote or favor increases in chlorophyll should be carried out, investigating the largely unknown mechanisms regulating plastid division and plastid number per cell (Mullet, 1988). Fundamental additional questions to be addressed are: Is this augmented chlorophyll concentration conducive to an increased photosynthetic rate of graminaceous chlorophyllic cells? If so, could these increases in chlorophyll and photosynthesis be mediated or facilitated through increases in the chloroplast stromal pH (Pier and Berkowitz, 1989), osmo or HSP's protection, or some other unknown mechanism? Finally, it is also important to evaluate the response of isogenic plants regenerated from the chlorophyllic cells to water stress in terms of chlorophyll accumulation. Studies are currently being conducted to address this issue.

Acknowledgments

The authors gratefully acknowledge Dr. Jeffrey Creque and Blanca Moreno Gómez for helpful comments and suggestions to the manuscript. This research was funded by Consejo de Ciencia y Tecnología del Estado de Guanajuato (CONCyTEG) under Project Nos. 03-24-k118-041 and 01-24-201-142.

References

Aguado-Santacruz GA, Cabrera-Ponce JL, Ramírez-Chavez E, Rascón-Cruz Q, Herrera-Estrella L, Olalde-Portugal V. Establishment, characterization and plant regeneration from highly chlorophyllous embryogenic cell cultures of blue grama grass, *Bouteloua gracilis* (H.B.K.). Lag ex. Steud. Plant Cell Rep 2001;20:131–6.

Aguiar M, Lauenroth W. Local and regional differences in abundance of co-dominant grasses in the short grass steppe; a modeling analysis of potential causes. Plant Ecol 2001;156:161–71.

Antolín MC, Sánchez-Díaz M. Effects of temporary droughts on photosynthesis of alfalfa plants. J Exp Bot 1993;44:1341–9.

Arnon DI. Copper enzymes in isolated chloroplasts. Plant Physiol 1949;24:1–15.

Bergmann L. Wachstum grüner Suspensionkulturen von *Nicotiana tabacum* var. "Samsun" mit CO_2 als Kohlenstoffquelle. Planta 1967;74:243–9.

Blumwald E, Tel-Or E. Salt adaptation of the cyanobacterium *Synechococcus* 63ll growing in a continuous culture (turbidostat). Plant Physiol 1984;74:183–5.

Bohnert HJ, Shen B. Transformation and compatible solutes. Sci Hort 1999;78:237–60.

Bokhari UG. The influence of stress conditions on chlorophyll content of two range grasses with contrasting photosynthetic pathways. Ann Bot 1976a;40:969–79.

Bokhari UG. Influence of temperatures, water stress, and nitrogen treatments on chlorophyll and dry matter of western wheatgrass. J Range Manage 1976b;29:127–31.

Borrell AK, Hammer GL, Henzell RG. Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield. Crop Sci 2000;40:1037–48.

Boyer JS. Plant productivity and environment. Science 1982;218:443–8.

Bray JR. The chlorophyll content of some native and managed plant communities in central Minnesota. Can J Bot 1960;38:313–33.

Bressan RA, Hasegawa PM, Handa AK. Resistance of cultured higher plant cells to polyethylene glycol-induced water stress. Plant Sci Lett 1981;21: 23–30.

Burton GW, Ingold KU. ß-carotene: an unusual type of lipid antioxidant. Science 1984;224:569–73.

Chang C-C, Locy RD, Smeda R, Sahi SV, Singh NK. Photoautotrophic tobacco cells adapted to grow at high salinity. Plant Cell Rep 1997;16:495–502.

Critchley C. Stimulation of photosynthetic electron transport in a salt-tolerant plant by high chloride concentrations. Nature 1982;298:483–5.

Dalton CC, Street HE. The influence of applied carbohydrates on the growth and greening of cultured spinach (*Spinacia oleracea* L.) cells. Plant Sci Lett 1977;10: 157–64.

Dean C, Leech RM. Genome expression during normal leaf development. I. Cellular and chloroplast numbers and DNA, RNA and protein levels in tissues of different ages within a seven-day old leaf. Plant Physiol 1982;69:904–10.

Edelman J, Hanson AD. Sucrose suppression of the chlorophyll synthesis in carrot tissue cultures: the role of invertase. Planta 1971;101:122–32.

Ellis JR, Jellings AJ, Leech RM. Nuclear DNA content and the control of chloroplast replication in wheat leaves. Planta 1983;157:376–80.

Evans JR. Photosynthesis and nitrogen relationships in leaves of C_3 plants. Oecologia 1989;78:9–19.

Garg AK, Kim J- K, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 2002;99: 15898–903.

Hanson AD, May AM, Grumet R, Bode J, Jamieson GC, Rhodes D. Betaine synthesis in chenopods: localization in chloroplasts. Proc Natl Acad Sci USA 1985;82: 3678–82.

Harms CT, Oertli JJ. The use of osmotically adapted cell cultures to study salt tolerance in vitro. J Plant Physiol 1985;120:29–38.

Holaday AS, Ritchie SW, Nguyen HT. Effect of water deficit on gas exchange parameters and ribulose 1,5-bisphosphate carboxylase activation in wheat. Environ Exp Bot 1992;32:403–9.

Iraki NM, Bressan RA, Carpita NC. Extracellular polysaccharides and proteins of tobacco cell cultures and changes in composition associated with growth-limiting adaptation to water and saline stress. Plant Physiol 1989;91:54–61.

Kingsbury RW, Epstein E. Salt sensitivity in wheat. Plant Physiol 1986;80:651–4.

Lauenroth WK, Sala OE, Coffin DP, Kirchner TB. The importance of soil water in the recruitment of *Bouteloua gracilis* in the shortgrass steppe. Ecol Appl 1994;4:741–9.

Leonardi A, Heimovaara-Dijkstra S, Wang M. Differential involvement of abscisic acid in dehydration and osmotic stress in rice cell suspension. Physiol Plant 1995;93:31–7.

Locy RD, Chang C-C, Nielsen BL, Singh NK. Photosynthesis in salt-adapted heterotrophic tobacco cells and regenerated plants. Plant Physiol 1996;110:321–8.

Mall LP, Billore SK, Misra CM. A study on the community chlorophyll content with reference to height and dry weight. Trop Ecol 1973;14:81–3.

Marin E, Nussaume L, Quesada A, Gonneau M, Sota B, Hugueney P, Frey A, Marion-Poll A. Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. EMBO J 1996;15:2331–42.

Mathis P, Kleo J. The triplet state of *ß*-carotene and of analog polyenes of different length. Photochem Photobiol 1973;18:343–6.

Mullet JE. Chloroplast development and gene expression. Ann Rev Plant Physiol Plant Mol Biol 1988;39: 475–502.

NDong C, Danyluk J, Wilson KE, Pocock T, Huner NPA, Sarhan F. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analysis. Plant Physiol 2002;129:1368–81.

Nieuwhof M, van de Dijk SJ. Differences between genotypes of tomato (*Lycopersicon esculentum* Mill.) in net photosynthesis, light absorption by leaves, chlorophyll content and specific leaf fresh weight under low-energy conditions. Neth J Agri Sci 1988;36: 396–9.

Parry MAJ, Andralojc PJ, Khan S, Lea PJ, Keys AJ. Rubisco activity: effects of drought stress. Ann Bot 2002;89: 833–9.

Pego JV, Kortstee AJ, Huijser C, Smeekens SCM. Photosynthesis, sugars and the regulation of gene expression. J Exp Bot 2000;51:407–16.

Peters JL, Schreuder MEL, Verduin SJW, Kendrick RE. Physiological characterization of a high pigment mutant of tomato. Photochem Photobiol 1992;56: 75–82.

Pier PA, Berkowitz GA. The effects of chloroplast envelope-Mg^{2+}, cation movement, and osmotic stress on photosynthesis. Plant Sci 1989;64:45–53.

Price A, Lucas PW, Lea PJ. Age dependent damage and glutathione metabolism in ozone fumigated barley: a leaf section approach. J Exp Bot 1990;41: 1309–17.

Rauzi F, Dobrenz AK. Seasonal variation of chlorophyll in western wheatgrass and blue grama. J Range Mange 1970;23:372–3.

Ristic Z, Cass DD. Chloroplast structure after water and high-temperature stress in two lines of maize that differ in endogenous levels of abscisic acid. Int J Plant Sci 1992;153:186–96.

Robinson SP. Osmotic adjustment by intact isolated chloroplasts in response to osmotic stress and its effect on photosynthesis and chloroplast volume. Plant Physiol 1985;79:996–1002.

Rogers SMD, Ogren WL, Widholm JM. Photosynthetic characteristics of a photoautotrophic cell suspension culture of soybean. Plant Physiol 1987;84:1451–6.

Rosenow DT, Quisenberry JE, Wendt CW, Clark LE. Drought tolerant sorghum and cotton germplasm. Agric Water Manage 1983;7:207–22.

Sabbah S, Tal M. Development of callus and suspension cultures of potato resistant to NaCl and mannitol and their response to stress. Plant Cell Tiss Org Cult 1990;21:119–28.

Schwab K, Heber U. Thylakoid membrane stability in drought tolerant and drought-sensitive plants. Planta 1984;161:37–45.

Steel RGD, Torrie JH. Principles and Procedures of Statistics. New York: McGraw-Hill; 1960.

Streb P, Feierabend J. Oxidative stress responses accompanying photoinactivation of catalase in NaCl-treated rye leaves. Bot Acta 1996;109:125–32.

Takabe T, Incharoensakdi A, Arakawa K, Yokota S. CO_2 fixation rate and rubisco content increase in the halotolerant cyanobacterium, *Aphanothece halophytica*, grown in high salinities. Plant Physiol 1988;88:1120–4.

Thomas H, Smart CM. Crops that stay green. Ann Appl Biol 1993;123:193–219.

Vierling E, Mishkind ML, Schmidt GW, Key JL. Specific heat shock proteins are transported into chloroplasts. Proc Natl Acad Sci USA 1986;83:361–5.

Weigel P, Lerma C, Hanson AD. Choline oxidation by intact spinach chloroplasts. Plant Physiol 1988;86: 54–60.

Widholm JM. Properties and uses of photoautotrophic plant cell cultures. Int Rev Cytol 1992;132: 109–75.

Willenbrink ME, Hüsemann W. Photoautotrophic cell suspension cultures from *Mesembryanthemum crystallinum* and their response to salt stress. Bot Acta 1995;108:497–504.

Winicov I, Seemann JR. Expression of genes for photosynthesis and the relationship to salt tolerance of alfalfa (*Medicago sativa*) cells. Plant Cell Physiol 1990;31:1155–61.

Winicov I, Button JD. Accumulation of photosynthesis gene transcripts in response to sodium chloride by salt-tolerant alfalfa cells. Planta 1991;183: 478–83.

Yamane K, Kawasaki M, Taniguchi M, Miyake H. Differential effect of NaCl and polyethylene glycol on the ultrastructure of chloroplasts in rice seedlings. J Plant Physiol 2003;160:573–5.

Yokota A, Kawasaki S, Iwano M, Nakamura C, Miyake C, Akashi K. Citrulline and DRIP-1 protein (ArgE homologue) in drought tolerance of wild watermelon. Ann Bot 2002;89:825–32.

Zhang J, Klueva NY, Wang Z, Wu R, Ho TD, Nguyen HT. Genetic engineering for abiotic stress resistance in crop plants. In Vitro Cell Dev Biol-Plant 2000;36: 108–14.