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Light-dark changes in proline content of barley leaves under salt stress

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Abstract

Proline accumulation in leaves of barley (*Hordeum vulgare* L. cv. Alfa) seedlings treated with 150 mM NaCl was promoted in the light and suppressed in the dark. The light/dark changes of proline content was enhanced with each 12 h light/12 h dark cycle and the proline content increased steadily. Root and shoot concentrations of Na^+ and Cl^- in salt treated plants increased about 10 to 25 times as compared to the control. The content of these ions and the content of malondialdehyde were higher in the shoot of seedlings exposed to salt stress for 4 d in the light in comparison with the seedlings exposed to NaCl for 4 d in darkness. Light stimulated both ions and proline accumulation in the leaves and has no effect in the roots. Oxygen uptake was higher in the seedlings kept 4 d in the light which have higher endogenous free proline content. Chlorophyll fluorescence measurements showed that the photochemical activity of PS 2 slightly decreased as a result of salt stress and was not influenced by light regimes during plant growth.

Additional key words: chlorophyll fluorescence, *Hordeum vulgare*, ions accumulation, malondialdehyde, NaCl, oxygen uptake.

Introduction

When plants are subjected to salt and drought stress, they can survive and grow due to ion transport and compartmentation, and by synthesis and accumulation of osmotic agents: glycine-betaine, proline, polyols. Proline accumulation is an early response to salt stress. It has been found that salt-tolerant cultivars show a stronger and faster accumulation of proline than sensitive ones (Igarashi *et al.* 1997). Proline accumulation is due to *de novo* synthesis, as observed by Rhodes *et al.* (1986). The stimulation of proline biosynthesis under salt and water stress has been shown to be associated with an increase of the D1-pyrroline-5-carboxylate synthetase (P5CS) and the P5CS corresponding mRNA (Savoure *et al.* 1995, Yoshiba *et al.* 1995). Some plants accumulated several time more proline in the light than in darkness (Fedina and Popova 1996, Hayashi *et al.* 2000). Many studies in plants have focused on the roles of proline in defense mechanisms against impairments caused by osmotic stress. Although a positive correlation between the accumulation of proline and osmotolerance in plants was found (Handa *et al.* 1986, Kavi Kishor *et al.* 1995) the roles of proline in osmoprotection in plants remain controversial. It has been established that proline not only acts as a mediator of osmotic adjustment (Handa *et al.* 1986), but also as a stabilizer of subcellular structures (Schobert and Tschesche 1978), a scavenger of free radicals (Saradhi *et al.* 1995), a sink for energy (Saradhi and Saradhi 1991), and a major constituent of cell wall structural proteins in plants in morphogenesis (Nanjo *et al.* 1999). According to Saradhi *et al.* (1995) UV radiation induced proline accumulation has an important role in a protecting plants against UV radiation promoted peroxidative processes.

In the present study light/dark changes of proline content of barley seedlings under salt stress are reported. The influence of the light on proline content and accumulation of Na^+ and Cl^- ions in the shoot and in the roots of treated plants was investigated. Chlorophyll fluorescence and oxygen uptake in the dark were also measured.

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Abbreviations: Chl - chlorophyll; F_0 - chlorophyll fluorescence of dark adapted leaf; Fm - maximal fluorescence; Fv - variable fluorescence; MDA - malondialdehyde; qN - non-photochemical fluorescence quenching; qP - photochemical fluorescence quenching; PPFD - photosynthetic photon flux density; PS 2 - photosystem 2; TBA - thiobarbituric acid; TCA - trichloracetic acid.

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Materials and methods

Plants: Seeds of *Hordeum vulgare* L. cv. Alfa germinated for 2 d at 25 °C. Seedlings were then transferred to pots containing distilled water. After 3 d 150 mM NaCl was added. The seedlings were grown under 12-h photoperiod using white fluorescent lamps (PPFD 160 μmol m^{-2} s^{-1}), relative humidity 60 %, and day/night temperature of 25/20 °C. Samples for analysis were taken at the end of each light and dark cycle. After starting the salt stress some seedlings were transferred into continuous light and the other into continuous darkness for 4 d. In the other sets of experiments NaCl treated seedlings were grown 2 d in the light and 2 d in the dark and *vice versa*.

Proline was determined by the method of Bates *et al.* (1973). Approximately 0.5 g of plant material was homogenized in 10 cm^3 of 3 % aqueous sulphosalicylic acid and the homogenate was centrifuged at 2 000 *g* for 5 min. Two cm^3 of the extract reacted with 2 cm^3 of acid-ninhydrine and 2 cm^3 of glacial acetic acid for 1 h at 100 °C. The reaction mixture was extracted with 4 cm^3 toluen. The chromophore containing toluene was separated and the absorbance read at 520 nm (*Specol 10*, Jena, Germany).

Malondialdehyde was determined by the method of Esterbauer and Cheeseman (1990). 150 mg barley leaves was homogenized in 3 cm^3 of 0.1 % TCA (4 °C) and centrifuged at 10 000 *g* for 15 min.. To 1 cm^3 of the supernatant 2 cm^3 of TCA-TBA-HCl reagent (15 % m/v) trichloracetic acid; 0.375 % m/v thiobarbituric acid; 0.25 M hydrochloric acid) were added. This solution was boiled 15 min in water bath centrifuged at 2 000 *g* for 5 min and the absorbance read at 352 nm.

Oxygen uptake rates were determined using a leaf disk electrode (*Type LD2/2*, *Hansatech*, Norfolk, UK).

Chlorophyll fluorescence induction of leaf disks was measured with a pulse amplitude modulation fluorometer (*PAM 101-103*, *H. Walz*, Effelrich, Germany) as described by Schreiber *et al.* (1986). The initial fluorescence yield in weak modulated light (PPFD 0.075 μmol m^{-2} s^{-1}), F_0, and maximum total fluorescence yield emitted during a saturating white light pulse (1 s, PPFD over 3500 μmol m^{-2} s^{-1}, by *Schott KL 1500* light source), Fm, were determined. The leaf disc (1 cm diameter) was then illuminated with continuous red radiation (125 μmol m^{-2} s^{-1}). When the measuring light was applied alone, a modulation frequency of 1.6 kHz was used, otherwise the modulation frequency was set to 100 kHz. The short pulses (with 20-s interval) on the background of a red radiation were used to obtain the fluorescence intensity Fm' with all photosystem (PS) 2 reaction centers closed in any light adapted state. Induction kinetics were registered and analyzed with a program *FIP 4.3*, written by Tyystjarvi and Karunen (1990).

Chlorophyll content was determined according to Lichtentahaler (1987).

Ions determinations: Cl^- was measured by silver ion titration by the method of Cotlove (1963). Na^+ was determined by flame photometry.

Statistics: Experimental data were processed statistically and significance of differences was evaluated by Student *t*-test.

Results

In 7-d-old barley seedlings subjected to 150 mM NaCl for 5 d proline content decreased near to the level of unstressed plants after 12 h of the first dark cycle (Fig. 1). On the second light cycle it became higher again. The light/dark change of proline content in the stressed plants was enhanced with each cycle and the proline content increased steadily. It was found that proline accumulation in salt stressed barley seedlings was promoted by light and suppressed in the dark. In order to determine if proline decrease in the dark period is due to its translocation from leaves to the roots we measured light/dark changes of proline content in the roots. In the roots proline accumulation both in the light and in the dark was not remarkable and was not influenced by light (Fig. 1). Some of the stressed seedlings were kept into continuous darkness and the other into continuous light for 96 h. Proline content in the salt stressed seedlings under continuous light is 3-fold higher than in the seedlings in the dark. Root and shoot concentrations of Na^+ and Cl^- (Table 1) in salt treated plants increased about 10 to 25 times as compared to the control plants. It was established that proline accumulation depends on ions content but the light has effect on this process too (Table 1). Light stimulated both ions accumulation and proline accumulation. The production of malon-dialdehyde was higher in the shoots of seedlings exposed to salt stress during 4 d in the light compared to seedlings exposed 4 d in the dark (Fig. 2). Oxygen uptake in the dark, as a measure of dark respiration, was stimulated by high endogenous proline content in stressed leaves (Fig. 2). After 4 d salt treatment in the light proline content in the leaves and oxygen uptake were higher than

in the leaves after 4 d salt treatment in the dark. Since PS 2 is a main target of many stress factors we investigated its functional activity in the salt stressed barley seedlings by means of chlorophyll fluorescence. Our results showed that salt stress slightly decreased the primary photochemical activity of PS 2, monitored by the ratio Fv/Fm (Table 2). The same result was observed for the photochemical fluorescence quenching qP, which estimate the relative change of PS 2 photochemical yield depending upon the concentration of open PS 2 reaction centers. The tendency for decreasing the non-photochemical fluorescence quenching qN, was also found (Table 2). Salt treatment of barley seedlings under different light regimes resulted in low decrease of chlorophylls and carotenoids contents (Table 3).

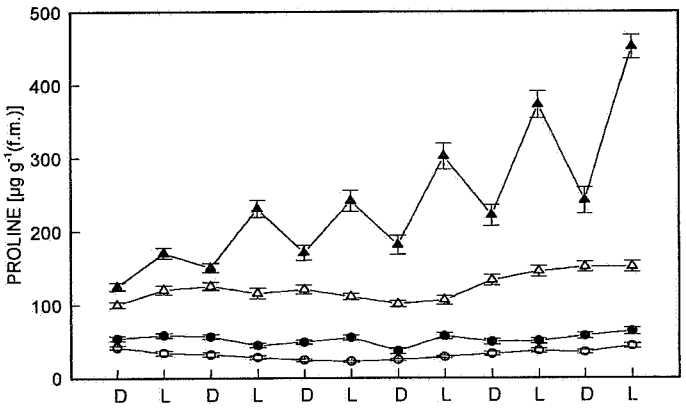

Fig. 1. Change of proline contents in the leaves (*triangles*) and roots (*circles*) of barley treated with 150 mM NaCl (*closed symbols*) in comparison with these in control ones (*open symbols*). Samples were taken at the end of light (L) or dark cycle (D). Means ± SE of 3 separate experiments.

Table 1. Proline, Cl^- and Na^+ contents in the leaves and roots of barley treated with 150 mM NaCl under different light regimes. Control (4 d 12 h L/12 h D) values were: proline - 88 ± 5.8 μg g^{-1}(f.m.); Cl^- - 1.8 ± 0.2 g kg^{-1}(d.m.); Na^+ - 2.6 ± 0.28 g kg^{-1}(d.m.) in the leaves and Cl^- - 2.6 ± 0.23 g kg^{-1}(d.m.); Na^+ - 3.8 ± 0.28 g kg^{-1}(d.m.) in the roots. Control values of plants in the other light regimes were similar. Means ± SE of 3 separate experiments. All values were significantly different from respective controls ($P < 0.001$).

Variant	Proline [μg g^{-1}(f.m.)]	Cl^- [g kg^{-1}(d.m.)] leaves	roots	Na^+ [g kg^{-1}(d.m.)] leaves	roots
2 d 12 h L/12 h D	220 ± 16	26 ± 1.4	35 ± 2.9	17 ± 1.3	25 ± 1.7
4 d 12 h L/12 h D	512 ± 32	42 ± 3.4	34 ± 3.2	26 ± 1.9	33 ± 2.8
2 d L	386 ± 28	25 ± 2.7	23 ± 1.7	17 ± 1.6	27 ± 2.4
4 d L	1560 ± 41	44 ± 3.9	33 ± 3.1	25 ± 2.7	37 ± 3.5
2 d D	180 ± 12	18 ± 1.6	33 ± 2.8	13 ± 1.1	40 ± 3.8
4 d D	580 ± 35	31 ± 2.8	32 ± 3.4	20 ± 1.8	41 ± 4.2
2 d D + 2 d L	880 ± 33	35 ± 3.2	33 ± 2.6	21 ± 2.3	36 ± 3.3
2 d L + 2 d D	520 ± 27	35 ± 3.4	36 ± 3.8	23 ± 2.1	42 ± 3.9

Table 2. Chlorophyll fluorescence parameters measured in leaf disk from control and treated with 150 mM NaCl barley seedlings under different light regimes. Values are means ± S E of 3 separate experiments.

Treatment		F_0	Fv	Fv/Fm	qN	qP
12 h D/12 h L	Control	0.048 ± 0.004	0.191 ± 0.01	0.796 ± 0.01	0.262 ± 0.01	0.948 ± 0.01
	NaCl	0.042 ± 0.003	0.154 ± 0.01	0.771 ± 0.01	0.206 ± 0.02	0.936 ± 0.01
4 d L	Control	0.052 ± 0.006	0.196 ± 0.02	0.781 ± 0.02	0.243 ± 0.02	0.944 ± 0.01
	NaCl	0.053 ± 0.004	0.164 ± 0.01	0.751 ± 0.02	0.205 ± 0.02	0.932 ± 0.01
4 d D	Control	0.051 ± 0.004	0.165 ± 0.01	0.759 ± 0.01	0.229 ± 0.01	0.922 ± 0.01
	NaCl	0.046 ± 0.003	0.164 ± 0.01	0.749 ± 0.02	0.227 ± 0.03	0.911 ± 0.02

Table 3. Contents of chlorophyll and carotenoids [mg g^{-1}(f.m.)] in barley seedlings treated with 150 mM NaCl under different light regimes. Means ± SE of 3 separate experiments.

Treatment		Carotenoids	Chl *a*	Chl *b*	Chl *a*/Chl *b*
12 h L/12 h D	Control	0.605 ± 0.05	0.997 ± 0.09	0.440 ± 0.03	2.26
	NaCl	0.534 ± 0.03	0.933 ± 0.08	0.390 ± 0.04	2.39
4 d L	Control	0.606 ± 0.06	1.071 ± 0.09	0.430 ± 0.05	2.46
	NaCl	0.653 ± 0.07	1.142 ± 0.10	0.475 ± 0.03	2.41
4 d D	Control	0.495 ± 0.05	0.920 ± 0.09	0.358 ± 0.02	2.56
	NaCl	0.420 ± 0.04	0.750 ± 0.08	0.305 ± 0.03	2.45

Discussion

During salt stress proline accumulation in the leaves of barley seedlings was stimulated by light and suppressed in the dark. It was established that proline synthesized in the light period is not translocated from the leaves to the roots. Obviously it is degraded or converted to the other metabolites. Proline might be incorporated into protein or

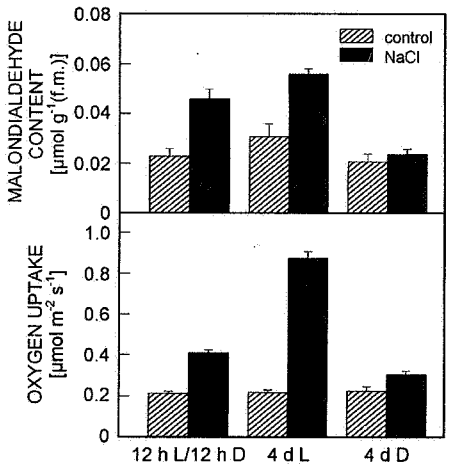

Fig. 2. Malondialdehyde content and oxygen uptake in barley seedlings treated with 150 mM NaCl under different light regimes: 12 h light (L)/12 h dark (D); 4 d L; 4 d D in comparison with untreated plants. Means ± SE of 3 separate experiments.

oxidized to other amino acids, organic acids and CO_2. Since energy is not generated photosynthetically in the dark, it is conceivable that proline accumulated in the light is used as a substrate of the cell respiration. The role of proline in the cells is not clear so far, in spite of many investigations on proline accumulation. We demonstrated that during salt stress in alga (Fedina and Benderliev 2000) and drought stress in barley leaves (Fedina and Popova 1996) proline accumulation was stimulated by light. According to Sanada *et al.* (1995) degree of stress increases in the light and plants accumulate proline as an osmolyte. Like proline, the extent of NaCl promoted free radical generation was higher in the light than in the dark. This lead us to believe that NaCl induced proline accumulation have an important role in protecting plants against salt promoted peroxidative processes. Proline might have the capacity to scavenge and/or to reduce the production of free radicals. The data in the present study show that light definitely influenced accumulation of Na^+ and Cl^- ions. There is a relationship between ions content and proline accumulation in stressed seedlings, but light additionally has effect on proline content. We observed that oxygen uptake is stimulated several times by accumulated proline. These results suggest that proline might be used as a substrate for dark respiration to supply energy to compartmentation of ions into vacuole in the dark. Stewart (1972) reported that proline was used as a carbon source for Krebs cycle in excited bean leaves in the dark. We found that salt stress slightly decreased the photochemical activity of PS 2. The decline in the intrinsic efficiency of PS 2 photochemistry, monitored as Fv/Fm, was due to a decrease in the maximum chlorophyll fluorescence yield Fm, whereas the dark fluorescence yield F_0 was not influenced. Fm values represent the reduction degree of the PS 2 on acceptor side (Q_A, Q_B, PQ pool). Q_A reduction is controlled by two factors: the rate of electron transport affecting the photochemical reaction of the intersystem electron carrier pool and the quantum distribution of the excitation light within the photosynthetic apparatus affecting the balance in activity of PS 1 and PS 2. Moreover, we also found a tendency for decreasing the non-photochemical fluorescence quenching qN. The qN mechanism is a part of the back regulation system which is related to the proton gradient and controls the primary light reactions. The decline in qN in stress conditions was related to decreased proton gradient formation across the thylakoid membrane (Pastenes and Horton 1996). Since light and dark reactions of photosynthesis are tightly coupled, it could be proposed that lower CO_2 assimilation at salt stress conditions (Fedina and Popova 1996) will cause a decreased demand for NADPH and ATP in the chloroplast, which may cause a down-regulation of the photosynthetic electron transport system. It should be

noted that the reduction in PS 2 activity in our experimental conditions was not significant. It could be proposed that proline acting as a scavenger of free radicals (Saradhi *et al.* 1995) could protect the photosynthetic apparatus from salt stress. Free radical generation is one of the initial cytochemical responses of plants stress. Malondialdehyde is a major cytotoxic product of lipid peroxidation and acts as an indicator of free radical production. Changes of proline content in the light and darkness suggested that it have some other functions in addition to serving as an osmolyte. It is possible that proline accumulated in the light to be used as a substrate for dark respiration.

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