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Response of NaCl-Adapted and Unadapted *Azolla pinnata-Anabaena azollae* Complex to Salt-Stress: Partial Photosynthetic Processes and Respiration

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Abstract

Chl *a* and *b* content in adapted complex, *Azolla pinnata-Anabaena azollae* was almost unchanged in response to 60 mM NaCl, while the values changed in the unadapted complex, resulting in an altered chl *a/b* ratio of the latter. Salinity increased the photosynthetic O_2 evolution in unadapted as well as adapted complex. However, the stimulation was rapid in the unadapted complex and reached its optimum within 12 h, whereas a gradual increase was evident in the adapted complex. Exposure of the unadapted complex to 60 mM NaCl resulted in enhanced PS II (27%) and PSI (10%) electron transport activity, while a decrease of 45% was evident in the whole chain activity. The complex adapted to 60 mM NaCl showed a reduction of 22% in PSI electron transport activity relative to that of the control, but appeared to adjust the PSII and whole chain electron transport activity. The effect of NaCl on *in vitro* electron transport activity showed that the PSII electron transport activity was most sensitive to NaCl, followed by PSI and cyt b_6-*f* activity. A moderate salinity of 20 mM NaCl was found stimulatory to PSI electron transport activity. The unadapted complex stressed with NaCl respired at higher rates in comparison to the control and the adapted complex incubated in the presence of NaCl.

Keywords: *Azolla pinnata-Anabaena azollae*, chlorophyll content, photosynthetic O_2 evolution, electron transport activity, respiration, NaCl

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1. Introduction

Azolla, a small aquatic heterosporous fern having the cyanobiont *Anabaena azollae* in symbiotic association, floats freely on the surface of water. It has a global distribution and occurs in freshwater habitats of tropical, subtropical and warm-temperate regions. *A. pinnata* used in the present investigation is found in most of Asia and the coast of tropical Africa (Van Hove, 1989).

To-day's concern of environment conservation and renewable sustainable resources combined with increased productivity and environmentally sustainable agricultural practices has made *Azolla-Anabaena* symbiosis agronomically outstanding. This is due to the ability of *Azolla-Anabaena* symbiosis to fix dinitrogen at substantial rates combined with high productivity (doubling time approximately 2 days) (Watanabe et al., 1977). Among the types of organisms that fix dinitrogen, legumes and *Azolla-Anabaena* symbiosis have the highest rates per unit area and the latter is capable of fixing dinitrogen even at higher rates (1100 kg N ha^{-1} $year^{-1}$) than the former (400 kg N ha^{-1} $year^{-1}$) (Hall et al., 1995). Rice paddy fields are an ideal environment for *Azolla* as both require flooded habitat. In addition, Wagner (1997) has counted many uses of *Azolla-Anabaena* symbiosis; animal feed, human food, a medicine, hydrogen fuel, production of biogas, water purifier, weed control and reduction of ammonia volatilisation after chemical nitrogen application and called it "green gold mine".

While nitrogen is an important limiting nutrient, salinization of the soil due to extensive irrigation of high water-demanding crops like rice and sugarcane is one of the major agricultural problem limiting crop productivity. Salinity reduces the growth of glycophytes (salt-sensitive species) which is often accompanied with the decreased rate of photosynthesis (Robinson et al., 1983; Seemann and Critchley, 1985), but the extent of this decrease depends upon the concentration of salt and the plant type. *Azolla-Anabaena* symbiosis, although offering immediate prospects for improving agricultural productivity, their resistance to salinity has rarely been tested (Moore, 1969; Zimmerman, 1985; Rajarathinam and Padhya, 1988, 1989; Rai and Rai, 1999). We have previously reported (Rai and Rai, 1999) that *Azolla pinnata* is extremely sensitive to NaCl and cannot withstand NaCl concentration beyond 30 mM and have adapted the complex giving stepwise transfers to grow at 60 mM NaCl.

Most of the crop plants can tolerate the salinity of about 50–100 mM NaCl (Downton, 1984). The *Azolla-Anabaena* association is different in the sense that it can tolerate NaCl concentration below 30 mM and is thus, highly sensitive to NaCl. Secondly, the energy (ATP), reductant and the photosynthates for N_2-fixation (to the cyanobiont) is provided by the host, ie., *Azolla* (Ray et al., 1978). Consequently, the host photosynthetic process is highly burdened in the case of *Azolla-Anabaena* complex than any other plant.

In the complex, the cyanobiont *A. azollae* accounts for 7.5–15% of the total chlorophyll (Peters and Mayne, 1974) and contributes as little as 2–5% of the association's total photosynthetic capability (Kaplan and Peters, 1988). Since the total isolation of *A. azollae* from the host is very difficult, in the present experiment we have not taken in account the contribution of individual partners but the complex as whole. Thirdly, the general growth of the complex is found to be comparatively more sensitive to NaCl than the N_2-fixation activity (Rai, 1998). Since the growth response is generally the outcome of net assimilation or photosynthetic activity, it is desirable to know the mechanism(s) involved in the response to the salt of such a highly sensitive plant. The reports, so far, available on the photosynthetic response of the plants to NaCl range above tolerance level of 50 mM NaCl (Seemann and Critchley, 1985). No report is available on the plant types incapable to grow at concentrations beyond 30 mM NaCl or the complex like *Azolla-Anabaena*. The role that salt adaptation plays in such a salt-sensitive plant in causing differences in photosynthetic processes/metabolism has not been worked out. Therefore, in an attempt to understand the mechanism(s) by which the salt-adapted plants allow the cells to survive and overcome salt-stress and to know whether the photosynthetic machinery of such a highly salt-sensitive plant responds to NaCl in the same way or differently, the present study was performed on component processes of photosynthetic pathway and respiration. To approach this problem we have selected unadapted *A. pinnata* (wild strain) which is highly salt-sensitive and the adapted one capable to grow at 60 mM NaCl (Rai and Rai, 1999) to study the partial photosynthetic events responsible for salt-toxicity.

2. Materials and Methods

Plant material and growth condition

Azolla pinnata R. Br. var. *imbricata* was collected from the rice paddy field of Banaras Hindu University campus. The nutrient solution and procedures for the growth and maintenance of the fronds in batch cultures of unadapted and the fronds that are adapted to 60 mM NaCl have been described previously (Rai and Rai, 1999). The NaCl-adapted complex was maintained in 60 mM NaCl for more than seven months prior to use in experiments. Saline treatment to the unadapted fronds was imposed by adding NaCl (final concentration 60 mM) to the nutrient solution. Control refers to the wild fronds grown in standard nutrient solution without NaCl, while stressed denotes for the wild (unadapted) fronds exposed to 60 mM NaCl. The solution was changed twice a week to keep the plants in clean state and to minimise nutrient depletion. Cultures of 12 to 15 day old were used for the experiment.

Growth estimation

Fronds were collected and rinsed in aerated iso-osmotic solution of sorbitol to remove the adhering ions, blotted dry on filter paper and weighed to represent their fresh weight (FW). Dry weight (DW) was determined by placing the samples in a weighing dish and dried in a hot air oven at 90°C for 24 h to a constant weight.

Extraction and estimation of chlorophyll

A. pinnata blotted dry on filter paper was ground in 80% acetone and allowed to stand in the dark at 4°C overnight for complete extraction, and then was centrifuged at 10,000 x g for 5 min. Amount of chlorophyll *a* and *b* in the clear supernatant was determined using the redetermined specific absorption coefficients of Lichtenthaler (1987).

Photosynthetic O_2 evolution/uptake

Measurements of O_2 evolution and uptake were made using an oxygen electrode enclosed in an air-tight reaction vessel and connected to an oxygen analyzer (Digital oxygen system, model-10, Rank Brothers, UK). A known amount of *A. pinnata* fronds (approximately 100 mg fresh weight) was placed in the reaction vessel containing 4.5 ml of the nutrient solution. The temperature of the vessel was maintained at 26°C by circulating water in the jacketed chamber around the reaction vessel. Light was provided using a projector lamp. O_2 evolution was recorded for 3 min and expressed as μmol g fresh wt^{-1} h^{-1}.

Isolation of chloroplasts

Exponentially grown *A. pinnata* fronds were harvested, washed twice with tricine-NaOH buffer (50 mM, pH 7.5) and stored at 4°C overnight. The fronds were then transferred into a tube containing chilled (N-[2-hydroxyethyl] piperazine-N'[2-ethanesulfonic acid]) (HEPES)-NaOH buffer (0.5 M, pH 7.5) and placed in an ice-bath. The fronds were sonicated (Sonicator XL 2020, Heat Systems Inc., USA) at power output adjusted to 80% of the maximum for a period of 30 seconds. The sonicated samples were examined under a microscope for the degree of cell breakage. Isolation and washing of chloroplasts were done by centrifugation. The cell-free preparation was than transferred into a beaker and stored in an ice-bath for 2–3 h before use.

Electron transport activities

The different components of photosynthetic electron transport activity was determined as described by Droppa et al. (1987).

The reaction mixture contained 50 mM HEPES buffer (pH 7.5), 0.1M D-sorbitol, 4 mM $MgCl_2$, 20 mM NaCl, 10 mM K_2HPO_4, 2 mM EDTA and chloroplast equivalent to 101 µg chlorophyll per sample. Whole chain electron transport activity was measured by the addition of $K_3Fe(CN)_6$ together with 2 mM NaN_3. For PS II electron transport activity 0.25 mM p-benzoquinone (pBQ) was added. PS I electron transport activity was measured by using 40 µM 2,6-dichlorophenol-indophenol (DCPIP) and 2 mM ascorbate (Izawa, 1980). The uncoupled electron transport rate was measured by adding 10 mM methylamine to the samples.

Respiration

Rate of respiration (defined as O_2 consumption in the dark) was determined by measuring total O_2 consumed in the dark for a given period of time minus non-specific O_2 uptake. To determine the non-specific O_2 uptake, the consumption of O_2 was measured in the presence of antimycin A (2 mM) (Grant, 1978).

3. Results

Chlorophyll content

Chl *a* content remained almost unchanged throughout the incubation period in control and adapted fronds, while in NaCl-stressed fronds followed a declining trend immediately after incubation (6 h) and reduced by 38% at 48 h (Table 1). Similarly, Chl *b* content also did not show any noticeable change in control and adapted complex. However, the complex stressed with NaCl exhibited a drastic reduction of 40% at the initial phase of incubation (6 h), attempted to recover by 12 h, but then declined (57% at 48 h of incubation to that of initial value).

Chl *a/b* ratio was slightly lower in adapted fronds (1.46 to that of 1.63 in control), probably because of the lower chl *a* content. In contrast to control and adapted fronds, the ratio (chl *a/b*) in NaCl-stressed unadapted fronds increased by 1.5 fold during the initial incubation period (6 h), but then approached the initial value by 12 h, only to shoot up again (43% at the end of incubation, 48 h). It is surmised that the presence of NaCl in the nutrient solution affected the chlorophyll content and purturbed the chl *a/b* ratio of the unadapted fronds but not of the adapted.

Oxygen evolution

Incubation of parent fronds in fresh nutrient solution increased the rate of O_2 evolution after 8 h (Fig. 1). When these parent fronds were incubated in medium

Table 1. Chl *a*, *b* content (mg.g FW^{-1}), and its ratio in *Azolla pinnata-Anabaena azollae* complex incubated in standard nutrient solution (control), supplemented with 60 mM NaCl (stressed) and the NaCl-adapted complex (incubated in the presence of 60 mM NaCl). Values are means of five replicates ± SD.

Time (h)	Chl *a*			Chl *b*			Chl *a/b*		
	Control	Stressed	Adapted	Control	Stressed	Adapted	Control	Stressed	Adapted
0	0.33 ±0.006	0.33 ±0.006	0.296 0.01	0.203 ±0	0.203 ±0	0.203 ±0	1.625 ±0.02	1.625 ±0.02	1.458 ±0.09
6	0.312 ±0.008	0.295 ±0.003	0.310 ±0.008	0.199 ±0.002	0.121 ±0.009	0.213 ±0.005	1.56 ±0.03	2.438 ±0.09	1.455 ±0.05
12	0.307 ±0.002	0.297 ±0	0.289 ±0.01	0.204 ±0.009	0.184 ±0.004	0.192 ±0.003	1.504 0.09	1.614 0.04	1.50 ±0
24	0.327 ±0.004	0.213 ±0	0.294 ±0.01	0.216 ±0.001	0.15 ±0	0.199 ±0.004	1.513 ±0.14	1.42 ±0	1.477 ±0.04
48	0.349 ±0.01	0.206 ±0.003	0.283 0.008	0.219 ±0.009	0.088 ±0.001	0.197 ±0.002	1.593 ±0.17	2.34 ±0.06	1.43 ±0.02

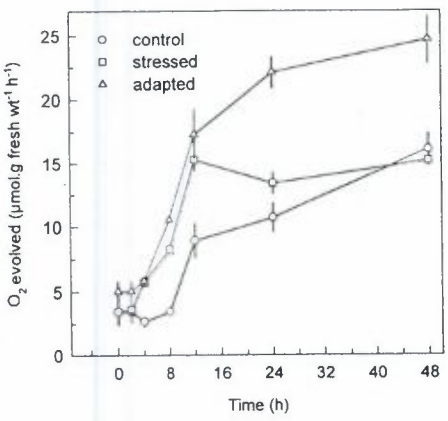

Fig. 1.

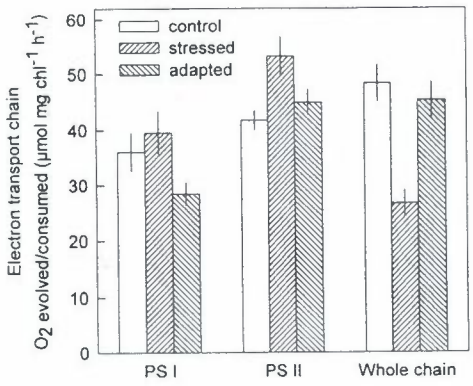

Fig. 2.

Figure 1. Photosynthetic O_2 evolution by control, stressed and adapted (60 mM NaCl) *A. pinnata* in response to incubation time. Control fronds were incubated in standard nutrient solution, while medium for stressed was standard nutrient solution + 60 mM NaCl, and in the case of adapted fronds full strength Hoagland's solution + 60 mM NaCl with Na^+/Ca^{2+} ratio of 30. The fronds were incubated under standard growth conditions in light. Samples were withdrawn at regular intervals (0–48 h) and O_2 evolution measured as in Materials and Methods. Values are mean of five replicates ± SD.

Figure 2. Electron transport chain activity (PSI, PSII and whole chain) of chloroplast isolated from control, stressed and adapted *A. pinnata*. Incubation conditions were the same as in Fig. 1. After 48 h of incubation, fronds were collected and used for the isolation of chloroplasts and the electron transport activity measured as in Materials and Methods. Values represent mean ± SD of five replicates.

containing 60 mM NaCl, an increase in the rate of O_2 evolution was observed even at 4 h and attained its optimum by 12 h, after that the rate stabilised. The adapted fronds in the presence of NaCl showed a gradual increase in O_2 evolution rate and saturated somewhere near 48 h. Further, the adapted fronds displayed the highest rate for O_2 evolution compared to the control and stressed fronds. It was also evident that the rate of photosynthesis increased maximally within 4 to 12 h in the presence of 60 mM NaCl (stressed and adapted fronds), whereas in control plants maximum increase was observed between 8 to 12 h of incubation (Fig. 1).

Electron transport chain (in vivo)

PS II electron transport activity was higher by 27% in stressed, followed by

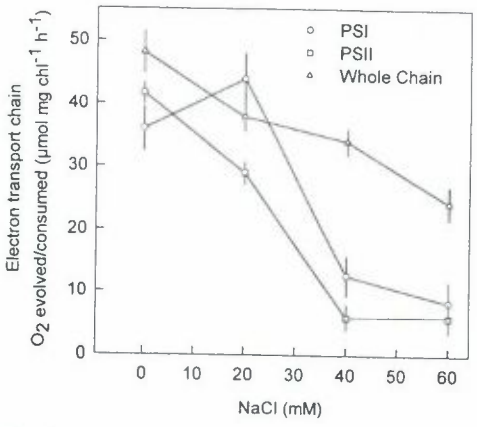

Fig. 3.

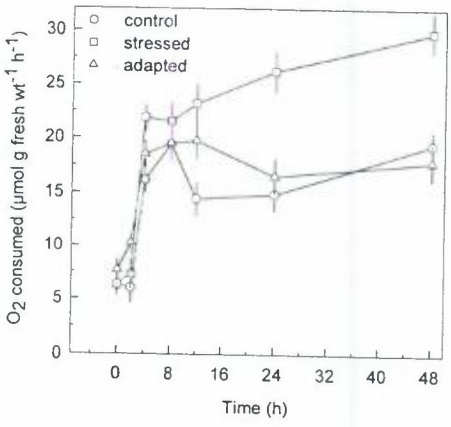

Fig. 4.

Figure 3. *In vitro* electron transport chain in isolated chloroplasts of *A. pinnata* in presence of various NaCl concentrations. *A. pinnata* grown on standard nutrient solution was used for chloroplast isolation. Values are mean ± SD of five replicates.

Figure 4. Rate of respiration (O_2 consumption) in control, stressed and adapted (60 mM NaCl) *A. pinnata* in response to incubation time (0–48 h). The incubation conditions were identical to those in Fig. 1. Data represent mean ± SD of five replicates.

adapted fronds (8%) in comparison to control (Fig. 2). The data on whole chain electron transport indicated minimum value (55% of the control) for stressed fronds, and almost identical rate for the control and adapted ones. In contrast, PSI electron transport activity showed highest value for stressed fronds (110%) and minimum (79%) for the adapted in comparison to the control. This reveals that the exposure of the unadapted complex to NaCl increases PSII and PSI electron transport activity and decreases the whole chain activity, while in adapted fronds there is an inhibition (22%) of PSI electron transport chain activity with almost no change in PSII and the whole chain electron transport activity.

Electron transport chain (in vitro)

In absence of NaCl, the rate of electron transport activity was maximum for the whole chain, followed by PSII and PS I (Fig. 3). Addition of NaCl in the reaction mixture inhibited the activity of all the three electron transport chains. PSII electron transport activity appeared to be highly sensitive to NaCl and was reduced by 30% at 20 mM NaCl and 86% at 40 and 60 mM NaCl.

Whole chain electron transport activity was also inhibited but less than the PS II activity (21–50% at 20–60 mM NaCl). NaCl at 20 mM stimulated the activity of PS I electron transport chain by 22%, but higher concentrations, 40 and 60 mM inhibited the activity drastically as in the case of PS I, indicating that a moderate salinity (20 mM NaCl) is stimulatory to PSI activity of the fronds.

Respiration

Respiration rate of the parent fronds incubated in fresh standard nutrient solution increased rapidly and was more than double of the initial rate at 8 h of incubation, then it was almost steady till the end of incubation (Fig. 4). Exposure of the parent to 60 mM NaCl enhanced the rate of O_2 consumption more markedly than that in the absence of NaCl, and in contrast to the control, the increasing trend in NaCl-stressed continued till the end of incubation approaching 3.75 fold higher than the rate observed at the beginning of incubation. The fronds adapted to 60 mM NaCl showed almost the same trend as that of the control with slightly higher values. This indicated that the parent fronds when stressed with NaCl respired at 54–70% higher rate in comparison to the parent incubated in absence of NaCl and adapted fronds growing on 60 mM NaCl.

4. Discussion

The results reveal that the response of chl *a* and *b* to salinity in unadapted *A. pinnata-A. azollae* complex differed markedly. A marginal reduction in chl *a* content (10%) during the first 12 h of exposure progressed with the length of exposure time, resulting in 38% reduction by 48 h. On the other hand, chl *b* showed a rapid decrease in the first stage (6 h), proceeded with rapid recovery (12 h), only to be followed by a rapid decline. Possibly, the chlorophyll degradation induced in the latter phase is the result of salt build-up. Presence of NaCl disturbed the chl *a/b* ratio of the unadapted fronds, indicating that the photosynthetic apparatus in the unadapted fronds was impaired by salt-stress. The NaCl-adapted fronds as expected, exhibited almost no disturbance in the chl *a* and *b* contents when growing under salinity. The value of chl *a/b* ratio in the adapted fronds was slightly lower than the control but remained unaffected by salinity. This indicates that the photosynthetic apparatus of the adapted fronds is conditioned to the salt stress. Seligmann and Amzallag (1995) have pointed out that the process of adaptation involves a transition towards new physiological mode integrating the perturbations.

When compared with control, salt stress during the first 12 h of exposure

induced an early and marked stimulation in photosynthetic O_2 evolution by unadapted fronds. In the later phase, it almost stabilised in the unadapted fronds, while the increase was continuous in the adapted. Stimulation of O_2 evolution has been observed in *Brassica juncea* at 200 mM NaCl (Alia and Saradhi, 1992). In *Acrostichum aureum* capable to grow up to 172 mM NaCl, the rate of O_2 evolution increased up to 120 mM NaCl. High oxygen evolving activity of NaCl-adapted fronds favours the observations of Murata et al. (1994) that the thylakoid membranes of NaCl-adapted cells have high oxygen-evolving activity. Higher photosynthetic rate of adapted plants compared to control may be attributed to larger investment of energy in the reorganized cellular metabolic processes as a result of salt adaptation.

Comparison of the salt-induced response of electron transport activities from unadapted and adapted fronds produced interesting differences. In unadapted fronds NaCl stimulated both the PSI (10%) and PSII (27%) electron transport activity but reduced the whole chain electron transport (45%). This indicated that the NaCl-induced damage was located near the linkage of PS II and I reaction centres, probably at Q_B and/or cyt b_6-f complex, the latter an assembly of several integral membrane proteins. *In vitro* analysis showed that NaCl inhibited all the photosynthetic electron transport chain activity, PSI, II and cyt b_6-f in decreasing order. Thus, the *in vivo* stimulation of both the PSI and II electron transport activity in unadapted fronds under salinity might be due to some protection mechanism provided by the intact cells. Stimulation in PSI activity at moderate salinity of 20 mM NaCl in the *in vitro* experiments favours the observations of Murakami et al. (1997) that Na^+ stress results in higher PSI/PSII stoichiometry. Reports available on the photosynthetic electron transport in response to NaCl are not consistent. Li and Ong (1998) reported an increased electron transport rate and primary photosynthetic reactions of PS II in *A. aureum* at 172 mM NaCl. The rate of electron transport chain in PSI and II was stimulated in *Medicago sativa* and *Amaranthus hypochondriacus* grown at 50 mM NaCl (Gonzàlez et al., 1990). *Sorghum bicolor* seedlings grown at concentrations lower than 100 mM NaCl also showed stimulation of electron transport (Masojidek and Hall, 1992). Stimulation of whole chain electron transport was also observed in *Brassica juncea* at 200 mM NaCl (Alia and Saradhi, 1992). On contrary, Guenther and Melis (1986) have concluded that salt-stress does not affect the rate of electron flow in spinach thylakoids (capable to grow at 200 mM NaCl). Mishra et al. (1991) found that in wheat both the activities of PS I and whole chain electron transport were not affected by salt stress (600 mM NaCl). Belkhodja et al. (1999) have also observed that the PS II efficiency in barley leaves did not change significantly with salinity.

Fronds adapted to 60 mM NaCl, though showing a decrease of 22% in PSI electron transport, appeared to adjust their PSII and whole chain electron

transport activity. The decrease in chl *a/b* ratio in adapted fronds indicates a reduced reaction centre in PSI and may be responsible for reduction in PS I electron transport. An increased PSII electron transport activity and reduced chl *a/b* ratio in adapted fronds indicates an increased PSII reaction centre. Probably, the requirement of extra energy under NaCl stress might have modified the thylakoid electron transport system of the adapted fronds. Molitor et al. (1990) have reported that Na^+ stress may cause regulation of the electron turnover capacity of the thylakoids.

Incubating the fronds in fresh nutrient solution either in saline or standard nutrient solution rapidly increased the rate of respiration during the initial incubation period. This is probably due to the increased demand of energy for the synthesis of structural components of the actively growing cells, which in addition to photosynthesis is met by the utilization of endogenous reserves. The unadapted fronds, when stressed with NaCl respired at a higher rate than the fronds incubated in standard nutrient solution or the adapted fronds in saline medium. This is because of the rapid utilization of the endogenous reserve to cope with unfavourable conditions for growth through increased respiration. Higher rate of respiration than the normal value in response to salinity has been described in many plants (Schwarz and Gale, 1981; Bloom and Epstein, 1984; Rawson, 1986; Li and Ong, 1998).

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