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Role of Calcium in the Osmoregulation under Salt Stress in *Dunaliella salina*

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Involvement of calcium in signal transduction of salt stress was investigated in 1.7 M NaCl adapted *Dunaliella salina*, extremely halotolerant, unicellular green alga. When hyperosmotic (3.4 M NaCl) or hypoosmotic (0.8 M NaCl) stress was treated, extracellular calcium was influxed in or intracellular calcium effluxed from *D. salina*, respectively, and these fluxes were proportional to the degree of stress. This might indicate indirectly that the change of calcium level occurred within the cells. In addition, the change of calcium flux was ahead of glycerol synthesis which has been known as the physiological response to salt stress. Osmoregulation was affected by extracellular calcium concentration, and increase of glycerol content as an osmoticum was inhibited about 50% by treatment of TFP and W-7 known as calmodulin specific inhibitors. Furthermore, in the case of the hyperosmotic stressed cells, the amount of 21 kD and 39 kD protein appeared to be calcium binding protein were increased. Among these, the 39 kD protein was detected only in the hyperosmotic stressed cells. The results obtained in the present work suggest that the possibility of calcium as a second messenger in the transduction of salt stress signal exists in the osmoregulation system of *D. salina*.

Keywords : *Dunaliella salina*, salt stress, osmoregulation, calcium, calcium-binding protein

All living organism has built-in regulatory mechanisms by which they transduce extracelluar signals into intracelluar events. The biochemical basis of transduction of extracellular signals has been extensibly studied in animal cells, where signaling systems use second messengers such as cAMP, calcium and cGMP (Berridge and Irvine, 1984; Rasmussen and Barett, 1984). In plants, efforts by plant physiologists to establish a second messenger role for cAMP in transducing extracellular signals have not been successful (Marme, 1982; Veluthambi and Poovaiah, 1984). But the importance of calcium in plant growth and development, including cell division, cell elongation, gravitropism, senescence, abscission, enzyme secretion and spore germination, has been known for years and recently enough evidences has accumulated to suggest that calcium acts as a second messenger in the transduction of extracelluar signals in plants (Marme, 1982; Hepler and Wayne, 1985; Poovaiah, 1985). Many enzymatic and physiological processes have been reported to be under the regulatory control of calcium. Furthermore, a number of physiological responses elicited by primary signals such as light, gravity and hormones can be induced by manipulating cellular calcium level (Ranjeva *et al.*, 1984). Therefore it is believed that changes in cytosolic calcium levels could lead to alterations in cellular processes which eventually result in a physiological response.

Though, there have been exhausted study on the mechanism of osmoregulation upon salt stress, it is not known exactly. The vital mechanisms for maintaining osmotic equilibrium in living cells are most easily studied in the extreme cases of halotolerant or halophilic microorganism. Eucaryotic unicellualr green algae *Dunaliella salina* (Volvocales, Chlorophyceae) can adapt to very wide range of salt concentrations (0.5-5.5 M NaCl) (Brown and Borowitzka, 1974) and thus affords a convenient model system for investigating the molecular mechanisms of osmoregulation. *D. salina* adjust to the external salinity

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by accumulating glycerol as osmolytes and compatible solutes (Ben-Amotz and Avron, 1973). At constant salinity, the turn over late of glycerol pool is relatively slow (Goyal *et al.*, 1986). But when subjected to a hypoosmotic or hyperosmotic shock, the cell reacts, within seconds, like osmometers, swelling or shirinking, respectively, due to very rapid water fluxes. This is followed by metabolic phases, which lasts for about a few hours (Ben-Amotz and Avron, 1973).

Even though the phenomena of glycerol synthesis or degradation following an osmotic shock has been well studied, little is known about the way in which glycerol is metabolized in the cells, and almost nothing is known about the signal triggering osmoregulation. However, in the golden brown algae *Poterioochromonas malhamensis*, osmotic regulation is mainly mediated by fluctuations in the pool size of isofloridoside and in this case, it was reported that calcium regulates the synthesis of isofloridoside (Kauss, 1983). In addition, change of cytosolic calcium level plays a important role in the osmoregulation of the epitherial cells of urinary bladder in animal (Wong and Chase, 1986). So it is expected that there is certain relationship between glycerol metabolism under salt stress and signal transduction system accompanying change of cytosolic calcium levels in *D. salina*.

In this communication, we attempt to elucidate the role of calcium in the signal transduction of osmoregulation in *D. salina*.

MATERIALS AND METHODS

Growth condition

Dunaliella salina was grown in media containing 1.7 M NaCl previously described (Pick and Chitlaru, 1991). Cultures were grown at 26℃ under continuous illumination with white fluorescent lamps at 3,800 lux with axenic air bubbling and maintained at the logarithmic growth phase.

Treatment of salt stress

Each of hypoosmotic and hyperosmotic shock was treated by the methods of Pick and Chitlaru (1991). Typically, hypoosmotic and hyperosmotic condition was formed by combining an equal volume of NaCl-free medium or 5 M NaCl cotaining medium with 1.7 M NaCl adapted suspension cells, repectively. This treatment reduced or increased the NaCl molarity of the medium from the normal of 1.7 M NaCl to 0.85 M or 3.4 M NaCl, respectively.

Measurement of Ca^{2+} flux

Efflux of Ca^{2+}

Ca^{2+} efflux was measured by detecting changes in ^{45}Ca efflux from cells following a hyper or hypoosmotic stress. Cells (approx. 2×10^7 cells/mL) were washed with growth medium, then incubated in growth medium containing $^{45}CaCl_2$ (1 μCi/mL) for 20 h at 26℃ in contiunous light. After the 200 μL of cell culture was washed with washing buffer I (NaCl 1.7 M, EGTA 10 mM, potassium phosphate 10 mM, pH 7.0) three times, salt stress was treated for 15 s. After centrifugation (15,000 rpm), the supernatent was used to measure radioactivity in liquid scintillation counter (Packard Tri-Carb 4530).

Influx of Ca^{2+}

We used the method of Pick *et al.* (1986) with some modifications. In separate experiments, cells were treated and incubated described above. For elimination of extracellular Ca^{2+} after salt stress, Dowex 50W ×8 minicolumn chromatography was performed. After packing 2 mL of the pre-equilibrated Dowex resin with washing buffer I to 5 mL of dispensible syringe, and washing with washing buffer II (glycerol 6.6 M, MOPS 10 mM, pH 7.0), 200 μL of cell culture under salt stress was loaded and eluted with 5 mL of washing buffer II, quickly. Radioactivities of eluates were measured with liquid scintillation counter (Packard Tri-Carb 4530).

Measurement of glycerol content

Glycerol was determined as follows (Pick and Chitlaru, 1989); the cells were washed twice in isotonic media. To 200 μL of the cell culture, 1 mL of periodate reagent (65 mg of $NaIO_4$ in 90 mL D.W., 10 mL of glacial acetic acid, 7.7 g of ammonium acetate) and 2.5 mL of acetylacetone reagent (247.5 mL of isopropanol, 2.5 mL of acetylacetone) in order were added and mixed. The mixtures were incubated

at 45℃ for 20 min. Optical density was determined at 410 nm and compared to calibration standards.

Treatment of TFP and W-7

Cells were prepared described above and preincubated for 2 h at 25 μM of TFP (trifluoperazine) or 50 μM of W-7 ([*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide), respectively. Then, salt was treated and glycerol content was measured.

Detection of Ca^{2+}-binding proteins by Autoradiography

SDS-PAGE (polyacrylamide gel electrophoresis) was performed using the discontinuous buffer system of Laemmli (1970) in gels containing 12.5% or 15% acrylamide. After SDS-PAGE, Elecrophoretic transfer of proteins to nitrocellulose membrane was carried out with an electrophoretic blotting apparatus (Hoefer TE50X) at 10 V/cm for 20 h at 4℃. The nitrocellulose membrane, with proteins transferred to it, was washed with a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl buffer (pH 6.5) and then incubated in the same solution with 1 mCi/L of $^{45}CaCl_2$ for 10 min at 25℃ by the method of Maruyama *et al.* (1984). The membrane was rinsed with 50% ethanol and dried for autoradiography.

Autoradiographs of ^{45}Ca-labelled proteins on the nitrocellulose membrane were obtaind by exposure of the dried membrane to Fuji-RX X-ray film for 3-7 d. After the above procedure, the proteins on the nitrocellulose membrane were then stained with Amido black 10B solution (0.1% in 45% methanol, 10% acetic acid) to estimate their respective intensities of bands.

RESULTS AND DISCUSSION

It is well known in plant and animal cells that extracellular stimuli change cytosolic Ca^{2+} level. In these cases, the changes of cytosolic Ca^{2+} level are seemed to be the first event to triggering intracellular physiological responses. In the cases of role of Ca^{2+} as a second messenger, it is known that Ca^{2+} should satisfy several criteria (Jaffe, 1980; Willianson, 1981). First, cytosolic Ca^{2+} level should respond to the extracellular stimuli and the physiological responses followed by the change of Ca^{2+} level. Second, artificial manipulation of Ca^{2+} level should induce the physiological responses. Third, the intracellular Ca^{2+}-sensing mechanism must exist and when it is inhibited, the physiological response should be inhibited. So, we attempt to elucidate the role of Ca^{2+} in the signal transduction of osmoregulation based on those criteria.

The changes of cytosolic Ca^{2+} level under salt stress

First of all, we examined the changes of cytosolic Ca^{2+} level according to salt stress. Despite the subsequent discovery of several other second messengers including cyclic nucleotides, products of inositol lipid hydrolysis, and products of tyrosine phosphorylation, Ca^{2+} still appears to be a centrally important activator in almost all cell types. Indeed, a key role for other messengers is often the modulation of Ca^{2+} fluxes. However, in the case of plants, it is not simple to measure the cytosolic Ca^{2+} level in direct (Poovaiah *et al.*, 1987), we measured the Ca^{2+} flux using $^{45}Ca^{2+}$ istope indirectly.

When hypoosmotic stress was treated, intracellular Ca^{2+} was effluxed from the cell about 3 times more than in the control (Fig. 1A). On the other hand, in the case of hyperosmotic stress, extracellular Ca^{2+} was influxed about 3 times more than in the control (Fig. 1B). These fluxes were proportional to the degree of the stress (Fig. 1C and D). This showed that the salt stress as an extracellular stimuli induced the changes of cytosolic Ca^{2+} level. In addition, the stress led to the highest influx pattern of Ca^{2+} at 10 s and then decreased gradually (Fig. 2). This indicate the rapid change of cytosolic Ca^{2+} level by extracellular stimuli and these results are similar to the previously reported Ca^{2+}-signaling system (Giloy *et al.*, 1991). In *D. salina*, the different patterns of cytosolic Ca^{2+} level according to the type of stress imply that a series of signal transduction mechanisms may exist between the changes of cytosolic Ca^{2+} level and the regulation of glycerol metabolism since hyperosmotic stress caused to synthesis of glycerol and in contrast, hypoosmotic stress led to degradation of glycerol (Ben-Amotz and Avron, 1973). Moreover, the results (Fig. 1C and D) suggest that cells have

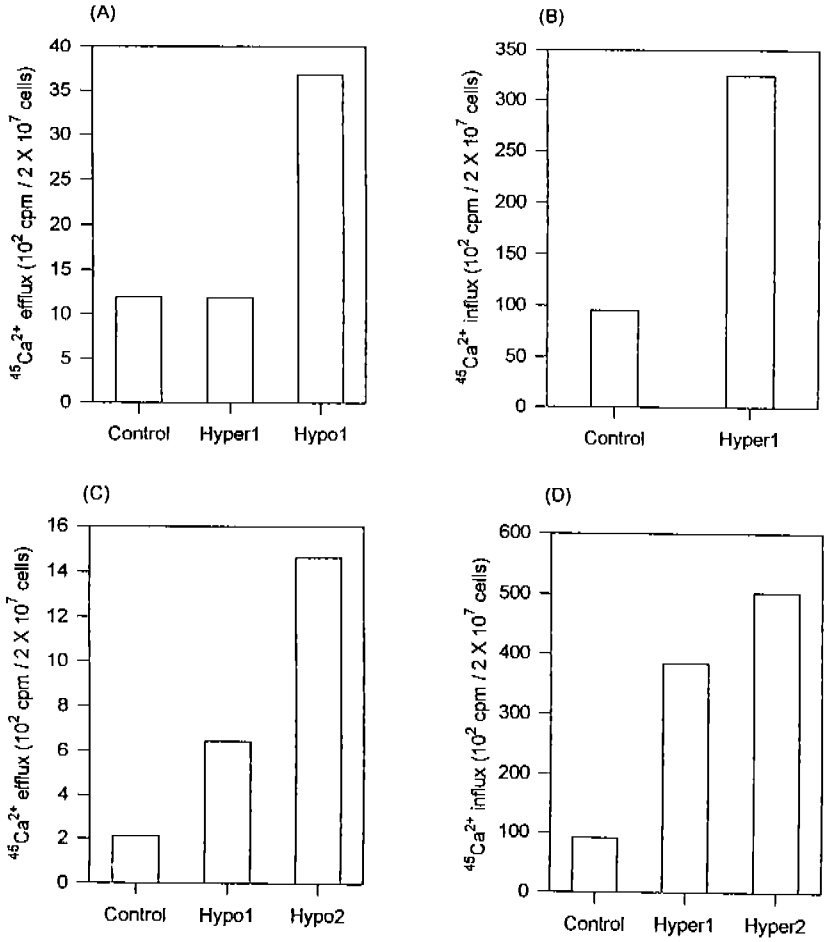

Fig. 1. Effects of osmotic stresses on $^{45}Ca^{2+}$-flux in *D. salina*. Cells were preincubated in the control medium contained $^{45}CaCl2$ (1 μCi/mL) for 20 h and were treated with the stress for 15 s. Panel A, effect of hypoosmotic stress on $^{45}Ca^{2+}$-efflux from cells; Panel B, effect of hyperosmotic stress on $^{45}Ca^{2+}$-influx into cells; Panel C, effect of hypoosmotic stress on $^{45}Ca^{2+}$-efflux from cells according to intensity of stress; Panel D, effect of hyperosmotic stress on $^{45}Ca^{2+}$-influx into cells according to intensity of stress. Control, 1.7 M NaCl contained medium; Hyper 1, treated to final 3.4 M NaCl; Hyper 2, final 5.1 M NaCl; Hypo 1, final 0.85 M NaCl; Hypo 2, final 0.56 M NaCl. Detailed methods are described in materials and methods.

a Ca^{2+} transporting system (Kubowicz *et al.*, 1982; Gross and Marme, 1978) which regulate Ca^{2+} flux by sensing the degree of salt stress. It is still unkown what mechanism could serve to recognize the degree of stress.

Changes of glycerol content and cytosolic Ca^{2+} level by salt stress

D. salina has no cell wall, so volume of cell is changed by alteration of extracellular osmotic pressure (Ben-Amotz and Avron, 1980). At this time, glycerol provides mostly the intracellular osmotic pressure for volume recovery (Ben-Amotz and Avron, 1983). So, we continued experiments to investigate the relation between changes of gylcerol contents and Ca^{2+} flux by salt stress. When hyperosmotic stress was treated, volumes of cells were recovered about 4 h and glycerol content was increased about 2 times than in the control (Fig. 3). Also, the change of Ca^{2+} flux was ahead of glycerol synthesis which has been known as the physiological response to salt stress (Fig. 2). To examine the effect of extracellular Ca^{2+} on glycerol synthesis according to salt stress, it was compared that the amounts of glycerol synthesis during hyperosmotic stress in 2.5 mM $CaCl_2$ contained normal medium and $CaCl_2$-free medium in time curves, respectively. As a result, gly-

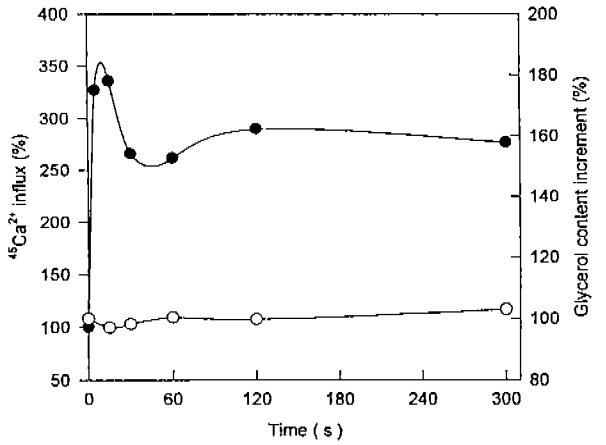

Fig. 2. Time-dependent changes of $^{45}Ca^{2+}$ influx and glycerol synthesis during hyperosmotic stress. Isoosmotic control was regarded as 100% and hyperosmotic stress was treated to final 3.4 M NaCl as described in materials and methods. ●−●, $^{45}Ca^{2+}$ influx; ○−○, glycerol synthesis.

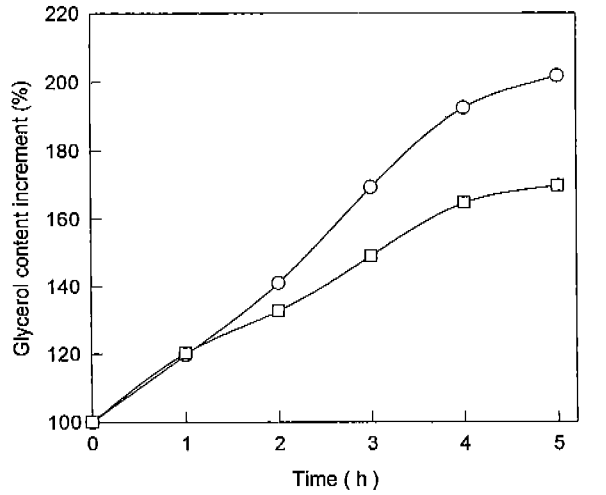

Fig. 3. Effect of extracellular calcium on glycerol synthesis during hyperosmotic stress. Isoosmotic control was regarded as 100% and hyperosmotic stress was treated to final 3.4 M NaCl as described in materials and methods. ○−○, calcium contained medium; □−□, calcium-free medium.

cerol synthesis was reduced about 30% in $CaCl_2$-free medium at 5 h than in the normal medium (Fig. 3). In the absence of external Ca^{2+}, it might be guessed that increase of cytosolic Ca^{2+} level by hyperosmotic stress is less than in the control, since there is no Ca^{2+} influx. Nevertheless, the fact that glycerol synthesis occured to some degree was guessed that Ca^{2+} efflux to cytosol from the intracellular organelles, that is, vacuole, ER, mitochondria, etc. (Dieter and Marme, 1980; Buckout, 1983; Schumaker and Sze, 1986) was served to signal in the synthesis of glycerol. But the result of the reduced glycerol synthesis in Ca^{2+} free media was also thought that the Ca^{2+} influx from exterior of cells could play a role in the signal to glycerol metabolism in hyperosmotic stress.

Effects of calmodulin antagonists

In most cases of Ca^{2+}- regulated signal transduction mechanisms, calmodulin as a calcium modulated protein play an important and essential role in coupling the change of cytosolic Ca^{2+} level to physiological responses (Cheung, 1980; Marme, 1982). It was widely known that Ca^{2+}-bound calmodulin undergo the conformational change, which caused active form, then regulate the activity of various enzymes (Klee, 1980; Poovaiah, 1985). To see whether calmodulin would participate between salt stress related changes of cytosolic Ca^{2+} level and glycerol metabolism as a osmoregulation, TFP (trifluoperazine) and W-7, known as calmodulin antagonists, were treated. In the previous experiment, the most reasonable concentrations were 25 μM and 50 μM of TFP and W-7, respectively (data not shown). Consquently, TFP and W-7 reduced glycerol synthesis about 50% at 25 μM and 46% at 50 μM under hyperosmotic stress, respectively (Fig. 4). These results suggest that clamodulin maybe involved. Calmodulin has been implicated in the volume recovery from osmotic stress in various cell types (Hoffman *et al.*, 1984; Foskett and Spring, 1985). Though it is difficult to conclude by these results, the calmodulin seems to take part in the signal transduction mechanism under salt stress.

Detection of salt stress related Ca^{2+}-binding proteins

It is widely known that calcium and Ca^{2+}- binding protein play a very important role in various cellular metabolism (Poovaiah and Reddy, 1987). And many reports show that various Ca^{2+}-binding proteins as well as calmodulin also exist in plants (Graziana *et al.*, 1984). Though the biochemical and physiological significance of Ca^{2+}-binding proteins are still unknown in detail, they seem to be related in the mechanism of signal transduction. In the fur-

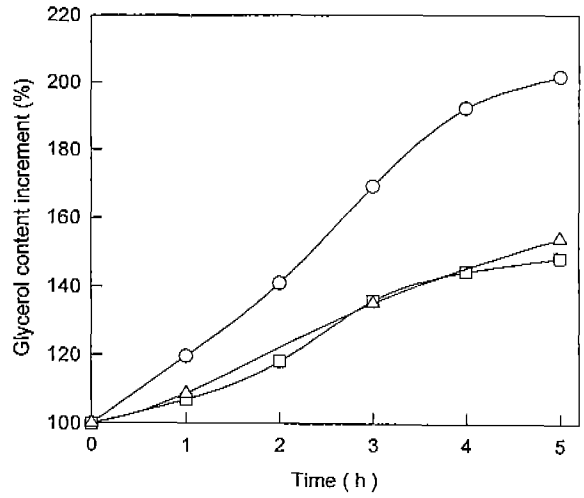

Fig. 4. Effects of calmodulin antagonists on glycerol synthesis during hyperosmotic stress. Isoosmotic control was regarded as 100% and hyperosmotic stress was treated to final 3.4 M NaCl as described in materials and methods. ○−○, Control; △−△, 25 μM of TFP treatment; □−□, 50 μM of W-7 treatment.

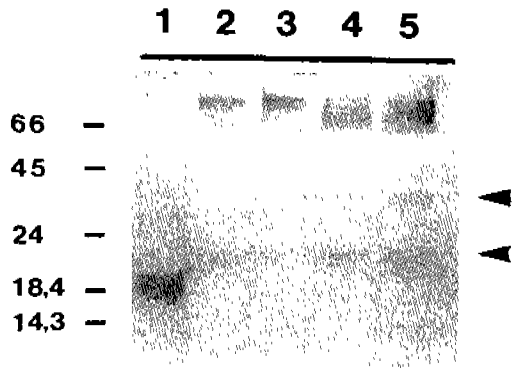

Fig. 5. Identification of calcium binding proteins using autoradiography on nitrocellulose membrane after SDS-PAGE. Calmodulin from bovine testis as a positive control (lane 1), same amounts of soluble proteins of control cells (lane 2, 3) and of hyperosmotic stressed cells (lane 4, 5). The positions of 39 kD and 21 kD calcium binding proteins are indicated by arrowheads top to bottom, respectively. Molecular size markers are indicated in left side.

ther research of Ca^{2+} signalling system induced by salt stress in *D. salina*, salt stress related Ca^{2+}-binding proteins were investigated using the method of Maruyama *et al.* (1984). Ca^{2+}-binding proteins were examined from soluble proteins of 5 h-hyperosmotic stressed cells and the control cells, respectively. In there with autoradiogram, we have detected two different Ca^{2+}-binding proteins. Their molecular weight were 21 kD and 39 kD, respectively. Interestingly, MW 21 kD was increased more in intensity in hyperosmotic stressed cells, and in contrast, MW 39 kD was detected only in hyperosmotic stressed cells (Fig. 5). The change of expression pattern of these Ca^{2+}-binding proteins by salt stress is very interesting. This means that these proteins can be participated in signal transduction of salt stress to intracellular response. In particular, the Ca^{2+}-binding protein of MW 39 kD might be regarded to relate to salt stress directly because it was detected only in salt stressed cells. However it is not certain whether the Ca^{2+}-binding protein of MW 39 kD is newly synthesized or undergo conformational change to bind calcium. It is our hope that further characterization of salt stress related Ca^{2+}-binding proteins will contribute to the understanding of the signal transduction mechanism in response to salt stress in *D. salina*.

We have preliminary indications suggesting that salt stress influenced the intracellular change of Ca^{2+} level and osmoregulation was affected by extracellular Ca^{2+} and calmodulin inhibitors, and Ca^{2+}-binding proteins related to salt stress. The results obtained in present work suggest that the possibility of calcium as a second messenger in the signal transduction of salt stress exists in *D. salina*.

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Salt Stress에 의한 *Dunaliella salina*의 滲透 助節 機作에서 Calcium 이온의 役割

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적 요

1.7 M NaCl에 적응시킨 초내염성 단세포 녹조류인 *Dunaliella salina*에서 salt stress에 따른 신호전달기작에 calcium 이온의 관여여부를 조사하였다. *D. salina*에 고삼투압성 stress (3.4 M NaCl)를 처리하였을 때 calcium이온이 세포 내부로 유입되었으며, 저삼투압성 stress (0.8 M NaCl)를 처리하였을 때는 반대로 calcium이온이 세포외부로 유출되었다. 그리고 이 과정은 고삼투압성 stress에 따른 세포내 생리적 변화인 glycerol 합성보다 시간적으로 선행하였을 뿐 아니라, stress 강도에도 비례하였다. Calcium이온이 연관된 신호 전달기작에서 key mediator로 알려져 있는 calmodulin을, 특이적 억제제인 TFP와 W-7을 처리하였을 때, 대조구에 비해 stress에 따른 glycerol 합성량이 약 50% 정도 감소하였으며, 또한 세포 외부의 calcium이온도 삼투조절기작에 영향을 주었다. 한편, 고삼투압성 stress를 처리한 경우, calcium-결합 단백질로 여겨지는 분자량 21 kD, 39 kD 단백질의 합성량이 증가하였다. 그 중에서 39 kD 단백질은 stress를 처리한 경우에만 검색되었다. 이상의 결과로부터, salt stress에 따른 *D. salina*의 삼투조절기작에 관한 신호 전달기작에서 calcium이 제2차 신호전달자로서 관여할 가능성이 존재한다고 여겨진다.

주요어 : *Dunaliella salina*, salt stress, 삼투조절기작, calcium, calcium-결합 단백질

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