Jpn. J. Crop Sci. 65(4) : 693—699 (1996)

Studies on the Mechanism of Salt Tolerance in *Salicornia europaea* L.*

III. Salt accumulation and ACh function

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Received March 2, 1996

Abstract : A basic understanding of the mechanism of salt stress tolerance in plants is crucial to the utilization of salt tolerant crop varieties. The present work was an investigation of NaCl accumulation at the organ level in *Salicornia* plants. Acetylcholinesterase (AChE) activity in various different organs was also determined, by chemical and histochemical methods, in order to determine the possibility that acetylcholine (ACh) functions in Na^+ and Cl^- transport between organelles. High NaCl accumulation occurred in roots and in lower stems. At 5 months after germination, *Salicornia* plants had accumulated approximately 160 nmol and 320 nmol/100 g fresh weight of Na^+ and Cl^- respectively in roots. High AChE activity was also observed in roots and in lower stems. The enzyme activity in stems was higher at nodes than internodes. Histochemically, AChE activity in roots was detected in the cortex, including endodermal cells around the vascular system, and strongly in endodermis, cortex and epidermis at the parting portion of lateral root from the main root. In stems, AChE activity was detected in endodermal cells around the vascular system and concentratively at the node connected with the branch in the stem. These results suggest the possibility that ion transport at the node connected with the branch in the stem, and the parting portion of the lateral root from the main root is facilitated by ACh function. Furthermore, excessive NaCl may be excreted through the epidermal cells of roots.
Key words : Accumulation of NaCl, AChE activity, ACh function, Halophyte, *Salicornia*, Salt tolerance.

***Salicornia europaea* L.の耐塩機構に関する研究**　第3報　塩の蓄積とアセチルコリン作用：桃木芳枝・小栗　秀・加藤　茂**・上村英雄（東京農業大学生物産業学部・**東京農業大学総合研究所）

要　旨：植物における耐塩機構を理解することは，耐塩作物品種の利用に重要なことである．本報では，アッケシソウにおける塩化ナトリウムの蓄積を器官レベルで測定した．さらに，Na^+ イオンおよびCl^- イオンの器官間輸送とACh作用との関係を検討するために，AChE活性を化学的定量および組織化学的検出によって器官レベルで測定した．アッケシソウにおいて高い濃度の塩化ナトリウムは，根と下位の茎に認められ，発芽5カ月後の根では，新鮮重100 g当たりNa^+ イオン160 nmol，およびCl^- イオン320 nmolがそれぞれ蓄積していた．アセチルコリン分解酵素（AChE）の活性は，同様に根と下位の茎で高く，また，どの部位の節も節間より高い活性を示した．さらに，組織化学的に検出されたAChE活性は，根および茎で顕著に認められた．根では，維管束系を囲む内皮を含む皮層細胞と，主根から側根の分岐部周辺に認められ，とくに，側根周辺の内皮，皮層および表皮細胞には強い反応が検出された．茎では，維管束に沿った内皮細胞と，主茎の側枝を持った節部で極めて強い反応が現れた．これらの結果から，主根からの側根の分岐部および主茎において側枝を持った節部でACh作用によるイオンの輸送が推定され，また，過剰の塩化ナトリウムが根の表皮細胞から排出されることが示唆された．
キーワード：アセチルコリン作用，アセチルコリン分解酵素，アッケシソウ，塩化ナトリウムの蓄積，塩生植物，耐塩性．

Palnts apparently rely on several mechanisms by which they adapt to salinity stress[6,7,11,21]. There are transport mechanisms for removal of Na^+ and Cl^- from anatomical structures, such as salt glands, bladders or specialized trichomes, in order to prevent the ions from reaching actively to growing cells[6,7]. In our previous work[18], *Salicornia* plants accumulated high concentration of Na^+ and Cl^- ions in tissues and high incipient plasmolytic concentrations of NaCl in epidermal cells. Also, osmotic pressure increased during the growth period. When the plants were grown under conditions of low salinity, the size of epidermal cells was about twice as large as epidermal cells of the wild plant. However, osmotic pressure was 35-50% lower than in wild plant cells. Binzel et al.[3] suggested that

* Part of this work was presented at the 200th meeting of Crop Science of Japan held in November, 1995.

smaller cells would inherently have an increased capacity to accumulate ions, perhaps mediated by enhanced transport capabilities as a function of the plasma membrane and tonoplast surface area to volume ratios. On the other hand, some halophytes maintain lower concentrations of NaCl than non-halophyres by apoplastic transport[22]. Ion transport between plant organs can increase as an adjustment of osmotic pressure for NaCl accumulation.

We have previously shown acetylcholine (ACh) to function as a gravitropic stimulus[13] and a leaf wilting and its recovery in heat stress[14,15] in various plants. These results improved our understanding of the gate control theory of ACh function[13], which states that ACh activates gates to open and close at plasmodesmata for transport of ions, water and hormones. If ACh could function in this manner, the presence of AChE would be required as a regulator of the plasmodesmatal junction between cells, tissues and organs for tranport of Na^+ and Cl^-.

The present work was investigated accumulation of Na^+ and Cl^- in different organs of *Salicornia* plants. Further, to test whether ACh could function in ion transport at the plasmodesmatal junction between cells, tissues and organs, AChE activity was determined by chemical and histochemical methods.

Materials and Methods

1. Plant materials

Plants of *Salicornia europaea* L. were collected in the Misaki area around the Lake Notoro-ko in the eastern region of Hokkaido, Japan. Samples were collected the end of June (3 months after germination), the end of July and the end of August, 1995. In 1995, *Salicornia* plants grew normally. The developmental stages of *Salicornia* plants during this growth period are described in detail in our previous paper[17,18]. The aerial portion of the plants was divided in upper, middle and lower parts as shown in Fig. 1.

2. Determination of $Na^+$and Cl^- ions

Accumulation of NaCl in selected organs was determined at different regions of stems, nodes, internodes, and roots. All plant materials were washed, cut and homogenized for 5 min in a blender with twice their weight of deionized water. The homogenate was filtered

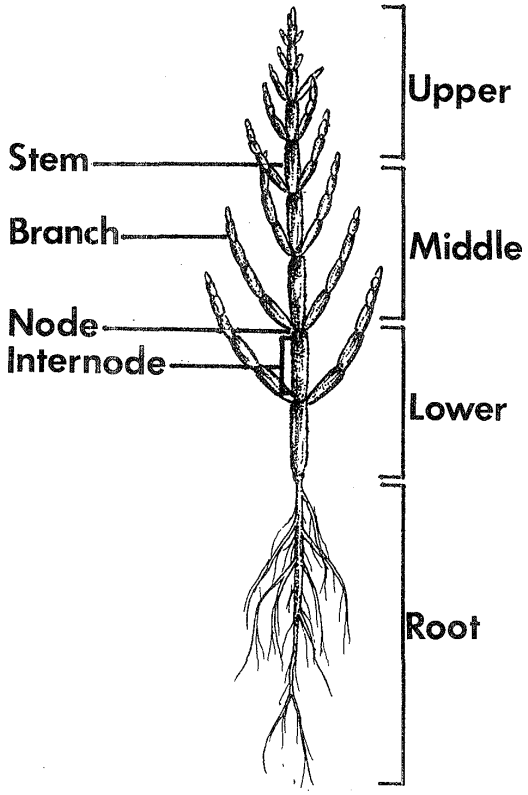


Fig. 1. The regions of organs measuring Na^+ and Cl^- ions and AChE activity in *Salicornia europaea* plants.

through 0.45 μm filter (Toyo Roshi Kaisha Ltd., DISMIC-25). Na^+ and Cl^- ions of the homogenate were analyzed by ion chromatography (Shimazu, IC-6A). The eluent for Na^+ was 5 mM NHO_3 solution and IC-Cl was used as the column. The Cl^- ion was eluted with 1 mM p-hydroxybenzoic acid and 1.1 mM N, N-diethylethanolamine solution using IC-Al as the column.

3. Determination of AChE activity by SH appearance

Determination of AChE activity was based on the colorimetric SH reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), as used by Asahi et al.[1] and Ellman et al.[5]. AChE activity was determined in stems at 3 regions and in roots. Harvested samples were cut 5 mm length and incubated for enzyme reaction. Details of reagent preparation and enzyme assay using acetylthiocholine iodide as a substrate of AChE were described in our previous paper[13]. Neostigmine (35 μM) was used as an inhibitor of AChE. Ten plants were used for each experiment.

4. Histochemical detection of AChE

The *Salicornia* plants were collected the end

of August. The cross sections were made from the middle part of stems and the upper part of main roots. The sections were made 100-110 μm thick with a Microslicer (Dosaka, DTK-1000). The preparation of reagents and enzyme assay were conducted following a modified method of Koelle[9,10], as described in detail in our previous papers[16,19]. The activity of AChE in plant cells was detected at the level of light microscope.

Results

Concentration of Na^+ and Cl^- ions in the tissues of *Salicornia* plants increased over a 2 month period from 50 to 90 nmol per 100 g fresh weight and 120 to 240 nmol per 100 g fresh weight, respectively (Table 1). High concentrations of Na^+ and Cl^- ions were found in roots and in lower region of stems. The concentration of Cl^- ions in all regions sampled was more than double concentration of Na^+.

AChE activity in various organs of *Salicornia* plants was measured by SH appearance following hydrolysis of acetylthiocholine is shown in Table 2. The highest AChE activity was found in roots. AChE activity in roots was 3-5 fold higher than that in the aerial portion of the plant at the youngest (3 months after germination). The enzyme activity in stems was highest in the lower region of stems at 5 months after germination. AChE activity at nodes in all regions was higher than at the internodes (Table 3). AChE activity was inhibited approximately 85% by 35 μM of neostigmine bromide as a inhibitor of AChE (data not shown).

Histochemical evidence of the AChE reaction in stems was found in whole endodermal cells between the cortex and stele around the vascular system (Fig. 2, A and B). In particular, evidence for the AChE reaction was concentrated at the node connected with branch in stem (Fig. 2, B). In roots, the AChE reaction was detected strongly in cortex, endodermis and epidermis around the lateral root (Fig. 2, D and E). Although whole endodermal cells between stele and cortex around vascular system showed AChE activity, the reaction was not equal with mixed strong and

Table 1. Concentration of Na^+ and Cl^- ions in various regions of *Salicornia* plants.

Month after germination	Concentration of ions (mmol $100g^{-1}$ fresh wt.)				
		Aerial portion			Root
	Ion	Upper	Middle	Lower	
3 (6/30)	Na^+	45±5	56±6	72±4	84±3
	Cl^-	116±8	143±4	166±6	180±5
5 (8/30)	Na^+	89±3	107±5	146+6	163+8
	Cl^-	242±9	260±6	294+10*	323+12
	Concentration of Na^+				
	Multiple comparison between O at each M level				
M.D. (0.05)[a]	M_3[b]	O_{Us}	O_{Ms}	O_{Ls}	O_R[c]
	M_5	O_{Us}	O_{Ms}	O_{Ls}	O_R
	Concentration of Cl^-				
	Multiple comparison between O at each M level				
M.D. (0.05)	M_3	O_{Us}	O_{Ms}	O_{Ls}	O_R
	M_5	O_{Us}	O_{Ms}	O_{Ls}	O_R

Values are means of 2 experiments ±SE.
Ten plants were used for each experiment.
*Part of stems were woody like and browny.
a) Minimum difference by Turkey's procedure (q-test) for the significance at the 5% level. Any two means underscored by the same line are not significantly different. Any two means not underscored by the same line are significantly different.
b) Month level. M_3, 3 months after germination; M_5, 5 months after germination.
c) Organ level. O_{Us}, Upper stem; O_{Ms}, Middle stem; O_{Ls}, Lower stem; O_R, Root.

Table 2. AChE activity in various regions of *Salicornia* plants measured by SH appearance following hydrolysis of acetylthiocholine.

Month after germination	AChE activity (SH pmol g^{-1} fresh wt. h^{-1})			
	Aerial portion			Root
	Upper	Middle	Lower	
3 (6/30)	53±4	53±5	71±10	265±29
4 (7/30)	198±8	203±9	240±14	287±30
5 (8/30)	202±9	205±8	250±14*	325±17

AChE activity

Multiple comparison between O at each M level

M.D. (0.05)[a]

M_3[b] $\underline{O_{Us}\ O_{Ms}\ O_{Ls}}\ O_R$[c]

M_4 $\underline{O_{Us}\ O_{Ms}\ O_{Ls}}\ O_R$

M_5 $\underline{O_{Us}\ O_{Ms}\ O_{Ls}}\ O_R$

Values are means of 3 experiments ±SE. Ten plants were used for each experiments.

*Part of stems were woody like and browny.

a) Minimum difference by Turkey's procedure (q-test) for the significance at the 5% level. Any two means underscored by the same line are not significantly different. Any two means not underscored by the same line are significantly different.

b) Month level. M_3, 3 months after germination; M_4, 4 months after germination; M_5, 5 months after germination.

c) Organ level. O_{Us}, Upper stem; O_{Ms}, Middle stem; O_{Ls}, Lower stem; O_R, Root.

Table 3. AChE activity in node and internode of stem in *Salicornia* plants.

Month after germination		AChE activity (SH pmol g^{-1} fresh wt. h^{-1})		
		Aerial portion		
		Upper	Middle	Lower
4 (7/30)	Node	196±5	208±14	281±4
	Internode	168±13	168±7	174±13

AChE activity

Multiple comparison between O at each S level

	S_U[b]		S_M		S_L	
M.D. (0.05)[a]	O_{IN}	O_N	O_{IN}	O_N	O_{IN}	O_N[c]

Values are means of 3 experiments ±SE.

Ten plants were used for each experiments.

a) Minimum difference by Turkey's procedure (q-test) for the significance at the 5% level. Any two means not underscored by the same line are significantly different.

b) Stem level. S_U, Upper stem; S_M Middle stem; S_L, Lower stem.

c) Organ level. O_{IN}, internode; O_N, Node.

Fig. 2. Histochemical evidence of AChE at the node connected with branch in the middle part of stems and the upper part of roots of *Salicornia* plants. The plants were collected the end of August (5 months after germination).

A, AChE-positive spots in node of stem (×40);

B, AChE-positive spots in the node connected with branch of stem (×200);

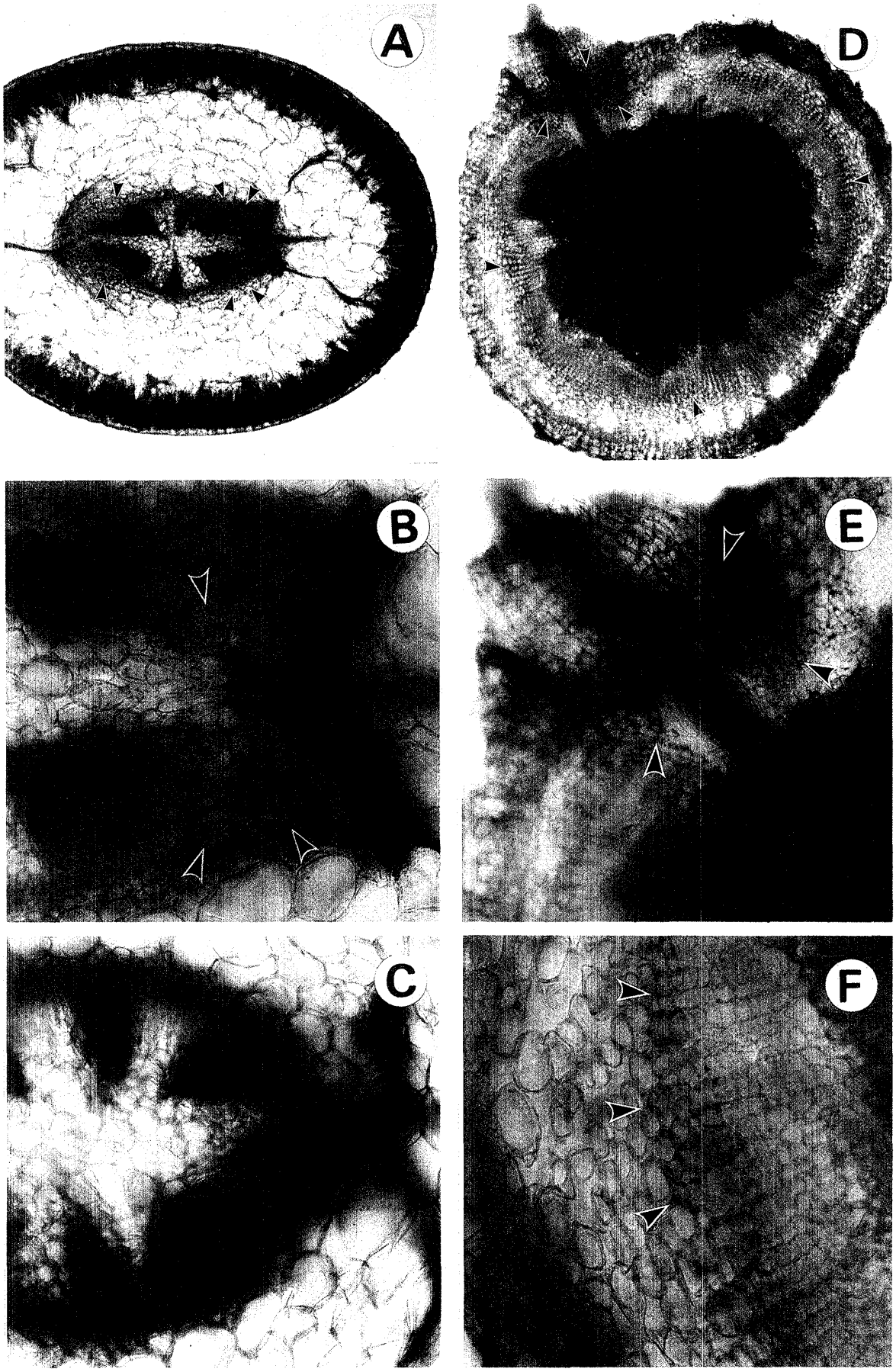
C, Negative reaction of AChE due to lacking substrate in the node connected with branch (×200);

D, AChE-positive spots in root (×70);

E, AChE-positive spots in endodermis, cortex and epidermis around the lateral root (×175);

F, AChE-positive spots in cortex including endodermal cells around the vascular system of root (×350).

The dark residues indicated by arrows show AChE-positive spots of copper sulfide after cytochemical reaction in tissues.



weak reactions (Fig. 2, F). The AChE activity in both of the organs appeared in the plasmamembrane associated with the cell wall and intercellular spaces (Fig. 2, B, E and F). No histochemical evidence of AChE was found in the tissue treated without substrate (Fig. 2, C).

Discussion

Under saline conditions, when salts reach to cells of plant organs, the cells are killed to limit the ion concentration in the cytoplasm. Plants may retain some mechanisms for prevention of salt accumulation in cells. Organic solutes such as glycinebetaine[4,8,12,20] or proline[2,23] may function as osmotically active solutes. On the other hand, Speer and Kaiser[22] suggest that apoplastic salt concentration can rise very rapidly following salinization due to the small volume of the cells encircled by cell walls. Binzel et al.[3] suggest ion transport between cells. *Salicornia* plants, under saline conditions, accumulated predominantly Na^+ and Cl^- ions[18]. The present work also showed accumulation of NaCl during the growth period in *Salicornia* plants. In contrast to the roots and lower stems, the upper and middle parts of stems maintained low NaCl concentrations. Furthermore, the concentration of Na^+ and Cl^- ions in roots and stems was high compared to other halophytes[18]. The results suggest ion transport between organs. Momonoki[13] proposed a working hypothesis of ACh in plants. Acetylcholine may function as a regulator of the plasmodesmatal junction between cells, tissues and organs. Acetylcholinesterase could exist to hydrolyze the released free ACh around the vascular system at the junction between organs. The AChE activity in roots of *Salicornia* plants at youngest stage was about 4-fold higher than aerial portion. As plants grew older, AChE activity in aerial potion increased to about 3-fold higher than the youngest stage. The enzyme activity increased in the stems located in the lower region of the plant. The AChE activity of nodes in all regions was also high compared that of internodes. By histochemical observation, the AChE in stems was detected to be concentrated in endodermis and cortex at the node connected with branch in stem. The extremely strong AChE activity in roots was detected in endodermis, cortex and epidermis around the lateral root. These results suggest the possibility that ion transport at the node connected with branch in stem and the parting portion of lateral root from the main root is facilitated by ACh function. Further, because of AChE activity detected in epidermis cells of root around the lateral root, it is speculated that ACh may function to exclude excessive NaCl from roots.

Acknowledgment

We would like to thank Dr. N. Engeseth, Plant Scientist, Department of Botany & Plant Pathology, Michigan State University for helpful comments and critical review of the manuscript.

References

1. Asahi, T., R. S. Bandurski and L. G. Wilson 1961. Yeast sulfate-reducing system. II. Enzymatic reduction of protein disulfide. J. Biol. Chem. 236 : 1830—1835.
2. Bar-Nun, N. and A. Poljakoff-Mayber 1977. Salinity stress and content of proline in roots of *Pisum sativum* and *Tamarix tetragyna*. Ann. Bot. 41 : 173—179.
3. Binzel, M. L., P. M. Hasegawa, A. K. Handa and R. A. Bressan 1985. Adaptation of tobacco cells to NaCl. Plant Physiol. 79 : 118—125.
4. Colmer, T. D., E. Epstein and J. Dvorak 1995. Differential solute regulation in leaf blades of various ages in salt-sensitive wheat and a salt-tolerant wheat x *Lophopyrum elongatum* (Host) A. Löve amphiploid. Plant Physiol. 108 : 1715—1724.
5. Ellman, G. L., K. D. Courtney, V. Jr. Andres and R. M. Featherstone 1961. A new rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7 : 88—95.
6. Flowers, T. J., P. F. Troke and A. R. Yeo 1977. The mechanism of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28 : 89—121.
7. Greenway, H. and R. Munns 1980. Mechanism of salt tolerance in nonhalophytes. Ann. Rev. Plant Physiol. 31 : 149—190.
8. Jolivet, Y., F. Larher and J. Hamelin 1982. Moregulation in halophytic higher plants : The protective effect of glycine betaine against the heat destabilization of membranes. Plant Sci. Lett. 25 : 193—201.
9. Koelle, G. B. and J. S. Friedenwald 1949. A histochemical method for localization cholinesterase activity. Proc. Soc. Exp. Med. 70 : 617—622.
10. ———— 1955. The histochemical identification of acetylcholinesterase in cholinergic, adrenergic

and sensory neurons. J. Pharmacol. Exp. Ther. 114 : 167—184.
11. Levitt, J. 1980. Responses of Plants to Environmental Stresses. Water, Radiation, Salt and Other Stresses. Vol. 2. Second edition. Academic Press, New York. 1—607.
12. Matoh, T., J. Watanabe and E. Takahashi 1987. Sodium, potassium, chloride, and betaine concentrations in isolated vacuoles of salt-grown *Atriplex gmelini* leaves. Plant Physiol. 84 : 173—177.
13. Momonoki, Y. S. 1992. Occurrence of acetylcholine-hydrolyzing activity at the stele-cortex interface. Plant Physiol. 99 : 130—133.
14. ———— and T. Momonoki 1992. The influence of heat stress on acetylcholine content and its hydrolyzing activity in *Macroptilium atropurpureum* cv. Siratro. Jpn. J. Crop Sci. 61 : 112—118.
15. ———— and ———— 1993. Changes in acetylcholine-hydrolyzing activity in heat-stressed plant cultivars. Jpn. J. Crop Sci. 62 : 438—446.
16. ———— and ———— 1993. Histochemical localization of acetylcholinesterase in leguminous plant, Siratro (*Macroptilium atropurpureum*). Jpn. J. Crop Sci. 62 : 571—576.
17. ———— and H. Kamimura 1994. Studies on the mechanism of salt tolerance in *Salicornia europaea* L. I. Changes in pH and osmotic pressure in *Salicornia* plants during the growth period. Jpn. J. Crop Sci. 63 : 518—523.
18. ————, S. Kato and H. Kamimura 1994. Studies on the mechanism of salt tolerance in *Salicornia europaea* L. II. High osmosis of epidermal cells in stem. Jpn. J. Crop Sci. 63 : 650—656.
19. ————, T. Momonoki and J. H. Whallon 1996. Acetylcholine as a signaling system to environmental stimuli in plants. I. Contribution of Ca^{2+} in heat-stressed *Zea mays* seedlings. Jpn. J. Crop Sci. 65 : 260—268.
20. Rohdes, D. and A. D. Hanson 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 44 : 357—384.
21. Sachs, M. M. and T. D. Ho 1986. Alteration of gene expression environmental stress in plants. Ann. Rev. Plant Physiol. 37 : 363—376.
22. Speer, M. and W.M. Kaiser 1991. Ion relations of symplastic and apoplastic space in leaves from *Spinacia oleracea* L. and *Pisum sativum* L. under salinity. Plant Physiol. 97 : 990—997.
23. Watad, A. A., L. Reinhard and H. R. Lerner 1983. Comparison between a stable NaCl-selected *Nicotiana* cell line and the wild type. K^+, Na^+, and proline pools as a function of salinity. Plant Physiol. 73 : 624—629.