Kasetsart J. (Nat. Sci.) 40 : 99 - 106 (2006)

Effects of Na^+, K^+ and Ca^{2+} Accumulation on the Expression of Ca^{2+}-ATPase Gene in Rice KDML 105

Wunrada Surach[1], Mingkwan Mingmuang[1] and Amara Thongpan[2]*

ABSTRACT

Having been exposed to 150 mM NaCl supplemented in the growth medium, the roots of KDML105 started to accumulate Na^+ in three hours but the leaves showed the amount of Na^+ change after one day. Addition of either 0.4 mM or 10 mM of $CaCl_2$ to the 150 mM NaCl supplemented medium, on the other hand, caused a small drop in Na^+ accumulation in the leaves and roots on day 8. K^+, however, significantly decreased in both leaves and roots as the result of NaCl in the medium. As for Ca^{2+} deposition in KDML105, NaCl supplemented alone in the medium had little effect but the combined NaCl and $CaCl_2$ caused the rise of Ca^{2+} in both leaves and roots.

The expression of Ca^{2+}-ATPase gene in the leaves of KDML105 seemed to be directly activated by the exposure to Na^+. The combined effect of Na^+ and Ca^{2+} also showed the drastic change in Ca^{2+}-ATPase expression, and rice, therefore, try to maintain Ca^{2+} homeostasis by producing more Ca^{2+}-ATPase mRNA especially in the leaves**.** Since the roots are not the main accumulation site of Na^+, the effect of NaCl alone on the Ca^{2+}-ATPase gene in the roots was not as distinctively seen. However, the addition of 10 mM $CaCl_2$ caused a large increase of Ca^{2+}-ATPase gene expression in the roots, while both Na^+ and Ca^{2+} accumulation seemed to affect more to the gene expression in the leaves.

Key words: Na^+, K^+, Ca^{2+}, rice KDML105, Ca^{2+}-ATPase gene, Ca^{2+}-ATPase mRNA

INTRODUCTION

Aromatic rice "Khao Dok Mali 105" (KDML105) is one of the most popular Thai rice, having high demand both for local consumption and international market. To have this rice cultivar widely propagated in all types of soil, i.e., high salinity, or under stress condition of drought, improvement has been continuously made on KDML105 through selection (BSU, 2001). In saline soil, NaCl can disturb ion homeostasis causing the imbalance of ions, i.e., Na^+, K^+ and Ca^{2+} which results in hyperosmotic stress and ion toxicity. So, plants have to acclimatize to survive in these conditions. One mechanism of acclimatization is to adjust osmotic pressure by ion compartmentation (Niu *et al*., 1995) where Ca^{2+}-ATPase is vitally needed.

In determining the levels of Na^+, K^+ and Ca^{2+} accumulated in different parts of KDML105 rice and how each type of ion could cause the change in Ca^{2+}-ATPase expression we would be able to understand the mechanism of salt tolerant rice and enable us to make a better plan to improve this rice cultivar.

1 Department of General Science, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

2 Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

* Cornesponding auther : e-mail.fsciart@ku.ac.th

Received date : 28/06/05 Accepted date : 19/12/05

MATERIALS AND METHODS

Plant materials

KDML 105 rice seeds (*Oryza sativa* L.) from Pathumthani Rice Research Center were germinated in pots containing sandy soil impregnated with Hoaglands' solution (Hoagland and Arnon, 1950) at room temperature for two weeks. Plantlets were transferred and grown hydroponically for a week in Hoagland's solution. The leaves were collected for RNA extraction.

Salt induction was carried out by adding 150 mM NaCl alone or having NaCl combined with either 0.4 mM or 10 mM $CaCl_2$ to Hoaglands' solution. Seedlings were maintained in this hydroponic culture for 8 days.

Measurement of Na^+, K^+ and Ca^{2+}

Plants were rinsed with distilled water to remove surface ions, then the shoots and the roots were separated, and oven dried at 80°C for 48 hours. The samples were chopped and digested in glass tubes containing 5 ml of concentrated HNO_3, H_2SO_4 and $HClO_4$ (5:2:1) and placed in a heat block at 180°C. After complete digestion, the sample volumes were adjusted to 50 ml with distilled water. The amounts of Na^+, K^+ and Ca^{2+} were determined using an atomic absorption spectrophotometer. Duncan's multiple range test was employed for test of significance.

Isolation of total RNA

Total RNA was extracted from the KDML105 leaves using guanidinium isothiocyanate according to the method described by Chomczynski and Sacchi (1987) with some modifications. Approximately one gram of the leaves was ground in liquid nitrogen and suspended in a 10 ml buffer solution containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, and 0.1 M β-mercaptoethanol. One millilitre of 2 M sodium acetate (pH 4.0), 10 ml of phenol and 2 ml of chloroform: isoamyl alcohol (24:1 v/v) were added and vortexed, followed by centrifugation at 3,500 g for 10 min. The supernatant was collected and mixed with an equal volume of cold isopropanol. DNA and RNA were allowed to precipitate at –20° C for 1 h, followed by centrifugation at 11,000 g for 10 min. The pellet was washed once using 1 ml of cold 70% ethanol and centrifuged at 5,000 g for 5 min. It was then dissolved in 500 μl of 0.1% DEPC-treated water and added with 500 μl of 6 M LiCl. The solution was left at 4°C overnight, followed by centrifugation at 11,000 g for 10 min. The precipitated RNA pellet was washed again in cold 70% ethanol as described above and dissolved in 0.1% DEPC-treated water. The purity and concentration of total RNA was determined using spectrophotometer at 260 and 280 nm. RNA was stored at –80°C until use.

cDNA synthesis

First strand cDNA was synthesized from the total RNA by the process of reverse transcription. Approximately 5 μg of RNA was mixed with 0.5 μg of oligo $(dT)_{12\text{-}18}$ primer and adjusted to the volume of 12 μl with distilled water. The mixture was heated at 70°C for 10 min and quickly cooled on ice. Superscript II $RNaseH^-$ kit solution (Invitrogen) containing 4 μl of 5X first strand buffer, 2 μl of 0.1 M dithiothreitol and 1 ml of reverse transcriptase (200 U) was added to the sample together with 1 μl of 10 mM dNTP. Then the mixture was incubated at 42°C for 50 min. The reaction was terminated by incubation at 70°C for 15 min. It was kept at –20°C until use.

Determination of the levels of gene expression using real-time PCR

Real-time PCR was performed using an ABI 7700 Sequence Detection System. The optimization of the real-time PCR reaction was performed according to the manufacturer's instructions (PE Applied Biosystems). The PCR conditions were standardized using core reagent

protocol. All reagents were provided in the core reagent kit, including AmpliTaq-Gold polymerase (PE Applied Biosystems). After optimization, nucleotide primers were used for the detection and quantification of Ca^{2+}-ATPase.

Gene expression of Ca^{2+}-ATPase was analyzed by Fluorescent PCR employing FAM labeled oligonucleotide probe. The primers and the probe were designed using ABI PRISM™ Primer Express™ (Applied Biosystems). A forward primer (5'-GCTGCTGGAATACGTG TTATGG-3') and a reverse primer (5'-TCACGGCATATCG CCTCTGCTGTTT-3') were applied to produce a single 110 bp PCR product. A probe (5'-TTAGTTCAAAGAGCTT CACAGGGA-3'), labeled with FAM (6-carboxylfluorocien) reporter at 5' end and TAMRA (6-carboxytetra-methylrodamine) quencher dye at 3' end, was used to produce fluorescent signal. Real-time PCR amplification was carried out in 5 μl of 10X PCR buffer, 6 mM $MgCl_2$, 0.5 mM dNTPs, 20 ?M of each primer for Ca^{2+}-ATPase forward primer and Ca^{2+}-ATPase reverse primer, 1 μl of cDNA sample, 1U AmpliTaq-Gold polymerase. Real-time PCR conditions consisted of an initial denaturation step of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Genomic DNA was used as a reference. Experiments were performed in triplicate. Ca^{2+}-ATPase transcripts were calculated by comparing to the reference generated by the controlled DNA. Ca^{2+}-ATPase expression was determined from the levels of mRNA using real-time PCR in the form of normalized genome equivalent (NGE).

RESULTS AND DISCUSSION

Na^+ accumulation

After transferring the three-week-old KDML105 to grow in the Hoaglands' medium containing 150 mM NaCl and supplemented with either 0, 0.4, or 10 mM $CaCl_2$, the amounts of Na^+ in the leaves and the roots were determined at 3 hours, 1 day, 2 days, 5 days, and 8 days periods. The results showed that Na^+ content in the leaves at the first three hours remained the same in all treatments (Figure 1A). The change in Na^+ accumulation was observed after 1-day growth and gradually increased from 0.8% to as high as 4.5% on day 8. $CaCl_2$ seemed to have positive effect on Na^+ accumulation in the leaves comparing to having only NaCl in the medium. In the roots, however, Na^+ accumulation profile was quite different from that of the leaves (Figure 1B). There was a jump in Na^+ level right at the first three hours of salt exposure. All the treatments with either 150 mM NaCl alone or having it combined with different amount of $CaCl_2$ made the Na^+ in the roots changed from 0.5% to 0.8%, but the increment of Na^+ at the later days of exposure was not drastically altered. The highest percentage of Na^+ accumulation was found on day 8 at 1.2%. The slight change of Na^+ in the roots could be the results of continuous transport of Na^+ to the leaves where the real accumulation was taking place. This type of high Na^+ accumulation in the leaves is also seen in cotton seedling treated with 150 mM NaCl (Binzel *et al*., 1987), in rice treated with 171 mM NaCl (Garcia *et al*., 1997), and in grass treated with 0-600 mM NaCl (Marcum, 1999). Although it was clearly seen that both NaCl and $CaCl_2$ caused the high Na^+ accumulation in the leaves of KDML105 in the beginning (up to day 5), but the longer exposure time of $CaCl_2$ with the 150 mM NaCl in the medium (day 8) seemed to lower the Na^+ accumulation than having 150 mM NaCl alone (Figure 1). This might be due to the inhibitory action of Ca^{2+} to the influx of Na^+ through cation channel and at the same time inducing the reaction of SOS (salt-overly-sensitive) protein system to eliminate extra Na^+ from the cells as well as blocking the influx of Na^+ through K^+ channel (Yokoi *et al*., 2002), which is also seen in citrus fruit, cotton seedling, and wheat (Cramer *et al*., 1985; Ben-Hayyim *et al*., 1987; Davenport *et al*., 1997).

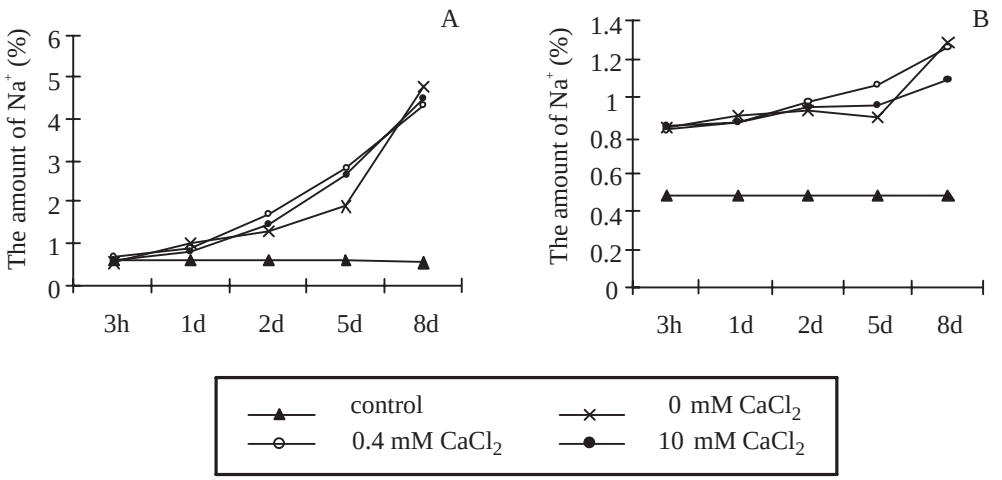

Figure 1 The levels of Na^+ in (A) the leaves and (B) the roots of KDML105 grown in Hoaglands' solution supplemented with 150 mM NaCl and different concentrations of $CaCl_2$ (0, 0.4 and 10 mM $CaCl_2$).

K^+ accumulation

In the control medium having neither NaCl nor $CaCl_2$, the K^+ accumulation in the leaves and the roots of KDML105 rice were at steady levels of 2.6% and 0.6%, respectively. However, having been exposed to either 150 mM NaCl alone or the combination of 150 mM NaCl with either 0.4 mM or 10 mM $CaCl_2$, the drop in K^+ accumulation were both observed at the low levels of 1.2% in the leaves and 0.4% in the roots (Figure 2A and B). There was only a slight change from the beginning of 3-hour exposure to the salt until at the end of 8-day period. The decrease of K^+ might result from the competitive effect of Na^+ in the medium with the flow of K^+ through inward-rectifying K^+ channel (Niu *et al*., 1995). At the same time, the maintenance of K^+ in rice at a certain level is necessary because of its several important roles in plants, i.e., enzymes activation, stomata opening, respiration and photosynthesis, homeostasis, as well as the growth of leaves and roots (Osothsapa, 2000). The additive effect of $CaCl_2$ and NaCl on K^+ accumulation was observed in both leaves and roots of KDML105 as also reported in cotton seedling (Cramer *et al*., 1985) but not at distinctive level

Ca^{2+} accumulation

The Ca^{2+} accumulation in the leaves of KDML105 was kept at rather steady levels from the beginning to the 8-day exposure of rice in the medium having either 150 mM NaCl alone or combining with different concentrations of $CaCl_2$, which was in the ranges of 0.8-1.2% (Figure 3A).

As for the roots, having only 150 mM NaCl or the combination with 0.4 mM $CaCl_2$ made the roots less susceptible to Ca^{2+} comparing to the control after the 3-hour exposure to the salt but not at later stages (day 5 and 8). However, the higher concentration of $CaCl_2$ (10 mM) gave an increase amount of Ca^{2+} to the ranges of 0.4% to 0.5% (Figure 3B). This result also agreed with the pattern of Ca^{2+} accumulation found in the leaves and the roots of cotton (Cramer *et al*., 1985). The importance of having Ca^{2+} at the same level in plant is as expected since Ca^{2+} plays a vital role in cellular homeostasis and signal transduction. The changes in Ca^{2+} content not only cause the change in membrane permeability, it also triggers the expression of Ca^{2+}-ATPase and hence protein synthesis (Osothsapa, 2000).

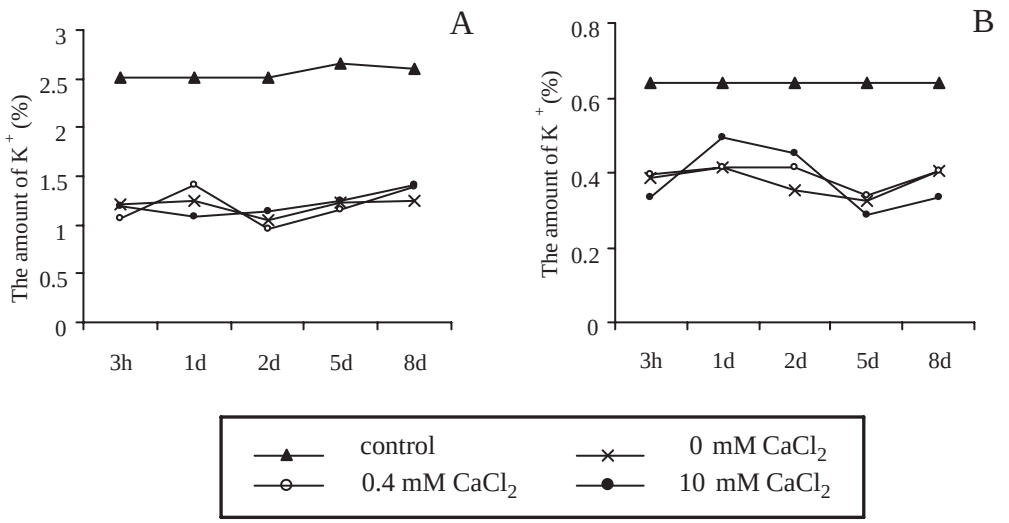

Figure 2 The levels of K^+ in (A) the leaves and (B) the roots of KDML105 grown in Hoaglands' solution supplemented with 150 mM NaCl and different concentrations of $CaCl_2$ (0, 0.4 and 10 mM $CaCl_2$).

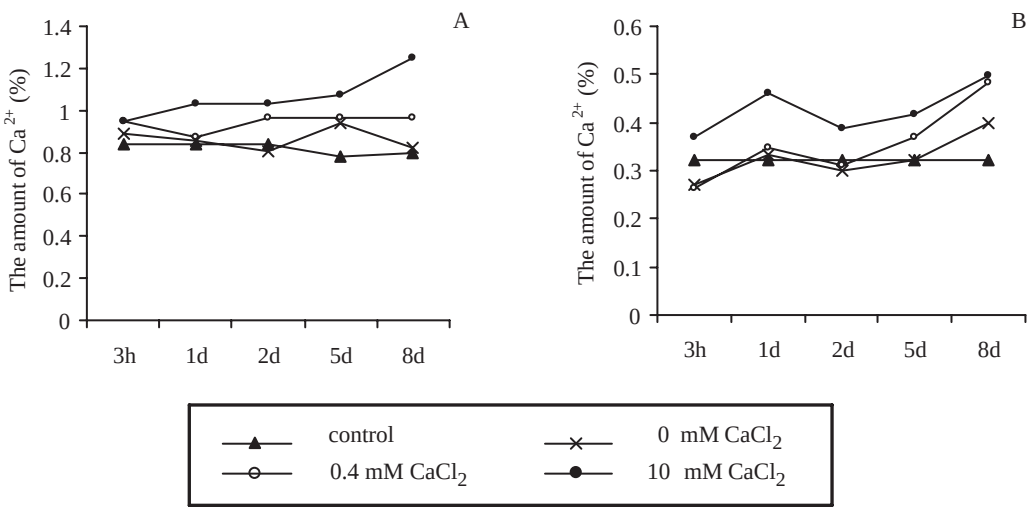

Figure 3 The levels of Ca^{2+} in (A) the leaves and (B) the roots of KDML105 grown in Hoaglands' solution supplemented with 150 mM NaCl and different concentrations of $CaCl_2$ (0, 0.4 and 10 mM $CaCl_2$).

Changes in Ca^{2+}-ATPase expression

Ca^{2+}-ATPase expression was determined from the levels of mRNA using real-time PCR in the form of normalized genome equivalent (NGE). Ca^{2+}-ATPase expression in the leaves of KDML105 was found to change at drastic levels after 3-hour exposure to 150 mM NaCl from 0.3 NGE to 2 NGE (7 folds), and even higher when 0.4 mM $CaCl_2$ was added (6 NGE, 19 folds). After one-day exposure to the salt, the levels of expression were all dropped to only 1.2 NGE (4 folds) but went up again on day 2 (2.2 - 5.6

NGE, 7-16 folds). Finally, all seemed to be at steady levels from day 2 to day 8, except those having 0.4 mM $CaCl_2$ treatment which leveled off on day 5 and day 8 (Figure 4A). The high fluctuation of Ca^{2+}-ATPase expression might result from the activating effect of NaCl to increase cytoplasmic level of Ca^{2+} (Niu *et al*., 1995) which in turn induces the signal transduction system in response to salt stress (Geisler *et al*., 2000). However, the activating effect was temporary and Ca^{2+} level needed to be controlled at a certain level to maintain the cellular functions. The high synthesis of Ca^{2+}-ATPase, therefore, is required to eliminate extra amount of Ca^{2+} in cytoplasm. $CaCl_2$ was also found to have accumulating effect on Ca^{2+}-ATPase expression as well as helping decrease the Na^+ accumulation in the cell at later period. These results agreed with the report of Wimmers *et al*. (1992) on the 310% increase in Ca^{2+}-ATPase mRNA of tomato leaves after one-day exposure to 50 mM NaCl. The other types of this change were also reported in different organisms, i.e., 190% increase of Ca^{2+}-ATPase mRNA found in *Arabidopsis* after one-day exposure to 100 mM NaCl (Geisler *et al*., 2000) and 10 folds increase of Ca^{2+}-ATPase in amoeba after having exposed to 80 mM NaCl for one month (Moniakis *et al*., 1995).

As for Ca^{2+}-ATPase expression in the roots, it was found that NaCl alone or combining with lower concentration of $CaCl_2$ (0.4 mM) had little effect on the change of Ca^{2+}-ATPase mRNA (Figure 4B). This is contrary to the 180% increase of Ca^{2+}-ATPase mRNA in the roots of tomato after having been exposed to 50 mM NaCl for one day (Wimmers *et al*., 1992). The less effect of NaCl in the roots of KDML105 could be the result of fast transportation of ions to different parts of the plant especially to the leaves where accumulation was more prominent and hence the distinctive change in Ca^{2+}-ATPase mRNA. However, the addition of high concentration of $CaCl_2$ (10 mM) could cause the change in Ca^{2+}-ATPase mRNA after one-day exposure to attain a very high level of 6.1 NGE (8 folds) and dropped to 4.3 NGE (5 folds) on day 2 (Figure 4B). Then on day 5, Ca^{2+}-ATPase mRNA level went to as high as 9.4 NGE (13 folds) before dropping to the normal level again on day 8. This observation could result from the saturated salt condition in the roots where its effect on Ca^{2+}-ATPase expression was unavoidable, but it could return to the original level when the salt was finally eliminated.

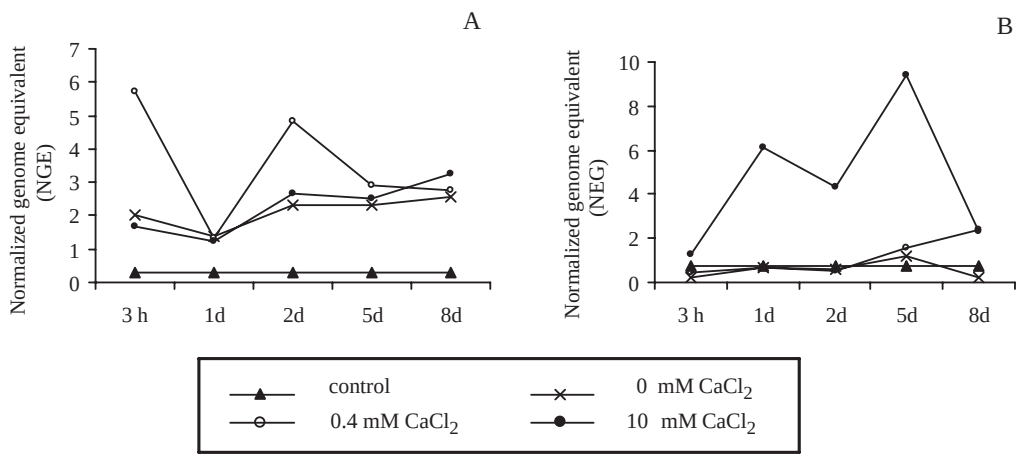

Figure 4 Ca^{2+}-ATPase in (A) the leaves and (B) the roots of KDML105 grown in Hoaglands' solution supplemented with 150 mM NaCl and different concentrations of $CaCl_2$ (0, 0.4 and 10 mM $CaCl_2$).

It was evident that the change in Ca^{2+}-ATPase expression as seen from the mRNA level was directly activated by the effect of NaCl and $CaCl_2$ and Ca^{2+} accumulation in cytoplasm (Niu *et al*., 1995). The change in Ca^{2+}-ATPase level was then inevitably needed to restore the Ca^{2+} homeostasis and made the rice survive in the high salt condition.

CONCLUSION

1. NaCl in the medium caused the increase of Na^+ in the KDML105 roots at the early stage of the exposure (3 hours) and kept at the same level throughout most of the experimental period except at day 8, while the Na^+ change in the leaves was observed after one-day exposure but continuously increased at distinctive level. Addition of $CaCl_2$ to the NaCl containing medium helped decreasing Na^+ accumulation in the leaves at day 8 but this effect was not distinctively seen in the roots.

2. NaCl showed a negative effect on the K^+ level in both leaves and roots of KDML105 from the early three hour after exposure to the salt. The change in K^+ over time, however, was not clearly seen. The early drop of K^+ level was mainly due to the competition of Na^+ with K^+ for the same K^+ channel.

3. NaCl alone had little effect on the Ca^{2+} levels in the leaves and the roots of KDML105 but having NaCl combined with $CaCl_2$ made a distinctive change in Ca^{2+} accumulation at both parts to a considerable level. However, this additive effect of $CaCl_2$ also caused the decrease in Na^+ level as a result of the inhibitory action of Ca^{2+} to the influx of Na^+ to the cell.

4. NaCl caused the drastic change in Ca^{2+}-ATPase mRNA of the KDML105 leaves. This effect was even greater when superimposed by $CaCl_2$ because both types of salt contributed to the Ca^{2+} accumulation in cytoplasm. The plant, therefore, tried to eliminate the surplus of Ca^{2+} by synthesizing more Ca^{2+}-ATPase even at the lower exposed amount of 0.4 mM $CaCl_2$. On the other hand, Ca^{2+}-ATPase mRNA in the roots of KDML105 was affected by $CaCl_2$ (10 mM) but not by NaCl alone**.** Since the roots were not the sites for Na^+ accumulation *per se*, fast transportation of Na^+ made the roots more stable and less likely to have high Ca^{2+}-ATPase expression at lower concentration of 0.4 mM of $CaCl_2$. However, the high concentration of 10 mM $CaCl_2$ was too much to be eliminated by a mere transportation. The roots, therefore, needed to synthesize more Ca^{2+}-ATPase to assist in coping with this salt stress condition.

ACKNOWLEDGEMENTS

This reserch was supported by the Rockefeller Foundation (grant # 98001), and Graduate School, Kasetsart University. We would also like to thank Pathumthani Rice Research Center for supplying KDML105 cultivar, and to Soil and Fertilization Analysis Unit, Research and Experimental Greenhouse for the use of ion analysis equipment.

LITERATURE CITED

Ben-Hayyim, G., U. Kafkafi and R. Ganmore-Neumann. 1987. Role of internal potassium in maintaining growth of cultured *Citrus* cells on increasing NaCl and $CaCl_2$ concentrations. **Plant Physiol.** 85: 434-439.

Binzel, M.L., P.M. Hasegawa, D. Rhodes, S. Handa, A.K. Handa and R.A. Bressan. 1987. Solute accumulation in tobacco cells adapted to NaCl. **Plant Physiol**. 84: 1408-1415.

Bioservice Unit (BSU). 2001. **Science and Technology with Thai Rice**. NSTDA, Bangkok, 158 p.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.

Anal. Biochem. 162: 156-159.

Cramer, G.R., A. L?uchli and V.S. Polito. 1985. Displacement of Ca^{2+} by Na^{+} from the plasmalemma of root cells. **Plant Physiol**. 79: 207-211.

Davenport, R.J., R.J. Reid and F.A. Smith. 1997. Sodium-calcium interactions in two wheat species differing in salinity tolerance. **Physiologia Plantarum** 99: 323-327.

Garcia, A.B., E.J. de Almeida, S. Iyer, T. Gerats, M.V. Montagu and A.B. Caplan. 1997. Effects of osmoprotectants upon NaCl stress in rice. **Plant Physiol**. 115: 159-169.

Geisler, M., N. Frangne, E. Gomes, E. Martinoia and M.G. Palmgren. 2000. The *ACA4* gene of Arabidopsis encodes a vacuolar membrane calcium pump that improves salt tolerance in yeast. **Plant Physiol**. 124: 1814-1827.

Hoagland, D.R. and I.D. Arnon. 1950. **The Water-Culture Methods for Growing Plants without Soil**. California Agricultural Experimental Station. Circular, 286 p.

Marcum, K.B. 1999. Salinity tolerance mechanisms of grasses in subfamily Chloridoideae. **Crop Sci**. 39: 1153-1160.

Moniakis, J., M.B. Coukell and A. Forer. 1995. Molecular cloning of an intracellular P-type ATPase from *Dicteostelium* that is up-regulated in calcium-adapted cells. **J. Biol.Chem**. 270: 28276-28281.

Niu, X., R.A. Bressan, P.M. Hasegawa and J.M. Pardo. 1995. Ion homeostasis in NaCl stress environments. **Plant Physiol.** 109: 735-742.

Osothsapa, Y. 2000. **Nutrition Element of Plant**. Kasetsart University, Bangkok. 424 p.

Wimmers, L.E., N.N. Ewing and B.A. Bennett. 1992. Higher plant Ca^{2+}- ATPase: Primary structure and regulation of mRNA abundance by salt. **Proc. Natl. Acad. Sci. USA**. 89: 9205-9209.

Yokoi, S., R.A. Bressan and P.M. Hasegawa. 2002. Salt stress tolerance of plants. **JIRCAS Working Rep**. 25-33.