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The Effect of NaCl Treatment on the Freezing Tolerance and Protein Patterns of Carrot Callus Suspension Culture

Soon-Ok Moon[1,2,3,*], Sook-Hee Park[1] and Bong-Heuy Cho[2]

[1]*Department of Genetic Engineering,* [2]*Division of Life Science, The University of Suwon 445-743*
[3]*Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea*
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Abstract : The growth, freezing resistance and electrophoretic protein patterns of carrot callus cultures were investigated following treatment with NaCl for various intervals at 20°C. Following 7 day exposure to 250 mM NaCl, freezing tolerance increased, which was measured by 2,3,5-triphenyl tetrazolium chloride (TTC) assay and fresh weight was reduced compared to control cells. Changes of electrophoretic patterns of total and boiling stable proteins were investigated using one or two dimensional gel system. Several proteins with molecular weight of 43 and 21 kDa increased by NaCl treatment. The most prominent change was detected in 21 kDa protein. The steady state level of this protein increased in NaCl treated cells, but decreased in control cells. Twenty one kDa protein was detected only in the NaCl treated cell when boiling stable protein was analyzed. The isoelectric point of 21 kDa protein was identified as 5.7. The timing of increase of 21 kDa protein was correlated to freezing resistance which implied the role of this protein in the induction of freezing resistance of the cell.

Key words : boiling stable protein, freezing tolerance, NaCl.

Exposure of temperate plants to low temperature activates a cold acclimation process that increases their freezing tolerance. Freezing of plant tissues results in the formation of extracellular ice crystals, which lowers the water potential in the cells or increases the osmotic pressure as a result (Hughes and Dunn, 1996). Drought or high salt stresses also induce dehydration of plant cells (Yamaguchi-Shinozaki and Shinozaki, 1994). In these severe environmental stresses, survival of plants depends on their ability to resist the osmotic stress induced by drought, salinity and temperature (Skriver and Mundy, 1990) and plants may respond to these environmental stresses with same physiological and biochemical changes. For example, osmotic adjustment of winter Brassica napus leaf cells were detectable during cold acclimation (Orr *et al.*, 1995) and increase of osmotic pressure has been observed during cold acclimation in suspension cultures of *Brassica napus* (Orr *et al.*, 1986). It is also known that drought stress can induce increased freezing tolerance in winter cabbage and rye (Cox, 1976; Siminovitch and Cloutier, 1982). ABA is thought to play an essential role in mediating the primary response to these environmental stresses (Chen and Gusta, 1983; Skriver and Mundy, 1990). Endogenous levels of ABA increase after exposure to low temperature (Lee *et al.*, 1991) and freezing tolerance increases following application of ABA to plants and cell cultures. ABA levels increase in tissues subjected to osmotic stress by desiccation, salt, or cold (Henson, 1984). Given this, it is hypothesized that drought and freezing tolerance might involve closely related genetic mechanism and gene products.

Biochemical and molecular studies have shown that cold acclimation may be mediated by the expression of specific genes (Yamaguchi-Shinozaki and Shinozaki, 1994). Recently, a number of low temperature responsive (LTR) genes have been characterized from a variety of plant species (Hughes and Dunn, 1996). Many LTR genes are also responsive to drought, salt, and ABA (Yamaguchi-ShinoZaki and Shinozaki, 1994). The function of most LTR proteins is unknown, although alignment of predicted amino acid has revealed regions within some of these proteins that are homologous to those induced by dehydration such as dehydrin and late embryogenesis abundant (LEA) families (Gilmour *et al.*, 1992; Neven *et al.*, 1993). Most characteristic features of LEA proteins, ABA or osmotic stress induced proteins are hydrophilicity (Dure *et al.*, 1989). The expression of these families of proteins under con-

*To whom correspondence should be addressed.
Tel : 82-331-220-2289, Fax : 82-331-220-2289.

trol of dehydration and the fact that they all appear to be very hydrophilic suggest that they may function in helping the plant to overcome the desiccation stress (Dure *et al.*, 1989).

The regulation of gene expression by dehydration induced by low temperature, drought, salt and ABA is critical to understanding the process of cold acclimation in plants. To better understand the role of proteins in acquisition of freezing tolerance, it is necessary to examine their expression not only at the transcriptional level, but also at the level of protein itself. Steady state level of protein can be more directly correlated with the physiological aspects of cold acclimation and freezing tolerance than can those of transcript. The objective of the present study was to observe the correlation of cold acclimation and salt stress to understand the mechanism of freezing tolerance. We have also characterized the proteins that are induced in callus culture in response to salt stress.

Materials and Methods

Cell culture and freezing tolerance measurement

Carrot callus suspension culture was maintained in Murashige and Skoog (MS) media supplemented with 2 mg/l of 2,4-D in continuous dark at 20℃. Cultures were subcultured every two weeks. Experiments on exposure to salt were performed by adding NaCl to a final concentration of 250 mM to log phase cells. Growth rate of suspension cells was determined by measurment of packed cell volume (PVC). Cells were collected in a 15 ml conical tube by centrifugation for 20 min at 2500 rpm (Hanil HA 300) and volume of collected cells was measured. Freezing tolerance was determined by 2.3.5-triphenyl tetrazolium chloride (TTC) assay (Chen and Gusta, 1983). After cells were placed on the filter paper in small petri dishes, 0.1 g of cells were transferred to microcentrifuge tube. One ml of distilled water was added to each tube and tubes were placed in freezer and the temperature was lowered to −20℃ by reducing the temperature 5℃/h and incubated further 1 h. Samples were thawed overnight at 4℃. Thawed cells were incubated in 1 ml of 2% TTC solution for 18 h at room temperature in the dark. After incubation, the cells were washed with distilled water and 1 ml of 95% ethanol was added. The reduced TTC was determined by measuring absorbance at 485 nm. Freezing resistance was expressed as the percentage of TTC reduction as compared to nonfrozen control.

Protein extraction and electrophoresis

Suspension cultured cells were collected by centrifugation for 20 min at 2500 rpm (Hanil HA 300) and ground with a mortar and pestle with protein extraction buffer (20 mM Tris-HCl, pH 7.4, 5% SDS, 7.5% 2-mercaptoethanol, 1 mM PMSF). The protein extracts were boiled for one min and centrifuged at 15000 rpm (microcentrifuge) to remove insoluble debris. Protein concentration was determined using Bio-Rad microassay kit (Bio-Rad, Hercules, USA) Prior to electrophoresis, an equal volume of loading buffer (100 mM Tris-HCl, pH 8.0, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 1.125% bromophenol blue) was added to protein extracts and boiled for 2 min. Protein samples were electrophoresed on 10% SDS polyacrylamide gels and stained with coomassie blue.

Preparation of boiling stable proteins and 2-D gel electrophoresis

Total cell proteins, obtained by method described above were precipitated by incubation with 5×V of ice-cold acetone. The pellet was dissolved in 50 mM Tris-HCl (pH 7.0). These soluble proteins were placed in boiling water for 25 min and allowed to cool on ice. Boiling stable proteins were recovered from supernatant after centrifugation at 15000 rpm (microcentrifuge) for 15 min. Boiling stable proteins were resolved by 2-D IEF/SDS-PAGE as described by O'Farrel (1977). IEF gels were prepared by using ampholyte (pH 3.5~10, Pharmacia, Uppsala, Sweden). Second dimension SDS gels (10% polyacrylamide) were stained with coomasie blue.

All reagents, except those described above, were purchased from Sigma (St. Louis, USA)

Results and Discussion

Freezing tolerance

Suspension culture of carrot, grown in the presence of 250 mM NaCl, showed the reduction of fresh weight in contrast to suspension culture grown in the absence of NaCl (Fig. 1). Larsson *et al.* (1989) indicated that an inhibition of shoot growth in osmotically stressed wheat seedlings was a result of decline of water content in the leaf tissue. The reduction of cell growth in NaCl treated culture may be result of dehydration of cell or water content. Dehydration, low temperature or high salt have been shown to induce freezing tolerance (Cox, 1976; Siminovitch and Cloutier, 1982; Cloutier, 1984) and same gene expression (Yamaguchi-Shinozaki and Shinozaki 1994), which suggests that responses to these stresses share common cellular components for the induction of freezing tolerance. To determine whether NaCl treatment induces freezing resistance in the carrot

suspension culture cells.

TTC assay was used (Chen and Gusta, 1983). It was reported that the degree of salt and freezing tolerance observed in the whole plant is also exhibited in callus tissue (Smith and McComb, 1981) which suggests that the mechanism of stress tolerance was operating at the cellular level.

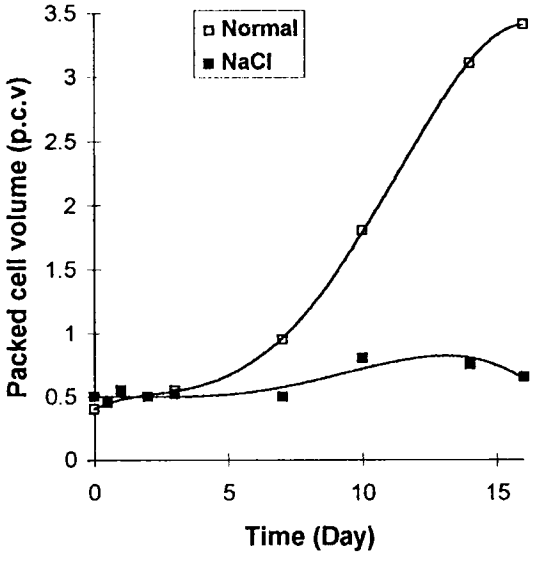

Fig. 1. The growth of carrot suspension culture cells. Suspension culture cells were incubated in the presence (■) or absence (□) of 250 mM NaCl at 20°C.

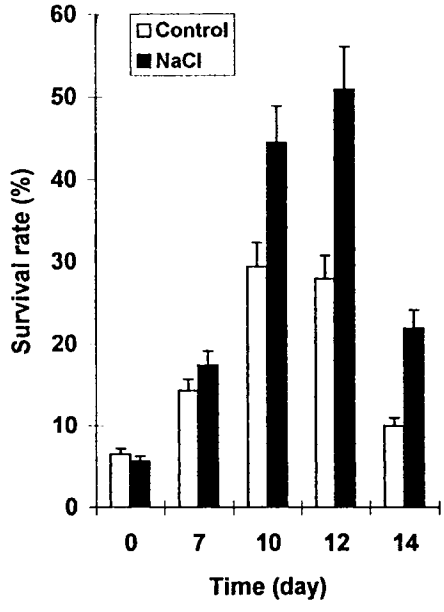

Fig. 2. Freezing resistance in carrot suspension culture. Log phase cells were incubated in the absence (□) or presence (■) of 250 mM NaCl for various periods. Surviving after freezing was determined by TTC assay as described in Materials and Methods. Each value and error bar represents the mean and standard deviation of three experiments.

NaCl treatment at 20°C increased freezing tolerance after 7 day compared to callus grown in the absence of NaCl. The maximal level increase in freezing tolerance was observed after 12 day incubation with salt, when 53% of total cells were survived during −20°C treatment (Fig. 2). The data indicate a correlation between salt treatment and the ability of carrot suspension culture to resist freezing temperature. Freezing resistance can be explained at least in part by increased dehydration tolerance.

Protein patterns in salt treated cells

To investigate the relationship between salt treatment, freezing resistance and protein patterns, we compared the protein patterns of salt-treated and control cells. Early studies of changes in protein profiles with cold acclimation reported increases in the synthesis of certain polypeptides that paralleled the acquisition of freezing tolerance and declines in the synthesis of these polypeptides as freezing tolerance was lost (Gilmour *et al.*, 1987; Guy and Haskell, 1987). Since radiolabeling studies examine only whether or not a protein is actively synthesized, they may not provide an accurate representation of the correlation between stress induced proteins and freezing tolerance. Recent evidence showed that both increase and decrease in the protein level of specific proteins appear to relate closely the gain and loss of the freezing tolerance (Kazuoka and Oeda, 1992; Moon *et al.*, 1994). Therefore, in the present study, steady state levels of proteins were examined. Fig. 3 shows the total cellular protien patterns from

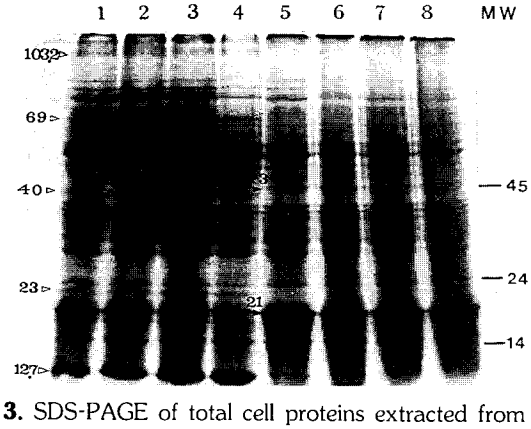

Fig. 3. SDS-PAGE of total cell proteins extracted from carrot callus suspension culture with (5~8) or without (1~4) 250 mM NaCl. Proteins were extracted after various incubation time from each condition (1, 5: 7 days; 2, 6: 10 days; 3, 7: 12 days; 4, 8: 14 days), separated by 10% polyacrylamide gel and stained by Coomassie blue. The arrowheads indicated the increased (◀) or decreased (◁) proteins.

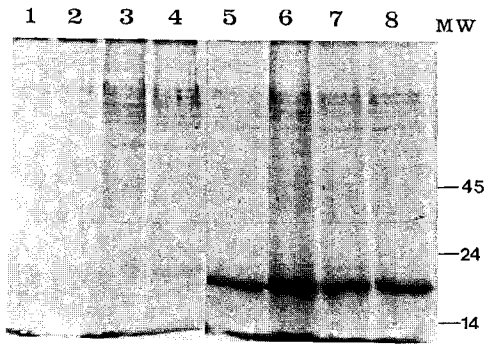

Fig. 4. SDS-PAGE of boiling stable proteins extracted from carrot callus suspension culture with (5~8) or without (1~4) 250 mM NaCl. Proteins were extracted after various incubation time from each condition (1, 5: 7 days; 2, 6: 10 days; 3, 7: 12 days; 4, 8: 14 days), separated by 10% polyacrylamide gel and stained by Coomassie blue. The arrow head indicated increased protein.

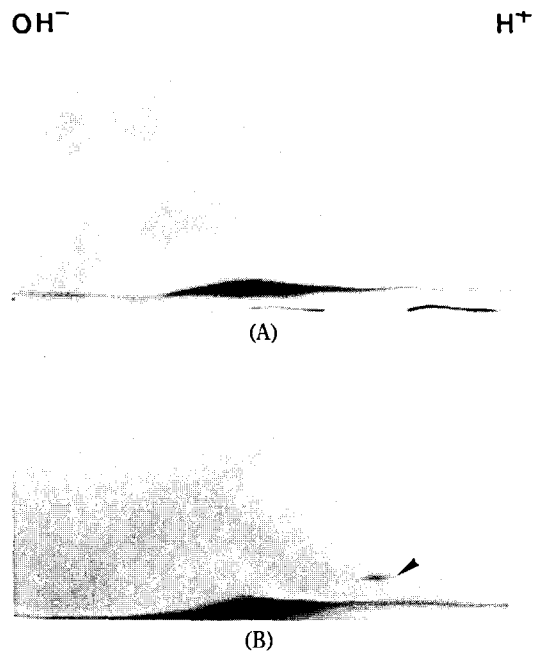

Fig. 5. Two dimensional polyacrylamide gel electrophoresis. Boiling stable proteins, prepared from 12 day incubated control (A) or 250 mM NaCl treated cells (B). The arrow head indicated increased protein.

suspension cells treated with or without 250 mM NaCl. The steady state level of several proteins (43, 21 kDa) increased and some (103.2, 69, 40, 23, 12.7 kDa) decreased in NaCl treated cells as compared with the control cells (Fig. 3). These results support that the reduction of water availability resulting from elevated level of salt is responsible for the changes in the steady state level of these proteins. Among these proteins, the level of 21 kDa protein increased in large amount between 7 and 14 day incubation period, as visible on coomassie blue stained gel, which was closely correlated with the increase of freezing resistance in NaCl treated cells (Fig. 2). Although the correlative studies cannot prove a role for proteins in the aquisition of freezing tolerance, our results would seem to suggest the involvement of 21 kDa protein in cold acclimation process.

Boiling stable proteins in salt teated cells

Many of the proteins induced in response to dehydration, caused either by freezing, drought, salt, or seed development, belong to the DHN/LEA/RAB family of proteins (Close *et al.*, 1989; Lin *et al.*, 1990; Close and Lammers, 1993). The main features of this protein family are hydrophilicity, remaining soluble after boiling and absence of Trp or Cys residues (Close *et al.*, 1989; Lin *et al.*, 1990; Close and Lammers, 1993). This property was used to enrich the NaCl-induced proteins by removal of nonboiling stable proteins. Proteins were extracted from the cells after exposure to 250 mM NaCl, boiled in aqueous solution and analyzed by SDS-PAGE. The protein band corresponding to the molecular size of 21 kDa was decreased in the control cells and increased in NaCl treated cells between 7 and 14 days (Fig. 4). To analyze this protein more accurately, boiling stable proteins, extracted from 12 day treated cells, were separated by 2-D IEF/SDS-PAGE. In control cells there was no detectable boiling stable protein in coomassie blue stained gel, but there was one polypeptide spot (PI, 5.7) which appeared in NaCl treated cells (Fig. 5). The timing of the increase of this protein in NaCl treated cells appeared to parallel somewhat the increase in freezing tolerance. However, its appearance in 14 day treated cells was not accompanied by sudden reduction of freezing tolerance. These observations imply that expression of NaCl responsive proteins is required for the development of increased freezing tolerance, but factors other than this protein may be necessary for freezing tolerance.

Molecular study of low temperature responsive genes has not provided information about the function of them. Although the function of most low temperature induced proteins is unknown, the common occurrence of boiling stable hydrophilic proteins among the low temperature response genes suggests that they may function in a cryoprotective role. For example, *Arabidopsis* kin 1 protein has been suggested to have cryoprotective activity and is hydrophilic and boiling stable (Kurkela and Franck 1990). Kin 1 is also induced by water stress and ABA, which has been suggested to be a common mediator for osmotic responses and cold acclimation in plants (Kurkela and Franck,

1990). Obviously, identification of the function of boiling stable 21 kDa protein requires the purification of this protein, isolation of genes encoding this protein and functional studies which include generation of transgenic plant overproducing this protein and antisense inhibition of this genes in transgenic plants. The relative significance of this salt induced 21 kDa protein is unknown, but the result from this study provides the basic information necessary to perform further studies related to the biochemical and molecular basis of freezing tolerance. Work is in progress to determine the relationship of this protein in freezing tolerance by inducing other method.

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