CHAPTER 14

ONE DIMENSIONAL SDS-PAGE POLYPEPTIDES PROFILE IN NaCl-SENSITIVE AND RESISTANT CELL LINES

14.1 INTRODUCTION

Salt-tolerant cell lines have been isolated from a wide range of plant species, but the underlying molecular mechanism(s) by which tolerance is conferred remains unclear (Tal, 1990). The understanding of gene expression changes during cellular tolerance to salt stress might lead to the identification of those genes which possibly control salt tolerance. The objective of identifying genes involved in salt tolerance has been approaches in several systems, mainly by comparisons of protein patterns from cells grown in the presence and absence of NaCl. The analysis of protein profiles has been carried out by *in-vitro* translation (Ostrem *et al.,* 1987), *in-vivo* radio labelling (Ramagopal, 1987; Gulick and Dvorak, 1987) as well as by investigating the steady state proteins by one and two-dimensional gel electrophoresis (Hurkman *et al.,* 1991). Such studies have shown that salt stress results in an increase in the synthesis of a few specific proteins and a decrease in the synthesis of others, with or without an induction of unique stress proteins (Hurkman *et al.,* 1991). The proteins/genes induced or increased with salt stress include Osmotin and NP 24 (Singh *et al.,* 1985, 1987; King *et al.,* 1986, 1988); Sal T (Claes *et al.,* 1990); rab 21 (Mundy and Chua, 1988); Gs_1 and Gs_2 (Hurkman *et al.,* 1991) and phosphoenol pyruvate carboxylase (Ostrem *et al.,* 1987). Some of these proteins have been purified and characterized too. However, their exact physiological role in salt-tolerance has not been understood as yet (Hurkman, 1991). In the present study, protein pattern of *Vigna radiata* cell lines which differ in salt resistance was compared in the presence or absence of salt with the view to understand the mechanism of regulation of gene expression during salt stress.

14.2 MATERIALS AND METHODS

Cell culture and selection

Callus was initiated from leaf explants of 7-d-old aseptically grown seedlings of *Vigna radiata* on PC-L2 (Phillips and Collins, 1979) medium supplemented with 0.5 mg l^{-1} NAA, 1 mg l^{-1} BAP and 0.5 mg l^{-1} 2,4-D. Callus was subcultured at four-week intervals on the same medium. Selection for salt-resistant cell lines was carried out at 300 mol m^{-3} NaCl as described in section 10 A. Stability of salt-resistance of the selected cells was also tested by growing them for two passages of one month each on NaCl-free PC-L2 medium and then tranferring them back to 300 mol m^{-3} NaCl.

Extraction of proteins

The proteins from salt-resistant and sensitive cells grown on normal medium and NaCl (300 mol m^{-3}) containing medium for 21 days, were extracted by the method of Zivy *et al.,* (1983).

The extraction buffer consisted of 30 mM Tris-HCl (pH 8.5), 1 mM ascorbic acid, 1 mM EDTA-Na, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride. Polyvinyl-polypyrrolidone was added to the tisse at the time of homogenization in the ratio of 50 mg per g fresh weight of the tissue. After homogenization of the callus tissue in the buffer (tissue to buffer ratio 1:5 w/v), the crude homogenate was spun at 12,000 xg for 15 min. at 4°C. The supernatant obtained was used for the extraction of pellet proteins. For the extraction of soluble proteins and the pellet was saved for the extraction of soluble proteins, the supernatant was recentrifuged at 12,000 xg for 10 min. at 4°C. The supernatant after this spun was collected and proteins were precipitated with eight volumes of prechilled acetone containing 10 mM 2-mercaptoethanol at -20°C for 3 h. This was followed by centrifugation at 17,000 xg for 15 min. The pellet was collected, air dried and dissolved in 500 µl Laemmli buffer (Laemmli, 1970). The Laemmli buffer contained 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% v/v glycerol and 5% v/v 2-mercaptoethanol. The pellet saved for the extraction of pellet proteins was also dissolved in 500 µl Laemmli buffer. The protein fractions dissolved in Laemmli buffer were subsequently denatured by heating them at 100°C for 5 min in a water bath. The denatured protein homogenate in each of the above samples, after bringing to room temperature, was centrifuged at 8000 xg for 5 min at 15°C. The supernatant was collected and an aliquot of it was used for the estimation of protein content. The rest of the supernatant was stored at -20°C for further analysis.

Quantification of proteins

The amount of proteins in the extract was estimated following the procedure of Bradford (1976). For removing the interferring agents, like 2-mercaptoethanol and SDS present in the Laemmli buffer employed for solubilizing proteins, an aliquot of the Laemmli buffer sample was precipitated with equal volumes of 20% w/v trichloroacetic acid for 2 h at 4°C. TCA-insoluble pellet obtained after centrifugation at 8000 xg for 5 min dissolved in 1 N NaOH for 2h at room temperature. The NaOH extract was then quantified for protein content by the Bradford (1976) method. The standard calibration curve was made using bovine serum albumin dissolved in 1 N NaOH.

Electrophoresis of proteins

Soluble and membrane (pellet fraction) proteins were subjected to one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (5-20% gradient gel) following the procedure given in Bio-rad manual (Bio-rad, USA), using LKB-Pharmacia (LKB-Pharmacia, Sweden) apparatus.

Gel staining

Silver staining was done according to the method of Damerval *et al.,* (1987). For coomassie staining, gels were placed in a staining solution prepared by dissolving 0.3% w/v coomassie brilliant blue R-250 dye in methanol : acetic acid : water (4:1:5 v/v) and then destained using methanol : acetic acid : water (4:1:5 v/v) mixture. The gels were stored in 5% glycerol solution at 4°C prior to photography. The molecular weight standards obtained from Sigma Chemical Co. (St. Louis, USA) (Table 1) were run in separate lanes of the slab get for calibration of the molecular weights.

14.3 RESULTS

Both coomassie blue and silver staining methods were employed for the resolution of polypeptides, separate by one-dimensional SDS-PAGE gel (5-20% linear gradient) (Plate 11A,B). The resolution of polypeptides was better with silver stain than coomassie blue. Hence, the detailed comparisons of constitutive and inducible polypeptides between salt-sensitive and resistant cell lines was made using silver staining (Table 2).

Constitutive differences

SDS-PAGE profile of the salt-sensitive and resistant calli in the absence of NaCl showed both qualitative and quantitative differences. Various polypeptides noted in the absence of salt in sensitive line. It was noted that 41.6 and 91.2 kD polypeptides are expressed by sensitive callus line, but not by resistant callus line. On the other hand, 44.6, 45.7, 54.9, 57.4, 100.0, 104.6, 109.6 and 131.8 kD polypeptides were more pronounced in sensitive callus line than resistant one.

Inducible differences

Inducible polypeptides in sensitive line

The salt-sensitive callus line on NaCl containing medium showed the synthesis of two new polypeptides of 34.6 and 45.7 kD. The synthesis of five other polypeptides with 10.0, 10.4, 12.0, 33.1 and 173.7 kD appeared to be inhibited. On the other hand, the amount of 125.8 kD polypeptide increased, while that of 131.8 kD decreased under salt stress in sensitive line.

Inducible polypeptides in resistant line

NaCl (300 mol m^{-3}) induced the synthesis of four new polypeptides of 34.6, 45.7, 79.4 and 91.8 kD and depressed the synthesis of 8 after polypeptides of 10.0, 12.0, 33.1, 125.8, 131.8, 138.0, 158.5 and 173.7 kD in resistant callus line. The accumulation of polypeptides of 19.9 to 31.6 kD decreased under salt stress in resistant callus.

Inducible polypeptides in salt-stressed sensitive and resistant callus lines

Both the sensitive and resistant lines were subjected to 300 mo
days, in order to characterize the relative pattern of polypeptides. Salt stress eit
or enhanced the synthesis of specific group of polypeptides in each cell line. NaCl ind
synthesis of two polypeptides of 10.4 and 79.4 kD in resistant callus line and 41.6, 69.2, 12
131.8, 138.0 and 158.5 in sensitive callus line. However, some polypeptides of 19.9, 21.8, 22.9, 26.3, 27.5, 28.8, 31.6, 43.6, 44.6, 54.9, 57.4, 100.0, 104.6, 109.6 and 125.8 kD were more abundant in salt stressed sensitive line.

14.4 DISCUSSION

The effect of salt stress on gene expression has largely been studied using seedlings or whole plants of barley (Hurkman and Tanata, 1987, 1988; Ramagopal, 1987 a,b, 1988 a,b) and wheat (Gulick and Dvorak, 1987). However, such studies except a few (Erickson and Alfinito, 1984; Singh *et al.,* 1985; King *et al.,* 1986) are not available in cultured cell systems. These studies suggest that certain polypeptides and translatable m-RNAs are enhanced, repressed or induced by salt stress. In the present study, three polypeptides of 34.6, 45.7 and 79.4 kD were identified as being salt induced, of which former two were detected in salt stressed calli of both the contrasting cell lines while the latter was only in salt stressed calli of salt-resistant line. These proteins have not been previously described and also appears to be uncommon in higher plants (see Table 3). The synthesis of these new polypeptides may be due to differences in genotype and experimental conditions related to the concentration of NaCl and culture method. The presence of 34.6 and 45.7 kD polypeptides in both salt-sensitive and resistant calli indicates that both lines express the genes which encode proteins of these masses. A unique protein of 79.2 kD has been detected only in salt-resistant cell line which shows cell growth in the presence of 300 mol m^{-3} NaCl. The synthesis of this protein is assumed to be closely related to cell multiplication inspite of injurious effect of salt. Such synthesis may play a role in the acquisition of tolerance to salt stress, may also represent a more general type of defence function.

The synthesis of 26.3 kD polypeptide in the control and salt stressed calli of both the contrasting cell lines of *V. radiata* may be analogous to the 26 kD protein observed in *Nicotiana tabacum, Hordeum vulgare* and *Oryza sativa* (Singh *et al.,* 1985; King *et al.,* 1986; Shirata and Takagishi, 1991). Similar to our results, no difference in the tomato species which differ in salt tolerance (King *et al.,* 1986). The five polypeptides with 158.5, 138.0, 131.0, 69.2 and 41.6 kD disappeared completely when tolerant cell line were grown on NaCl. Thus, the newly induced proteins as well as the ones whose synthesis was elevated by salinity treatment may be particularly important for salt tolerance.

Finally, it has to be borne in mind that the above analysis has been done with 5-20% linear gradient gel following silver staining. Though, this approach is quite common, it is not having the resolution to pin point those polypeptides which are altered in a subtle manner. In other words, the noted polypeptide differences might represent the major changes in polypeptide profiles and may have ignored the ones which are minor in nature. It would be important to make such an analysis using two dimensional (2-D) gel electrophoresis which is a more sensitive approach (Hurkman *et al.,* 1991; Singh *et al.,* 1985, 1987). Due to certain constraints, the 2-D analysis in the present study has not been made, but is worth in the following future studies. Additionally, it needs to be pointed out that the present analysis has been made on steady state proteins only. To mark polypeptides which are specifically altered by salt stress with steady state protein profiles is a difficult proposition because the various housekeeping proteins predominate in this kind of electrophoretic system. It will be important to carry the analysis of the contrasting callus lines using the approach of analysing the *de novo* synthesizing proteins by feeding the radio labelled amino acid precursors such as ^{35}S methionine or ^{3}H leucine. In such an event, it would also be possible to address to the question of analysing time dependent changes in the polypeptide profiles, an aspect which has not been addressed to in the present study. Therefore, it must be pointed out that the present attempt is of preliminary nature only. The polypeptide differences noted in the present study must be subjected to further rigorous investigations. The establishment of the callus lines which have a contrast in the salt response, is a very first step for analysing genes important in salt tolerance and this was successfully shown in this study. Hopefully, the present attempt will play a way for future attempts in discerning genes important in controlling this contrast.

With the recent developments in the techniques of protein engineering and recombinant DNA technology, it has been possible to obtain information on genes underlying the subtle polypeptide differences. For instance techniques have been developed to the excise the protein bands from the gel for their N-terminous microsequencing and for raising antisera. The availability of these two inputs can enable the researchers to screen gene libraries, in order to obtain information on gene clones responsible for synthesis of such polypeptides (King *et al.,* 1988; Claes *et al.,* 1990). The present study is a first step towards such an endeavour. It needs to be taken up in a more exhaustive manner in the future attempts.

Table 1. SDS Molecular weight marker proteins

Protein	Mol. wt. (kD)
α - Lactalbumin	14
Trypsin inhibitor, soybean	20
Trpsinogen	24
Carbonic anhydrase	29
Glyceraldehyde-3-phosphate dehydrogenase	36
Albumin, egg	45
Albumin, bovine	66
Phosphorylase b	97
β - Galactosidase	116
Myosin	205

Table 2. Changes in polypeptide levels of 3 week old calli of NaCl-sensitive and resistant cell lines grown in the presence or absence of NaCl.

	Protein M.Wt. (kD)	Sensitive on normal medium	Sensitive on salt medium	Resistant on salt medium	Resistant on normal medium
1.	173.7	+	-	-	+
2.	158.5	+	+	-	+
3.	138.0	+	+	-	+
4.	131.8	+ +	+	-	+
5.	125.8	+	+ +	-	+
6.	109.6	+ +	+ +	+	+
7.	104.6	+ +	+ +	+	+
8.	100.0	+ +	+ +	+	+
9.	91.2	+	+	+	-
10.	79.4	-	-	+	-
11.	69.2	+	+	-	-
12.	57.4	+ +	+ +	+	+
13.	54.9	+ +	+ +	+	+
14.	45.7	-	+	+	-
15.	44.6	+ +	+ +	+	+
16.	43.6	+ +	+ +	+	+
17.	41.6	+	+	-	-
18.	34.6	-	+	+	-
19.	33.1	+	-	-	+
20.	31.6	+ +	+ +	+	+ +
21.	28.8	+ +	+ +	+	+ +
22.	27.5	+ +	+ +	+	+ +
23.	26.3	+ +	+ +	+	+ +
24.	22.9	+ +	+ +	+	+ +
25.	21.8	+ +	+ +	+	+ +
26.	19.9	+ +	+ +	+	+ +
27.	12.0	+	-	-	+
28.	10.4	+	-	+	+
29.	10.0	+	-	-	+

Table 3. Summary of reports on changes in polypeptides associated with salt-stress in higher plants

Plant species	Tissue	Polypeptides (kD) that Induced or increased	decreased	No change	Technique(s) used	Reference
Nicotiana tabacum	cultured cells	20,26,32	-		SDS-PAGE	Erickson and Alfinito (1984)
N.tabacum	"	58,37,35.5, 34,26,21, 19.5,18	54,52,17.5, 16.5		SDS-PAGE	Singh et al., (1985)
N.tabacum	"	Osmotin (26 kD)	-	-	SDS-PAGE	Singh et al., (1987)
Lycopersicon esculentum	"	NP 24 (26 kD)	-	-	N-terminous sequencing, Oligonucleotide synthesis	King et al., (1988)
Oryza sativa	"	rab 21 (16.5 kD)	-	-	SDS-PAGE and differential screening	Mundy and Chua (1988)
O.sativa	"	Sal T (15 kD)	-	-	2D, Oligonucleotide synthesis	Claes et al., (1990)
O.sativa	Callus	26 & 27 kD (PI 6.1 & 6.4)	-	-	One-dimensional SDS-PAGE	Shirata and Takagishi (1990)
Zea mays	Callus	74,28.5 and 26.2	16.5, 30, 39, 39.5, 30	-	One-dimensional SDS-PAGE	Ramagopal (1986)
Hordeum vulgare	Root	45.4, 26	39.6,40.4, 45.6, 77.5 and 77	-	Two-dimensional (2-D) SDS-PAGE	Hurkman et al., (1989)
H.vulgare	Root culture	97,65, 31.8,26.7	29.5, 29	-	One-dimensional SDS-PAGE	Ramagopal (1988)
H.vulgare	Seedling root	31,26.8, 42.5,37.5	53.5, 51, 38.5, 25.6, 19.5	-	One-dimensional SDS-PAGE	Ramagopal (1987)
H.vulgare	Seedling root	28,45,60.5, 76.5,82.5, 26.5,96.5 45.0	25.5 and 46.5	-	1-D and 2-D SDS-PAGE	Ramagopal (1988)
H.vulgare	Seedling shoot	45,60.5, 76.5 & 82.5	-	-	-do-	Ramagopal (1988)
H.vulgare	Seedling embryo	92,89, 37.5, 32.0	42	-	-do-	Ramagopal (1988)
Medicago sativa	Salt adapted calli	31 & 37 kD	-	-	In-vitro translation of m-RNA and 1-D SDS-PAGE	Winicov et al., (1989)

Plate 11 (A & B)

One-dimensional SDS-PAGE polypeptide profiles of steady-state proteins of NaCl-sensitive and resistant callus lines of *Vigna radiata* cv. K-851 on medium with or without 300 mol m^{-3} NaCl after 21 days of culture. Electrophoretic separation was carried on 5-20% linear gradient gel. A sample containing 10 µg protein was loaded in each lane of the gel and the gels were visualised by silver staining and comassive brilliant blue R_{250} staining.

Lane 1. Low molecular weight marker proteins.

Lane 2. NaCl-sensitive callus line grown on medium containing 300 mol m^{-3} NaCl.

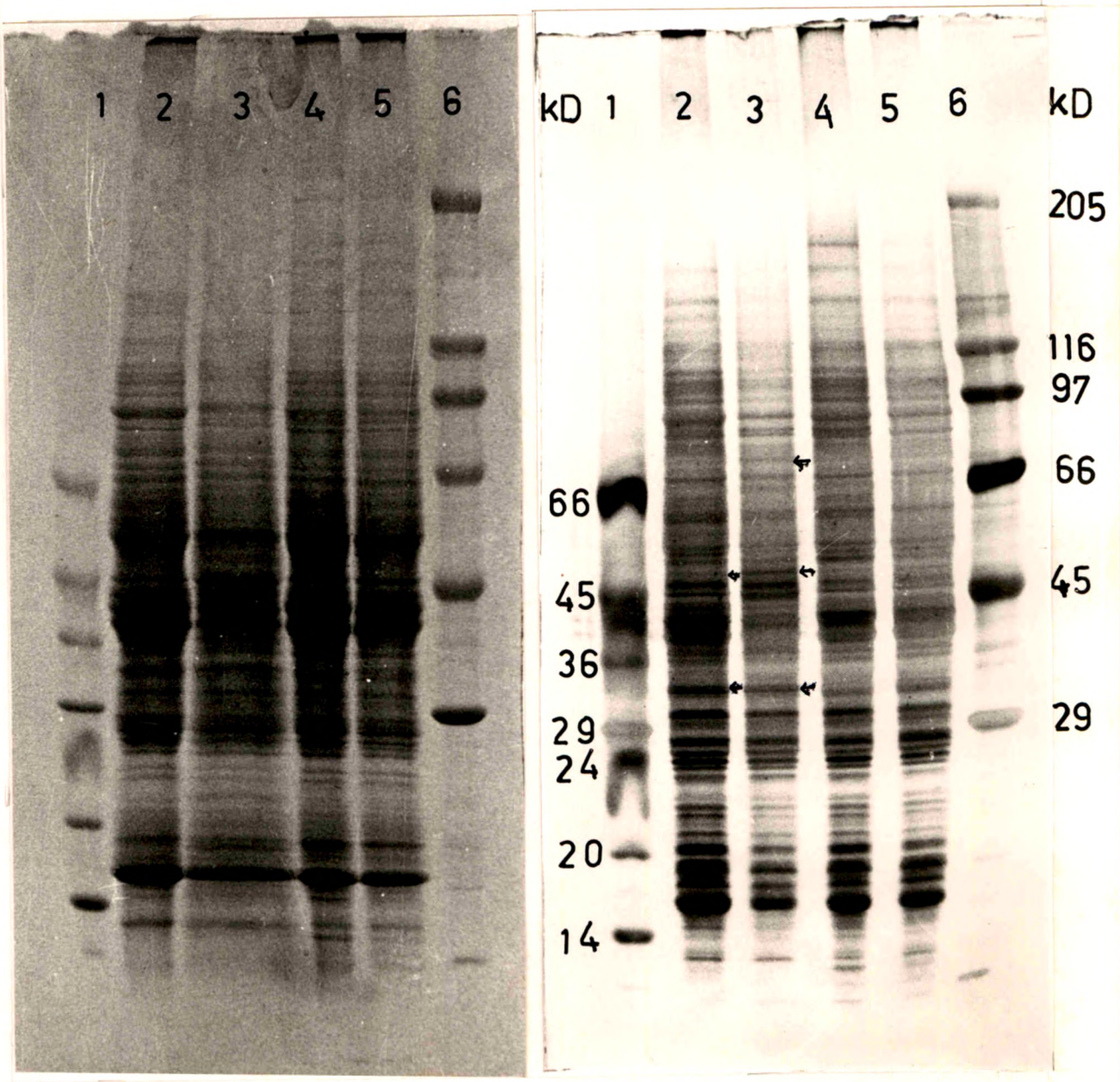
Lane 3. NaCl-resistant callus line grown on medium contaning 300 mol m^{-3} NaCl.

Lane 4. NaCl-sensitive callus line grown on normal medium (without NaCl).

Lane 5. NaCl-resistant callus line grown on normal medium (without NaCl).

Lane 6. High molecular weight marker proteins.

Arrows show the polypeptides appearing during NaCl stress.



A B

PLATE 11