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Messenger RNA induction in cellular salt tolerance of Alfalfa (*Medicago sativa*)

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ABSTRACT

A salt tolerant alfalfa Medicago sativa L. cell line (HG2-N1) has been selected for growth in 171 mM NaCl. The salt tolerance characteristic is stable and is retained after growth in absence of salt selection for two months. In vitro translation was used to compare mRNA composition from the salt tolerant HG2-N1 and parent salt sensitive HG2 cell lines grown in the presence and absence of 171 mM NaCl. The results suggest that the mRNA composition differs between HG2-N1 and HG2 in a number of RNA species. The salt tolerant HG2-N1 shows both increases and decreases in specific polypeptides as compared to HG2. Many of the enhanced polypeptide bands from mRNA in the salt tolerant HG2-N1 variant appear to be constitutively expressed, since they can be detected from HG2-N1 cells grown in presence and absence of NaCl, but the expression of a few bands may depend on the presence of added NaCl. Most enhanced polypeptides, which are detected from mRNA in the salt tolerant variant HG2-N1 (grown on NaCl) are different from polypeptide bands enhanced in the salt sensitive HG2 line as a result of 24 hour salt stress. Similar results were obtained from two dimensional analysis of in vivo labeled polypeptides. At least one isolated cDNA clone shows selective expression of mRNA in salt tolerant cells grown in NaCl. These results indicate that adaptive mechanisms for salt tolerance may differ in some aspects from acute stress mechanisms.

INTRODUCTION

Plant cell culture can be used effectively for selection of salt tolerance at the cellular level (Stavarek and Rains, 1984). The molecular mechanisms which allow for this change in salt tolerance are not completely understood. Although cellular salt tolerance is infrequently lost in culture (Chandler and Vasil, 1984), regeneration of salt tolerant whole plants from the selected cells has been difficult (McCoy, 1987). In order to develop alternate strategies to somaclonal variance selection for improving the salinity tolerance of alfalfa, we have chosen to identify the genes which are differentially expressed in the acquired cellular salt tolerance of alfalfa. The problem of differential gene expression in salt stress and tolerance has been approached in other systems by comparisons of protein patterns from cells grown in the presence and absence of NaCl either by in vitro translation (Ostrem et al.,1987) or in vivo synthesized protein profiles (Ramagopal, 1987; Gulick and Dvorak, 1987). Antibody studies have shown that a 26 kD protein (Singh et al., 1985) induced by NaCl in suspension cultures of tobacco is also expressed in whole plant roots (King et al., 1986), suggesting that at least for this protein, expression is similarly regulated in cell culture and roots.

We describe in this report the enhanced expression of several genes at the mRNA level from the salt tolerant alfalfa cells as measured by in vitro translation and in vivo polypeptide synthesis. We also present a comparison of alfalfa differential gene expression at the mRNA level between the selected tolerant cell line and the control salt sensitive HG2 cells grown under conditions of acute salt stress. We have isolated one cDNA clone from poly A+ RNA that selectively identifies an inducible mRNA species in the salt tolerant line grown in salt, but not the salt sensitive line under salt stress.

MATERIALS AND METHODS

Cell Culture and Selection. Callus cultures were initiated from immature ovaries (McCoy 1987) of diploid M. sativa, clone HG2 (McCoy and Bingham, 1977). Schenk and Hildebrandt (1972) medium (designated SH medium) supplemented with 2 mg/L kinetin plus 2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) was used throughout these experiments. Callus was subcultured at four week intervals on control medium or medium containing 0.5%

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Table 1
Stability of NaCl tolerance in variant HG2-N1 after maintenance in low salt medium.

Clone	Callus Fresh Weight (g)a		
	0 NaCl	86mm NaCl	171mM NaCl
HG2 (Parent line)	2.95±0.23	0.89±0.11	0.38±0.09
HG2-HG2-N1 (NaCl Selected)	3.33±0.19	3.86±0.42	3.98±0.24
HG2-HG2-N1 (4 wks. NONSEL)	4.21±0.35	3.72±0.56	3.51±0.29
HG2-HG2-N1 (8 wks. NONSEL)	4.36±0.52	3.43±0.61	3.92±0.33

a) Final fresh wt. (g)±S.D. after 28 days growth on SH medium ± indicated NaCl conc.

(86 mM) or 1% (171 mM) NaCl. Selection for salt tolerant cell lines was carried out by a step-up protocol from 0.5 to 1% NaCl as described by McCoy (1987). Stability of the salt tolerance phenotype was tested by growth of tolerant cell lines for at least two subcultures on control SH medium, followed by transfer back to 1% NaCl. The salt sensitive HG2 parent cell line was maintained on control SH medium. Cellular response to NaCl was determined by transfer of 200 to 250 mg of callus to 100x200 mm culture plates containing 50 ml of SH medium with or without NaCl with continuous illumination (approximately 30 umol quanta m-2 s-1) and approximately 60% relative humidity.

RNA purification and Cell Free Protein Synthesis. RNA was extracted from selected salt tolerant cells grown on SH ± 1% NaCl medium and HG2 control cells grown on SH medium or after 24 hr salt stress on SH medium plus 1% NaCl. Callus was ground in liquid nitrogen, lysed in 0.1 M NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 1% SDS and extracted with phenol/chloroform/isoamyl alcohol (49:49:2). The extract was precipitated with isopropanol and the pellet washed extensively with cold 2 M LiCl, 5 mM EDTA (Palmiter, 1974). Polyadenylated RNA was isolated by oligo dT chromatography (Aviv and Leder, 1973). Northern blot analysis was carried out on 10 ug total RNA separated on denaturing formaldehyde 1% agarose gels (Thomas, 1980). Prehybridization was overnight at 42oC in deionized formamide, 2xSSC, 0.1 M Na phosphate pH7.6, 5x Denhardts and 200 ug/ml denatured salmon sperm DNA. Hybridization with primer extended DNA probe was carried out at 37oC for 46 hours. Blots were extensively washed after hybridization in 2xSSC, 0.1% SDS for 30 min. at room temp, 30 min at 45oC and 15 min at 60oC. Autoradiography was with screens at -80oC.

In vitro translation was carried out using New England Nuclear wheat germ translation system and [35S]-methionine according to the manufacturers (NEN) specifications. The polypeptides were separated by SDS-PAGE electrophoresis according to the method of Laemmli (1970). The gels were stained with Coomassie blue R250 and the [35S] polypeptides were detected by autoradiography. The autoradiograms were scanned and data collected in a Varex Universal Interface analog-digital converter and analyzed with special software developed for this application (Waterborg et al., 1989). All scans were normalized to a major invariant protein peak near the top of the gel.

Protein Labeling in Intact Cells and Polypeptide Analysis. Callus cultures of HG2-N1 tolerant line grown on SH ± 1% NaCl and HG2 control cells grown on SH medium, or stressed for 24 hr on 1% NaCl, were gently dispersed in conditioned medium ± 1% NaCl at 300 mg/ml. Two ml cultures were labeled for 4 hr. under normal growth conditions with 200 uCi [35S] methionine (Sp. Act. 1134 Ci/mmol, NEN), with intermittent rocking. The cells were collected by centrifugation, washed once frozen in liquid nitrogen. Cell extracts were prepared and analyzed essentially by the procedures described by Mayer et al., (1987). Frozen cells were ground in liquid nitrogen in one half packed cell volume of IEF buffer containing: 2% 2D Pharmalyte 3-10 (Pharmacia); 300 mM NaCl; 1mM EDTA; 1mM EGTA; 2% Triton X-100; 5 mM ascorbic acid; 100 mM DTT; 10 ug/ml leupeptin and 10 ug/ml alpha 2-macroglobulin; treated with protamine sulfate and the supernatant adjusted to 9 M urea. Aliquots of the supernatant containing equal amounts of acid precipitable [35S] counts were applied to the first dimension acrylamide IEF gels containing 5% 2D Pharmalyte 3-10, 2% Triton X-100 and 9 M urea. Running conditions for IEF and the second dimension SDS (Laemmli, 1970) gel electrophoresis were identical to those described by Mayer et al. (1987). The gels were stained with Coomasie blue and treated for fluorography, and autoradiographed with screens for 6 to 14 days.

Construction and Screening of a cDNA Library from mRNA Isolated from the Salt Tolerant Variant. A cDNA library was constructed from poly A+ mRNA from the salt tolerant variant HG2-N1 grown in presence of 0.171 NaCl in lambda ZAP (Stratagene Cloning Systems). We have differentially screened 25,000 plaques of the unamplified library with duplicate filters using as probes cDNA made to mRNA from isogenic salt tolerant and salt sensitive lines (Maniatis et al., 1982). From the 61 plaques picked from the original screen, 30 showed strong differential hybridization patterns on the second plaque purification rescreen. These isolates represent about 0.1% of the mRNA population. Clone pA10, is one of those 30 clones.

RESULTS

Salt Tolerance Selection and Stability.

The NaCl tolerant cell line, designated HG2-N1 was selected from diploid M. sativa HG2 cells. As shown in Table 1, the parent HG2 callus growth is highly sensitive to added NaCl in the medium, since even 86 mM

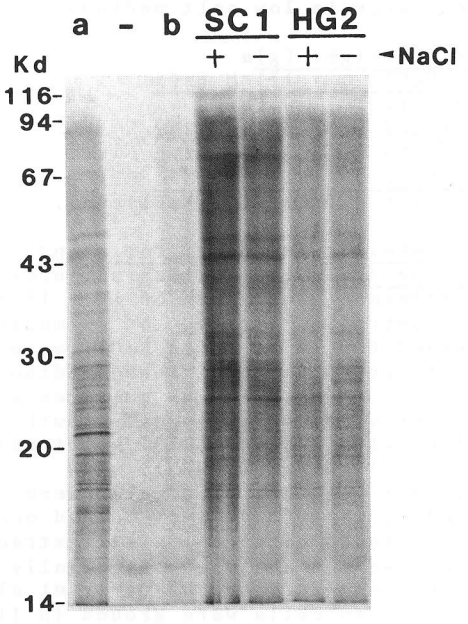

Figure 1 In vitro translation products from M. sativa HG2 and HG2-N1 (SC1) RNA. [35S]-methionine labeled translation products were denatured and analyzed on 12.5% acrylamide SDS gels. Lane a: yeast mRNA control; lane b: no added RNA. Lanes c through f: lug polyadenylated RNA from each culture. RNA preparations from HG2-N1 and HG2 cells grown on SH medium ± 171mM NaCl as indicated. Molecular weight markers in kD are indicated on the left.

Table 2
In vitro translation products from mRNA induced by salt stress or salt tolerance.

Polypeptides in k Daltons		
HG2 - Salt Stressed	HG2-N1 - Salt Tolerant	
+ NaCl	+NaCl	- NaCl
-	115	115
-	92	92
-	82	82
80	-	-
-	75	75
-	72	*
70	-	-
-	61	61
58	-	-
54	-	-
-	42	42
-	37	*
-	35	*
31-32	31	31
-	28	*
27	27	27
-	24	-
-	21	*
-	17	-

Results are compiled from 2 RNA isolates and multiple scans from several gels using 15, 12.5 and 10 % polyacrylamide.
*Indicates that the molecular size protein band characterized from mRNA in the presence of NaCl was not detected in the absence of NaCl.

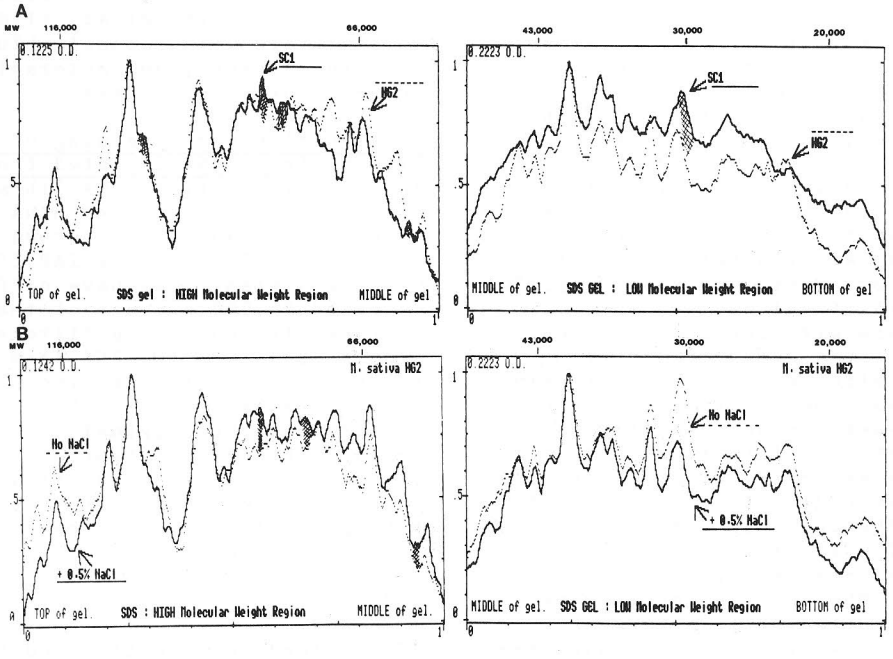

Figure 2 Computerized gel scans of in vitro translation products from autoradiogram in Figure 1. A: comparison of mRNA translation products from salt sensitive HG2 and salt tolerant HG2-N1 (SC1). B: comparison of mRNA translation products from salt sensitive HG2 grown in normal medium and under conditions of 24 hr salt stress.

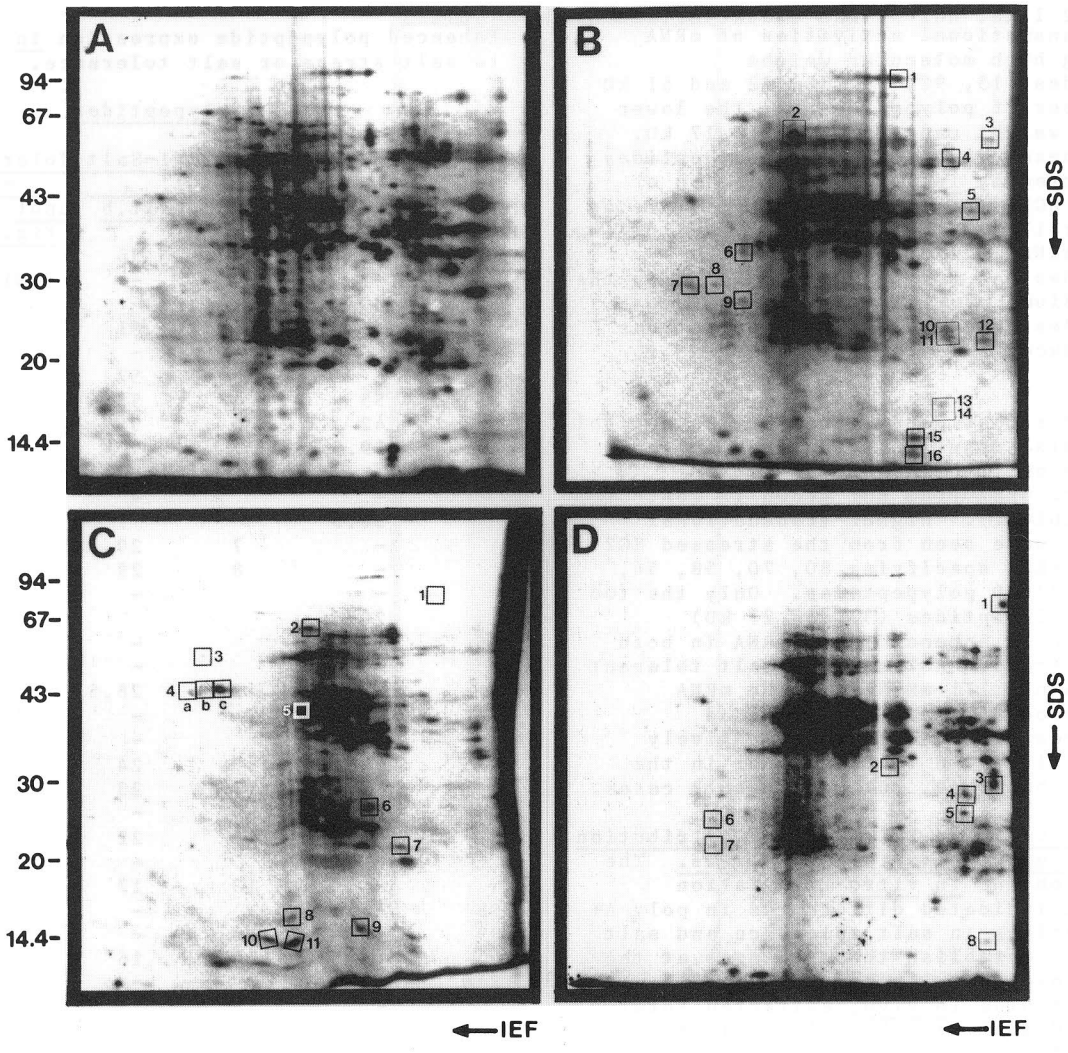

Figure 3 Two-dimensional IEF-SDS electrophoresis and fluorography of in vivo labeled polypeptides from: A, salt sensitive HG2 grown on SH medium; B, salt tolerant variant HG2-N1 grown on SH medium; C, 24 hr salt stressed HG2 grown on SH medium + 171mM NaCl; D, HG2-N1 grown on SH medium + 171mM NaCl. Equal acid precipitable counts (34,000) were applied to each IEF gel. The squares show polypeptides induced in HG2-N1 as compared to parent HG2 in panel B; polypeptides induced by salt stress of HG2 in panel C; and polypeptides induced by NaCl in HG2-N1 in panel D.

NaCl is lethal to greater than 95% of the cells. The selected line HG2-N1 grows equally well on control medium and medium containing 86 and 171 mM NaCl. The ability of HG2-N1 to grow in the presence of NaCl remained unchanged after 4 and 8 weeks growth on control SH medium. These results indicate that the NaCl tolerance HG2-N1 is a stable change of phenotype, which does not require the continued selective pressure of 171 mM NaCl to maintain expression of the tolerance characteristic.

Changes in poly A+ RNA in Salt Tolerance and Salt Stress. Differential gene expression between the two cell lines was analyzed at the mRNA level by in vitro translation of 1 ug of polyadenylated RNA isolated from each cell line after growth on SH medium + NaCl. The patterns of [35S]-methionine labeled polypeptides by in vitro translation are shown in Figure 1. Poly(A)+ RNA is actively translated in the wheatgerm system not only from HG2-N1 cells, but also from the HG2 parent line, even when the RNA has been isolated from HG2 cells which have been stressed with 171 mM NaCl for 24 hours, indicating mRNA persistence during the experimental stress period. The in vitro translated polypeptides range from 15 to 130 kD.

The patterns of in vitro translation products of all four mRNA preparations exhibited evident similarities. However, quantitative scan comparisons (Figure 2) of several gels with different acrylamide concentrations as well as in vitro translation of mRNA from two different RNA preparations show discrete differences in the polypeptide band patterns. These data are summarized in Table 2 which lists new or enhanced polypeptides from mRNA in stressed HG2 cells or the tolerant HG2-N1 grown with and without 0.171 M NaCl. All scans were normalized and compared to the

parent HG2 line. HG2-N1 was characterized by higher transnational activities of mRNA specifying high molecular weight polypeptides 115, 92, 82, 75, 72 and 61 kD and a number of polypeptides in the lower molecular weight range from 42 to 17 kD. Some of these new or enhanced polypeptides were detected by *in vitro* translation of mRNA from HG2-N1 cells grown in presence or absence of 171 mM NaCl (Table 2), indicating that the mRNA accumulation for these polypeptides was not affected by NaCl in the growth medium. For five of the enhanced polypeptides however, mRNA concentrations may be induced by added NaCl as shown in Table 2.

The salt stress conditions for the control HG2 line also resulted in changes in the population of translatable mRNA when compared to mRNA from HG2 grown in control medium (Table 2). Higher transnational activities were seen from the stressed HG2 cells for mRNA specifying 80, 70, 58, 54, 31-32 and 27 kD polypeptides. Only the two smallest polypeptides (31 and 27 kD) appeared to be enhanced from mRNA in both the salt stressed HG2 and the salt tolerant HG2-N1 grown ± 171 mM NaCl. The mRNA species encoding the other enhanced polypeptides appeared to be selectively expressed either in the stress or in the tolerance phenotype, but not in both cases.

Changes in Cellular Polypeptide Distribution in Salt Tolerance and in Salt Stress. The results from the *in vitro* translation experiment indicated differences in poly A+ RNA populations in salt tolerance and salt stress. To establish these changes at the cellular level, we labeled whole cells with [35S] methionine *in vivo*, extracted total protein and analyzed the polypeptides by two-dimensional IEF/SDS/polyacrylamide electrophoresis and autoradiography. Figure 3 shows the labeled polypeptide distribution for the salt sensitive HG2 cells (Fig. 3A) and salt tolerant HG2-N1 (Fig. 3B), both grown on normal SH medium. The *in vivo* labeled polypeptides, which are newly detectable in a comparison between the HG2-N1 line and the parent HG2 on SH medium, are marked by boxed areas in Fig. 3B. A similar comparison, indicating newly detectable polypeptides as a result of salt stress of the sensitive HG2 line, is shown by boxed areas in Fig. 3C. The fourth panel, Fig. 3D shows the distribution of polypeptides in the tolerant HG2-N1 grown on 171mM NaCl. The boxed areas in Fig. 3D mark only polypeptides which appear to be induced by NaCl in the tolerant variant.

The distribution and molecular weights of the newly synthesized proteins which result from salt tolerance and salt stress is summarized in Table 3. As in the case of *in vitro* translation data, we can see a constitutive change in expression of many of the new polypeptides detected in the tolerant variant present in both Fig.3B and 3D, but for the sake of clarity are only marked in Fig.3B). Others, boxed in Fig. 3D, are induced by NaCl in the tolerant HG2-N1, but are not detected in HG2 under

Table 3
Enhanced polypeptide expression *in vivo* due to salt stress or salt tolerance.

		New Polypeptides			
HG2-Salt Stress + NaCl		HG2-N1-Salt Tolerant -NaCl		HG2-N1-Salt Tolerant +NaCl	
Spot	k Dalt.a Fig.3C	Spot	k Dalt.b Fig.3B	Spot	k Dalt.c Fig.3D
-		1	96		96
-		-	-	N1	83
S1	80		-		-
-		2	61		61
S2	60		-		-
-		3	57		57
S3	55	4	55		55
S4	42	5	55		55
S5	39.5		-		-
-		6	34		34
-		-	-	N2	
	30.5				
-		7	29.5		29.5
-		8	29		29
-		-	-	N3	
	28.5				
S6	27		-		-
-		-	-	N4	27
-		9	26.5		26.5
-		-	-	N5	25
S7	24		-		-
-		10	24		24
-		11	23		23
-		-	-	N6	23
-		12	22		22
-		-	-	N7	21
-		13	17		17
S8	16.5		-		-
S9	16		-		-
-		14	16		16
S10	15.5		-		-
-		15	15		-
S11	14.5		-		-
-		16	14		14
-			-	N8	14

These results were compiled by comparing two-dimensional polypeptide patterns shown in Fig. 3. (-) represents no detectability. a) Polypeptides detected in NaCl stressed HG2 (Fig.3C), but not in normal HG2 (Fig. 3A). b) Polypeptides detected in HG2-N1 (Fig.3B), but not in HG2 (Fig.3A). c) All the polypeptides detected in HG2-N1 grown in NaCl (Fig.3D), but not in HG2 (Fig. 3A). Polypeptides numbered N1-N8 were detected in HG2-N1 grown in NaCl (Fig. 3D), but not in HG2-N1 grown in normal SH medium.

stress conditions. Salt stress induces different polypeptides in HG2, since most of these polypeptides are not detected in the tolerant HG2-N1 ± NaCl. These results suggest different adaptive mechanisms for salt stress and salt tolerance.

Detection of mRNA Selectively Induced in the Tolerant Variant. We have used a cDNA clone from poly A+ RNA, which was isolated by differential screening, to determine specific mRNA induction by NaCl in the tolerant variant. Northern blot analysis with our clone pA10, shows in Figure 4, that indeed this mRNA species is expressed in HG2-N1 grown in NaCl, is not seen in

HG2-N1 grown on normal SH medium or in HG2 + NaCl. This shows specific mRNA induction by NaCl in the tolerant variant.

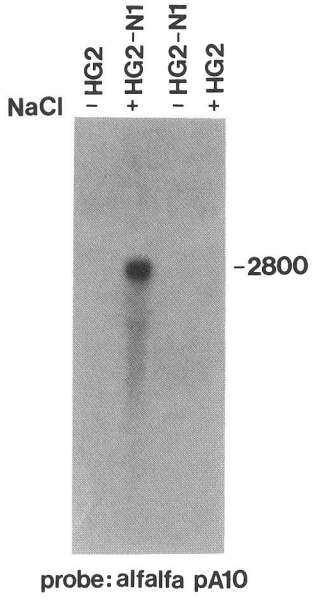

Figure 4 Northern blot analysis of salt induced mRNA expression in HG2 and HG2-N1 of alfalfa. 10 ug total RNA from HG2 ± 24 hr salt stress, or HG2-N1 grown continuously ± NaCl. Probe: alfalfa cDNA clone pA10.

DISCUSSION

The selection of a salt tolerant variant of diploid M. sativa HG2 has allowed us to compare differential gene expression at the mRNA level in two closely related cell lines of alfalfa, which differ in the cellular salt tolerance phenotype. We have chosen to focus at this time on those mRNAs which lead to increased transnational activities for specific polypeptides in line HG2-N1, in order to ultimately identify those genes which are activated in the tolerant phenotype of alfalfa.

Induction of specific polypeptides in response to salt stress is also found in tobacco and tomato cells (Singh et al.,1985; King et al.,1986). Whole plant comparisons of salt stress and tolerance also show changes in tissue specific regulation of mRNAs coding for individual polypeptides (Ramagopal, 1987; Gulick and Dvorak, 1987; Ostrem et al., 1987). Our analysis of alfalfa mRNAs and their in vitro translation products has identified mRNAs coding for polypeptides in the 17 to 115 kD range, which appear to be enhanced in the salt tolerant HG2-N1 cell line. The cellular growth experiments ± NaCl of the tolerant HG2-N1 cell line suggest that the increased expression of many mRNAs coding for the enhanced polypeptides is constitutively changed, i.e. they are not qualitatively affected by the presence of NaCl in the growth medium as measured in vitro or in vivo. However, some other polypeptides are NaCl inducible as demonstrated in Figure 4 and may be of particular importance for the tolerance phenotype. The somewhat unexpected finding was that with a few exceptions, stress and tolerance induced mRNAs and polypeptides seem to belong to different groups. These results suggest that adaptive mechanisms for salt tolerance may differ from acute stress mechanisms.

Possibly the 27 kD polypeptide (Table 2), encoded by mRNA from both the HG2 stressed and HG2-N1 tolerant alfalfa lines is similar to the 26 kD protein observed in tobacco, tomato and alfalfa (Singh et al.,1985; King et al., 1986); however, the two dimensional gel analysis presents a more complex pattern of polypeptides. To date very few stress proteins have been identified with known functions (Hanson and Jacobson, 1984; Ostrem et al., 1987). Therefore, we can not predict the functions of the enhanced alfalfa polypeptides in tolerance or stress. Further information in this area is likely to come from cDNA clones which detect mRNA species that are differentially expressed in the cellular salt tolerant phenotype. Experiments are in progress to characterize pA10 and identify other clones which correlate their expression with cellular salt tolerance in alfalfa.

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