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Gene expression in two different genotypes of alfalfa under salt stressed and unstressed conditions

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GENE EXPRESSION IN TWO DIFFERENT GENOTYPES OF ALFALFA UNDER SALT STRESSED AND UNSTRESSED CONDITIONS

by

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ABSTRACT

Gene expression in two different genotypes of alfalfa, salt-tolerant and salt-sensitive, was examined by studying differences in protein products coded for by poly(A^+) RNA isolated from shoot and root tissue. Plants were grown in hydroponics under unstressed or salt-stressed conditions. Two salinity levels (low salt:30 mM NaCl and 6 mM $CaCl_2$ and high salt:133 mM NaCl and 27 mM $CaCl_2$) and one unstressed control were applied. The salt-tolerant genotype showed higher biomass accumulation than the salt-sensitive genotype under both control and salt-stressed conditions. The difference in biomass accumulation between the two genotypes was greatest at the highest salt level. The effect of salt stress on gene expression was studied via in vitro translation of poly (A^+) RNA with [^{35}S]-methionine. The labeling pattern was similar in all treatments when analyzed by one dimensional SDS-PAGE. However, a two dimensional analysis (isoelectric focusing followed by SDS-PAGE) showed that salt-stress induced a number of new proteins and repressed several others.

INTRODUCTION

Salinity is an important limitation to crop production in many agricultural areas (Staples and Toeniessen, 1984). In order to meet the demand for increased food production it will be necessary to use irrigation water with higher salinity. It has been estimated that between 400 x 10^6 (Ponnamperuma, 1977) and 950 x 10^6 hectares of crop lands (Massoud, 1974) are adversely affected by soil salinity.

Little is known about the mechanisms of plant responses to salinity. The current state of knowledge in this area has been reviewed by Flowers, Troke and Yeo (1977), Greenway and Munns (1980) and Cheeseman (1988). A number of studies indicate that plant tolerance to salinity may involve changing some metabolic pathways via directed synthesis of salinity-induced proteins (Greenway and Munns, 1980). Hurkman and Tanaka (1987) has reported that the net synthesis of a number of barley (Hordeum vulgare L.) root proteins, as measured by the in vivo incorporation of [^{35}S]-methionine, changed during growth in 200 mM NaCl. The most striking change was a significant increase of label in two protein pairs that had pIs of approximately 6.3 and 6.5 and each pair consisted of proteins of approximately 26 and 27 KD. Gene expression at

the mRNA level was investigated in salt-tolerant and salt-sensitive genotypes of barley (Ramagopal, 1987). Ramagopal suggested that salinity triggered differential transcription of specific mRNAs depending upon genotype and tissue. In tomato (Lycopersicon esculentum VFNT), a cDNA clone has been isolated and characterized which codes for a salt-induced protein (King et al, 1988). The metabolic role(s) of these salt induced proteins remains unknown.

In this study, we investigated the effect of salt stress on the pattern of gene expression in alfalfa (Medicago sativa L.). Alfalfa has the highest livestock feeding value among all forage crops and is the primary perennial forage crop grown under irrigation in Arizona as well as in most arid and semiarid agriculture regions throughout the word (Barnes and Schaeffer, 1985). Variation in salinity tolerance has been observed in alfalfa (Allen et al, 1985), yet the underlying molecular and genetic factors responsible for this effect are not understood.

To begin to study salt tolerance in alfalfa at the molecular level, two genotypes of alfalfa were examined. One shows salt tolerance during germination and seedling growth (McKimmie and Dobrenz, 1987) while the other is salt sensitive. The plants were grown in a growth chamber in hydroponics culture under a level of salt stress that reduced

growth rate but did not cause gross modification to the phenotype, a state we have labelled "growth-maintaining stress adaptive". We consider this condition to reflect adaptive growth under stress as opposed to a stress-shocked "survival" mode. The salt-tolerant genotype was derived from the salt-sensitive genotype through several germination and seedling selection cycles (Dobrenz et al, 1983 and Mckimmie and Dobrenz, 1987).

Gene expression was studied by isolating poly (A^+) mRNA from roots and shoots of plants. The mRNA was translated in vitro and the protein products were analyzed by one dimensional SDS-PAGE gels and two dimensional electrophoresis. The experimental objectives of this research were: 1) to compare the levels of expression of genes in selected stress tolerant and nontolerant genotypes under both stressed and non-stressed conditions. One approach to this objective is to identify differences in protein products coded for by the mRNAs; 2) to make cDNA libraries for identifying candidate salt stress tolerance genes in alfalfa using the mRNAs from both genotypes.

The long range goals of the research were twofold: 1) to identify candidate salt stress tolerance proteins and cDNA clones as a first step towards understanding the metabolic basis of salt tolerant metabolism in alfalfa. 2) to

vertically integrate molecular biology into the ongoing effort to develop alfalfa cultivars and synthetic populations with enhanced salt tolerance. Candidate salt tolerance cDNAs may be identified directly by +/- screening or indirectly using immunological or sequence data obtained from proteins. These candidate cDNAs may then be used as probes in hybridization studies with alfalfa populations under continuous selection for enhanced salt tolerance. The availability of these selected populations allows us to look for cDNAs whose expression is enhanced, either at the DNA or RNA levels, in genotypes with enhanced salt tolerance. Identification of clones that give a positive response in these types of assays will simultaneously provide prima facie verification of a role for this gene in salt tolerance and give us a breeding tool with which to screen germplasm for this trait.

Therefore, this research provides a model for a system whereby molecular research may be integrated into existing physiological and genetic programs designed to select for an agronomically important trait.

LITERATURE REVIEW

It is becoming more important for human beings to modify plants to suit adverse environments in order to maintain sufficient yields for food. One of the these adverse environments involves salinity which presents a stress condition for many crop plants. Increasing salinity of soil or water threatens agriculture. One third to one half of the world's irrigated area has been already adversely affected to some degree by excess salinity (Norlyn, 1980; and Raloff, 1984). Although irrigated land accounts for only 15 percent of the world's total cropland, it produces 30 percent of the world's food supply (Wittwer, 1979). In addition, not all salinity problems are confined to irrigated croplands. It is a worldwide agricultural problem. In California about 1.2 million acres are so severely affected by salts as to be of no use for agriculture (Kelley, Norlyn and Epstein, 1979). In Australia the annual cost to agriculture resulting from salinity is estimated to be 32 million dollars (McWilliam, 1986). Approximately 40,000 ha of the Indian sub-continent are annually removed from crop production due to increasing soil salinity (Chapman, 1975). Meanwhile, the Earth's population is continually increasing and more food must be

provided. Therefore there is a stronger push for agriculture on marginal lands, often characterized by soils and water with a high degree of natural salinity. Salinity tolerance would therefore be a highly desirable characteristic to introduce into crop plants if yield potential could be maintained.

Salinity and Plants

Natural or artificial salinity represents a complex and widespread phenomenon. The type and degree of salinity varies as does plant response to it. In ocean water (about 35,000 ppm or -3.0 MPa), Na^+ and Cl^- ions are dominant. They are, however, usually not essential for plant growth or at most required in a minute amount. If salts are in excess in soil or water, they will inhibit plant growth or even kill the plants. Some irrigation sources as well as saline soils may contain toxic amounts of boron or other ions (Maas and Hoffman, 1977). Because the degree of impairment of plants by salt depends on species, variety, growth stage, and environmental factors, it is difficult to define saline conditions precisely. Current definitions are based on salt content alone or in conjunction with texture, morphology, or

hydrology (Northcote and Skene, 1972). The most widely accepted definition of a saline soil is one that gives an electrical conductivity (EC) in the saturation extract exceeding 4 mmho/cm (4dS/m) at 25•C (Mengel and Kirby, 1978).

Diversity of plant response to salinity is very great. Generally plants are classified into two groups; halophytes and nonhalophytes (Jennings, 1976). Halophytes are defined as plants growing on soils that contain soil solutions of at least -0.33 MPa (the equivalent of 70 mM monovalent salts) (Greenway and Munns, 1980). However, there are very great differences in response to salinity between species, and even below the species level in both halophytes and nonhalophytes (Maas and Hoffman, 1977).

Salt tolerance of plants may be defined generally as sustained growth of plants in an environment of NaCl or combinations of mixed salts, and can be measured by a number of criteria. Survival at high salt concentrations has been the fundamental selection criterion for barley (Rush and Epstein, 1976), wheat (Triticum aestivum L.)(Rush and Epstein, 1981), and tomato (Epstein and Noryln, 1977). Another method of measuring salt tolerance is the determination of growth or yield response under saline condition (Maas and Hoffman, 1977).

Salinity affects plant growth of different species in different ways. The halophytic species generally require a moderate level of soil salinity to maintain optimal growth conditions. Most halophytes reach optimum growth rates with 200 mM to 500 mM NaCl in the root environment (Greenway and Munns, 1980). Glycophytes, in contrast, generally reach their maximum growth rates in non-saline conditions. The glycophytes represent a broad range of sensitivity to salinity. Sugar beets (Beta vulgaris L.), for instance, have exhibited increased fresh weights, dry weights, and sugar yield in response to NaCl application (Milford, Cormock and Durrant, 1977; Draycott and Farley, 1971). Salinity also affects the growth of alfalfa plants. Smith, Dobrenz and Schonhorst (1981) indicated that Cl^- was much more toxic than Na^+ ion in reducing plant growth.

It has been suggested that there is a relationship between salinity and the synthesis or accumulation of compatible, organic solutes in the plants. The primary role most often attributed to the compatible solutes in the salt stressed plants is a contribution to the osmotic balance for intracellular osmotic adjustment and a protective effect on enzymes in the presence of high electrolytes in the cytoplasm (Greenway and Munns, 1980). Solutes which increase at high salinity in many species include glycinebetaine, proline

(Storey and Jones, 1977), and various types of sugars (Bernstein and Ayers, 1953). Exogenous glycinebetaine and proline enhanced the salt tolerance of Marin Mink Barley embryos in culture (Lone and Jones, 1986). They also found that exogenous proline resulted in a significant exclusion of Na^+ and Cl^- from the shoot tissue without any apparent effect on root tissue absorption.

The high concentration of Na^+ and Cl^- ions has been shown in some studies to inhibit the in vitro activity of cytoplasmic enzymes isolated from halophytes and nonhalophytes (Flowers et al, 1977). Much evidence indicates that inorganic ions are stored in the vacuole to avoid injuring cellular metabolic process, and compatible solutes are synthesized and accumulated in the cytoplasm to balance the lowered osmotic potential of the vacuole (Stewart and Lee, 1974; Flowers et al, 1977). Unlike the inorganic ions the compatible organic solutes do not interfere with the activity of cytoplasmic enzymes (Pollard and Jones, 1979).

In general, plants follow two strategies in response to saline conditions. Some plants in seasonal or transient salinity environments can avoid the adverse effects of salt by rapid completion of ontogenesis, leaf shedding or low stomatal conductance to reduce water vapor loss. Some plants alter their physiological and biochemical processes to adapt

to saline environments. Unfortunately, we know very little about this mechanism for salt tolerance. However recent research is focusing on this aspect of salt stress.

The Breeding and Genetics of Salt Tolerance

One of the earliest accounts of variability in salt tolerance and its inheritance was made by Lyon (1941) in a study conducted at the United States Salinity Laboratory. In his research, Lycopersicon pimpinelifolium was less sensitive than Lycopersicon esculentum to $NaSO_4$ salinity as measured by root dry weight, average fruit weight, and mean weight per fruit. The interspecific F1 hybrid derived from these two species exhibited the characteristics of the sensitive parent.

Around 1952, intraspecific differences were noted in the germination and yield responses of certain barley and wheat varieties at high salinities (Ayers, Brown and Wadleigh, 1952). Subsequent studies with lettuce (Lactuca sativa L.), onion (Allium cepa L.), carrot (Daucus carota L.), and green beans (Phaseolus vulgaris L.) indicated only minor intraspecific differences among these horticultural species

(Ayers, Wadleigh and Bernstein, 1951; Bernstein and Ayers, 1951; and Bernstein and Ayers, 1953). This led Bernstein (1961) to conclude, "For most crops, the related uniformity in salt tolerance among varieties suggests little likehood of improving salt tolerance by any (genetic) combination of the commonly available varieties." He suggested the use of "wild" germplasm resources to introduce the necessary variability into such species.

However, there is genetic diversity for response to salinity within crop species. Ayers, Brown and Wadleigh (1952) have reported varietal differences for salt tolerance in wheat and barley. Distinctions in salt response between commercial cultivars have also been found in rice (Oryza sativa L.) (Pearson, Ayers and Eberhard, 1966), wheatgrass (Agropyron cristatum L. Gaertn.) (Shannon, 1978), grain sorghum (Sorghum bicolor L. Moench) (Taylor, Young and Rivera, 1975), soybean (Glycine max Merrill) (Abel and Mackenzie, 1964), sugarbeet (Marschner, Yun and Kuiper, 1981), alfalfa (Greub, Drolsom and Rohweder, 1985), bermuda grass (Cynodon dactylon L. Pers.) (Dudeck et al, 1984) and Paspalum turf (Dudeck and Peacock, 1985). In a study of salt-induced sterility in rice, Akbar and Yabuno (1977) found that rice varieties subjected to salinity responded quite differently from one another during flowering. They classified three

types of salt-induced sterility. F1 crosses between the relatively salt-tolerant 'Jonah 349' and the salt-sensitive 'Magrolia' varieties were highly tolerant to salinity. The F2 population included some salt-tolerant and salt-sensitive individuals.

Whole plant breeding systems have met with some success in improving the response of crop plants to salinity. It is promising that a halophytic mode of adaption was introduced into the cultivated tomato by crossing it with a related species from the Galapagos (Kelley, Norlyn and Epstein et al, 1979). Alfalfa is considered as moderately salt sensitive but there is variability among cultivars. Some salt-tolerant alfalfa plants have been derived from germination and seedling selection (Stone et al, 1981; Smith, Dobrenz and Schonhorst, 1981; Dobrenz et al, 1983 and Mckimmie and Dobrenz, 1987).

The application of tissue or cell culture techniques provides another method for improving the response of crops to salinity stress. Salt tolerance of undifferentiated callus tissue can be evaluated by adding salt to a culture medium (Croughan, Stavarek and Rains, 1978; Smith and McComb, 1981). Many cell lines have been selected for tolerance to NaCl (Hasegawa, Bressan and Handa, 1980; Nabors, et al, 1975 and Nabors, et al, 1980). They include Nicotiana sylvestris, N.

tabacum L., Capsicum annum L. (Kochba et al, 1982), Coffea arabica L. (Yasuda, Maegawa and Yamaguchi, 1982) and Colocasia esculenta L. (Nyman, Gonzales and Arditti, 1983). Successful selection of salt-tolerant alfalfa cell lines has been accomplished using cell suspension culture techniques with a 1% (171 mM) NaCl media (Croughan, Stavarek and Rains, 1978). The selected salt-tolerant lines performed poorly in non-saline media, suggesting a salt requirement similar to that seen in halophytes. It appears that the cell culture system can be used to select cells tolerant to salt. However, the limitation is regeneration from cell culture and retention of the salt tolerant trait in regenerated plants.

In conclusion, the effects of salinity in plants have been studied for nearly a century, but there is little knowledge of the genetics of plant response to salinity and it is unlikely that a simple one gene relationship with total salt tolerance will be found.

Molecular Biology of Plants Under Salinity Stress

Recent advances in molecular biology and genetic engineering technology have made it possible to isolate,

manipulate, and study the expression of specific stress-induced genes. Many laboratories have been using these techniques to study the mechanisms of salt tolerance in halophytic and nonhalophytic plants. Altered gene expression due to salinity has been demonstrated in barley (Ramagopal, 1987 and Hurkman and Tanaka, 1987), spring wheat (Gulick and Dvorak, 1987), and tomato (King et al, 1988). Additionally there are many salt adaptive cell lines from various plant species. Salinity can trigger or regulate the expression of selected mRNAs of roots and shoots but patterns differ in different barley genotypes (Ramagopal, 1986). The molecular mass and pI of the induced proteins ranged from 20 to 27 KDa and pI6.1 to pI7.6 using both in vivo and in vitro translation experiments. Gulick's (1987) work on wheat showed that twice as many genes are affected by salt stress in Chinese Spring as in the amphiploid, although Chinese Spring was exposed to slightly lower salinity than the amphiploid. This suggested that a number of genes in Chinese Spring wheat that were affected by the altered metabolism resulting from salt stress have little or nothing to do with salt tolerance. Because the amphiploid is more salt tolerant than Chinese Spring, the most important question is whether any of the genes regulated by salt stress in the amphiploid are causally related to its superior salt stress tolerance.

The observations of Hurkman and Tanaka(1987) indicate that the response of barley roots to NaCl does not involve the synthesis of unique proteins, but rather involves the modulation of the net synthesis of a wide range of constitutive proteins. The labeling of the 26 to 27 KDa proteins increased significantly in response to NaCl treatments and decreased when the plants were transferred to nutrient solution without NaCl. In cultured tobacco cells adapted to grow in media containing high levels of salt, a 26 KDa polypeptide increased significantly (Ericson and Alfinito, 1984 and Singh et al, 1985).

One explanation for the observed changes in net protein synthesis induced by NaCl may be that the translation of the mRNAs is inhibited or stimulated to varying degree by increased cytoplasmic NaCl concentration. It is a possible that the 26 to 27 KDa proteins may represent shock-induced prcteins rather than proteins that allow barley to grow or survive better in the presence of salt. Therefore there may be a difference between expression of genes induced by shock and genes where expression is altered only after adaptation (Singh et al, 1985).

A tomato cDNA clone coding for a protein induced by exogenous NaCl has been isolated and characterized from a tomato root cDNA library (King, et al, 1988). The mature

polypeptide sequence, as deduced from the nucleotide sequence, revealed a protein with a molecular weight of 24 KDa. This protein has been named NP24. It is slightly basic and has an unusually high number of cysteine residues (King, Hussey and Turner, 1986). Northern blot analyses showed that the abundance of mRNA for NP24 was at least 100-fold greater in log phase tomato suspension culture cells grown in medium with NaCl than in cells grown in the control medium. The protein is not secreted, and is localized within the cytoplasm or the soluble fraction of the nucleus, vacuole, or microbodies.

MATERIAL AND METHODS

Plant Material and Salt Treatments

Two different genotypes of alfalfa were used. Both generously provided by the laboratory of Professor A. K. Dobrenz (Department of Plant Sciences, University of Arizona). One of the genotypes, from the cultivar Mesa-Sirsa, is salt sensitive. The other is salt tolerant, selected from an alfalfa germplasms, AZ-GERM SALT-I (Dobranz et al, 1983), and represents two cycles of recurrent selection for seedling salt tolerance (McKimmie and Dobrenz, 1987). The two genotypes were furnished coded as "A" and "X", so that all experiments were conducted without knowledge as to which was salt tolerant.

These genotypes were vegetatively propagated by cutting. The mature stems of "A" and "X" were cut to approximately 10 cm with a razor blade. Each cutting contained 2 nodes. The leaf of the top node was kept while the leaf of the bottom node was cut off. The bottom end of the cutting was moistened with water and gently dipped with Rootone F (Union Carbide Agricultural Products Company, Inc.). All the cuttings were placed upright in a tray (40 X 60 X 10

cm) containing prewetted perlite and placed in a greenhouse under a fine mist condition for rooting. After about 20 days, plants with 3-5 cm roots were transferred to tapered 21 cm containers and allowed to grow for two weeks with daily watering. Plants of approximately equal size were selected for hydroponic culture in an environmental growth chamber.

Hoaglands Nutrient Solution was used at half strength. Each of six buckets was filled with 14 liters of the diluted Hoaglands solution. There were 3 buckets for each genotype and with four plants per bucket. The plants were suspended in the buckets by foam plugs in a styrofoam raft. The environmental conditions in the growth chamber were adjusted for optimum growth in order to minimize interaction between salt and other types of biophysical stresses (Table 1).

After 10 days to adapt to the hydroponics, the plants were salinized over a 10 day period. The experiment involved three treatments:

1. Unstressed control (0.5X Hoaglands solution only).
2. Low salt stress (-0.18 MPa or 30 mM NaCl and 6 mM $CaCl_2$ in the 0.5X Hoaglands solution).
3. High salt stress (-0.80 MPa or 133 mM NaCl and 27 mM $CaCl_2$ in the 0.5X Hoaglands solution).

Table 1. Environmental conditions in the growth chamber during hydroponic culture.

Time	Temp. (°C)	Humidity*	Lamp ($\mu E/M^2 \cdot Sec$)
06:00	18	0	350
08:00	20	0	350
10:00	23	0	350
12:00	26	0	350
14:00	22	0	350
16:00	18	0	350
18:00	15	0	0
:	15	0	0
:	15	0	0
06:00	18	0	350

*Indicates chamber humidifier not operational.

Salts were added into the culture solution in 1/10 increments over 10 successive days. The plants were grown for one more week after salinitation then harvested and the biomass of roots and shoots was measured. All tissues were frozen in liquid nitrogen and stored at - 70°C.

There were three replications in the "A" genotype and two replications in "X" genotype. Each replication had 12 plants. Additional replications were not possible due to the loss of use of the growth chamber.

Extraction and Purification of RNA

The procedure for isolation of mRNA was optimized by Danon, et al. (personal communication, 1987). Twenty gm of frozen tissue was ground in a mortar under liquid nitrogen to a fine powder and transferred into 50 ml of 10 mM Tris-Cl, (pH8.8), 50 mM NaCl, 6% PAS (p-aminosalicylic acid, Sodium salt, Sigma), 1% TNS (tri-isopropylnaphthalene sulphonic acid, Na salt, Kodak), 6% isobutanol. The mixture was homogenized with a Polytron mixer (Brinkmann) for 3 min at power 7. Immediately, an equal volume of phenol : chloroform : isobutanol (50:50:1) solution was added and homogenized again

for 15 sec.

The mixture was shaken vigorously for 20 min at room temperature and centrifuged at 4,000g (RCF) for 15 min. The top aqueous phase was collected and the phenol phase was re-extracted with 25 ml of 10 mM Tris-Cl, pH8.8, 3% PAS, 0.5% TNS, 3% butanol, vigorously shaken for 20 min at room temperature and spun down as before. The first and second aqueous phases were combined and re-extracted with 2/3 volume of the phenol : chloroform : isobutanol by shaking for 20 min, and spun down as before. The RNA was precipitated with 1/10 volume of 3.0 M NaOAc, pH 6.0 and 2.5 volumes of pre-cooled ethanol for 6 hrs or overnight.

The precipitate was recovered by pelleting at 5,000g at 4°C for 10 min. The supernatant was decanted and the pellet allowed to dry completely. The pellet was resuspended in 80 ml of 10 mM Tris-Cl, pH8.0, 1% sarkosyl and 1 mM EDTA. One quarter volume of 10 M LiCl was added (2.0 M final conc.) and the solution mixed well. RNA was precipitated at 4°C for 6 hrs or overnight. The RNA was pelleted at 12,000g at 4°C for 20 min. The dry pellet was suspended with 80 ml of 10 mM Tris-Cl (pH8.0), 0.5% sarkosyl, 1 mM EDTA. One quarter volume of 10 M LiCl was added and the RNA precipitated at 4°C. After 6 hrs, the precipitate was pelleted and resuspended with 6.5 ml of 10 mM Tris-Cl, pH8.0, 0.1% SDS, 1 mM EDTA and

precipitated with NaOAc/ethanol as before.

Total RNA was purified further via CsCl gradient density ultracentrifugation. The pellet was dissolved in 50 mM Tris-Cl (pH7.5), 0.2% SDS, and adjusted to 0.4 M NaCl. The solution was warmed to 65°C and solid CsCl (0.5 g/ml solution) was added while stirring at 65°C for 10 min. The solution was layered onto 5.7 M CsCl in 100 mM EDTA (adjusted pH to 7.0 with KOH) and ultracentrifuged for 24 hrs (Beckman, L8 - 55, 35K rpm in an sw50.1 rotor, at 20°C). The RNA pellet was washed with cold ethanol by vortexing, repelleted at 12,000g for 15 min and allowed to dry. The RNA was suspended in 6 ml of 10 mM Tris-Cl (pH7.5), 0.2% SDS, 1 mM EDTA, made 0.3 M with 3 M NaOAC (pH5.5) and precipitated with 2.5 volumes of ethanol at -20°C overnight. The precipitate was pelleted at 12,000g and resuspended with 2 ml DEPC distilled H_2O. A_{260}, A_{280}, and A_{320} of the RNA was determined. Total RNA was calculated as A_{260} reading x 40 μg/ml. This value and the fresh source tissue weight were used to calculate the yield of mRNA per gram of tissue.

Poly (A^+) mRNA was isolated from total RNA as follows. The RNA in DEPC diH_2O was added to 2 ml of buffer (100 mM Tris-Cl, pH7.5, 2 mM EDTA, 0.8 M NaCl) then adjusted to 0.2% SDS with 20% stock solution. The RNA solution was incubated at 65°C for 5 min and passed through a column filled with 500

mg of oligo dT cellulose (Pharmacia, Molecular Bio. Division) that had been pre-saturated with the first wash buffer (50 mM Tris-Cl, pH7.5, 1 mM EDTA, 0.4 M NaCl, 0.2% SDS) overnight at room temperature. The RNA solution was collected and passed over the column two times. The column was washed with 30 ml of the first wash buffer, 10 ml of the second wash buffer (50 mM Tris-Cl, pH7.5, 1 mM EDTA, 0.1 NaCl, 0.01% SDS) and 1 ml of the third wash buffer (identical to the 2nd except without SDS). The Poly(A^+) RNA was eluted with 9 ml of 50°C elution buffer (10 mM Tris-Cl, pH7.5, 1 mM EDTA) and collected with 6 fractions of 1.5 ml each. The RNA content in each fraction was determined via a spot test (5 μl + 5 μl dilute ethidium bromide solution). The RNA containing fractions were combined in a 25 ml tube and precipitated with NaOAc/EtOH as before. The ethanol precipitate of the Poly$(A)^+$ RNA was suspended in 100 μl - 200 μl sterile water. A_{260}, A_{280}, and A_{320} were read and content of poly (A^+) RNA, concentration per μl, and percent of total RNA were calculated. The RNA was stored at minus 80°C.

In Vitro Translation of Poly (A^+) RNA

The isolated poly (A^+) RNAs were translated with a nuclease-treated, rabbit reticulocyte in vitro translation (IVT) lysate from Promega Biotec (Madison, WI) according to instructions supplied by Promega. A 25 μl reaction mixture containing 1-2 μg of poly (A^+) RNA and 25 uCi of ^{35}S-methionine (New England Nuclear) was incubated for 60 min at 30°C. Incorporation into trichloroacetic acid-insoluble precitates was determined by spotting 1 μl of the IVT solution on a 3 mm filter disk. The disks were washed sequentially for 15 min at room temperature, 5 min in a boiling solution and 15 min in 4°C of 5% trichloroacetic acid (total volume in all phases = 10 ml/disk). The filters were washed with excess water and rinsed with acetone. After drying in a hood, the filters were dissolved in scintillation cocktail and counted using a Beckman LS8100 scintillation counter.

1-D SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out after the method of Laemmlli (1970). The system consisted of a stacking gel composed of

4% polyacrylamide in a Tris buffer (0.125 M Tris-Cl, pH 6.8, and 0.1% SDS). The resolving gel was 10% polyacrylamide in a buffer of 0.375 M Tris-Cl, pH8.8 and 0.1% SDS. The reservoir buffer was composed of 0.1% SDS, 0.1 M glycine and 0.025 M Tris, pH8.6. Gel dimensions were 190 mm length, 16 mm width, and 1.5 mm thickness. All gels were run at 12°C until the tracking dye migrated out of the gels.

After electrophoresis, gels were fixed with 7.5% acetic acid and 50% methanol, then treated for fluorography (Bonner and Laskey, 1974). The dried gel was placed onto a sheet of Kodak X-Omat AR X-ray film and kept at -80°C for 1-2 days. The developed film was scanned by a laser densitometer (LKB, Bromma, Ultrascan XL), and the data were analyzed with a software 2400 program (LKB, Gelscan XL, version 1.0).

Two-Dimensional Gel Electrophoresis

This technique was previously described by O'Farrell (1975) and O'Farrell, Goodman and Farrell (1977). The specific protocol used here was furnished by Bio-Rad. The first dimension separated proteins via isoelectric focusing

(IEF). The IEF gels were composed of 48.6 g urea, 28.8 ml diH_2O, 11.8 ml acrylamide/bisacrylamide (30% stock), 20.3 ml 10% triton, 4.5 ml Bio-Lyte 5/7 and 0.5 ml Bio-Lyte 3/10. Gels were in 130 x 1.5 mm tubes. 500,000 CPM of each sample was focused at 400 V for 18 hours followed by 800 V for 2 hours. IEF gels were extruded into equilibration buffer (62.5 mM Tris, 10% glycerol, 2.25% SDS, 5% 2-mercaptoethanol) and either used directly or frozen in a dry ice/ethanol bath and stored at -70°C. In either case gels were equilibrated by gentle shaking for 15 min in the equilibration buffer before loading onto the second dimension. The second dimension resolved proteins via SDS-PAGE gels (160 x 190 x 1.5 mm) and contained 12% polyacrylamide, 0.1% SDS and 0.375 M Tris-Cl, pH 8.8. The IEF gels were attached to SDS PAGE gels with 1% agarose dissolved in the equilibration buffer. Tank buffer was 0.1% SDS, 0.1 M glycine and 0.025 M Tris. The SDS gels were run at 35 mA/gel at 12°C until the bromophenol tracking dye reached the end of the gel, usually about 8 hours.

Fluorography

To increase the intensity of the radioactively labeled bands (1-D gel) or spots (2-D gel), fluorography (Bonner and Laskey, 1974) was done by soaking the gels for 2 hours in 50% methanol/7.5% acetic acid followed by 1 hour in enhance solution of 67% acetic acid/28% 2-ethoxy ethanol/5% 2-methyl naphthalene (melted) and 0.5% (w/v) ppo followed by 1 hour in water. The dried gels were exposed to Kodak X-ray films (X-Omat, XR-2) at -80°C for about 2 days (1-D gel) or 7 days (2-D gel).

The molecular weights of polypeptides were estimated with the aid of radioactively-labeled maker proteins (Amershan UK) by plotting the log of the molecular weight against the relative mobility of the polypeptide (Weber an Osborn, 1969).

RESULTS

Accumulation of Biomass in Hydroponic Culture

After the growth data was taken, we were informed that the "A" genotype was selected from AZ-GERM SALT-1, for salt tolerance at germination and early seedling growth. The "X" genotype, from the synthetic population Mesa-Sirsa, was considered salt sensitive at these stages. In this experiment, the treatments with NaCl and $CaCl_2$ (5:1) were carried out during vegetative growth of both genotypes. Salinity levels were suggested by Professor A. K. Dobrenz (personal communication) on the basis that the low salt treatment (-0.18 MPa) would mimic an Arizona crop production /irrigation situation where vegetative yield is reduced significantly due to reduced plant growth but not because of stunting or abnormal salt-induced phenotype. The high salt level (-0.80 MPa) was picked to simulate a more severe salt-stressed condition where stunting would occur.

Biomass accumulation of both genotypes was decreased with increased salinity. As expected, higher salinity resulted in lower biomass accumulation (Tables 2, 3). The salt levels produced the desired effects. In both genotypes,

Table 2. Fresh weight of the shoots and roots of the "A" (salt tolerant) genotype in three different treatments.[+]

Treatment	Replic.	No Salt	Low Salt	High Salt
Shoot	1	74.8	64.1	27.3
(gm/4 plants)	2	107.9	54.5	33.9
	3	69.1	38.9	23.3
Average (gm/plant)		21.0	13.1	7.0
Root	1	49.5	46.0	37.1
(gm/4 plants)	2	105.7	56.6	24.2
	3	51.3	41.5	36.7
Average (gm/plant)		17.2	12.0	8.2
Total Weight	1	124.3	110.1	64.4
(gm/4 plants)	2	213.6	111.1	58.1
	3	120.4	80.4	60.0
Average (gm/plant)		38.2[*]	25.1[*]	15.2[*]
% unstressed control		100.0	65.7	39.8

[*]No significant difference was found between No Salt and Low Salt treatment but means between No Salt and High Salt treatments were significantly different at the .05 level by t-test.

[+]The plants were grown in a growth chamber in nutrient solution for adaptation to the hydroponics for 10 days, then 1/10 amount of total mixture salt solution ($NaCl/CaCl_2$, 5:1) were added into the culture solution each day during a successive 10 day period (see text). The data was from plants harvested after they had grown in the designated water potential (-0.18 MPa or -0.80 MPa) for 10 days.

Table 3. Fresh weight of the shoots and roots of "X" (salt sensitive) genotype.[+]

Treatment	Replic.	No Salt	Low Salt	High Salt
Shoot	1 st	67.2	53.7	14.3
(gm/4 plants)	2 nd	56.9	18.8	17.6
Average (gm/plant)		15.5	9.1	4.0
Root	1 st	39.3	42.6	18.2
(gm/4 plant)	2 nd	66.1	33.4	34.0
Average (gm/plant)		13.2	9.5	6.5
Total Weight	1 st	106.5	96.3	32.5
(gm/4 plants)	2 nd	123.0	52.2	51.6
Average (gm/plant)		28.7[*]	18.6[*]	10.5[*]
% unstressed control		100.0	64.7	36.6

[*]No significant difference was found between No Salt and Low Salt treatment but means of No Salt and High Salt treatments were significantly different at the .05 level by t-test.

[+]The data were obtained under the same conditions as with the "A" genotype. One of three replications was discarded because insects damaged some of the plants.

the total fresh weight of the low salt treatment was about 66% of the unstressed control. Biomass of the high salt treatment was about 33% of control. The high salt treatment showed phenotypic abnormalities including stunting and slightly darkened color. The mean of total fresh weight was not significantly different between the control and low salt level. High salt significantly lowered vegetative growth.

Vegetative growth of the "A" (salt tolerant) genotype was much more vigorous than the "X" (salt sensitive) genotype under the control condition. Also, the "A" (salt tolerant) genotype accumulated a higher biomass under both the low salt and high salt stress (Figure 1). The effect of salt stress on the roots and shoots depended on the genotypes. Salt affected shoot and root growth differently. The "X" (salt sensitive) genotype shoot showed a much greater inhibition of growth under salt stress while "X" roots grew slightly better than "A" (salt tolerant) genotype roots. This is shown in Fig. 2 and Table 4. The ratio of the biomass yield of the "A" (salt tolerant) genotype shoot tissues to the "X" (salt sensitive) genotype shoot tissues is 1.35 under the control conditions. During the low salt stress, it reached at 1.44 and under high salt, it increased to 1.75 (Table 4). It is obvious that the difference in fresh shoot weight between genotypes was enhanced with increased salt concentration.

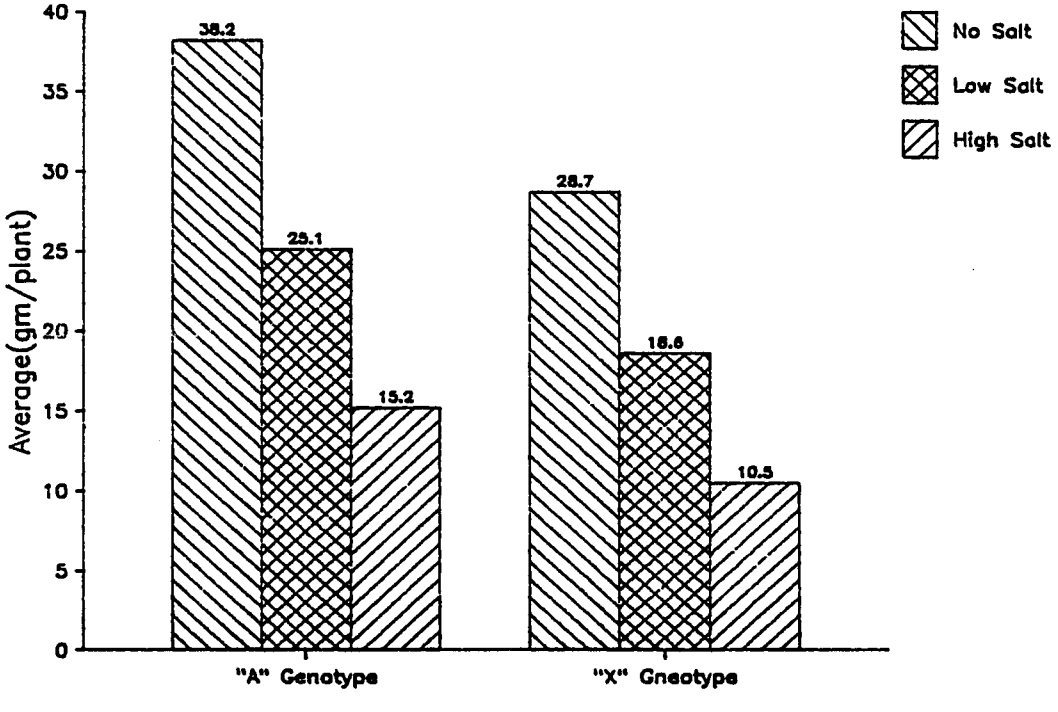


Fig 1. The schematic representation of total fresh weight of both genotypes under the three treatment conditions.

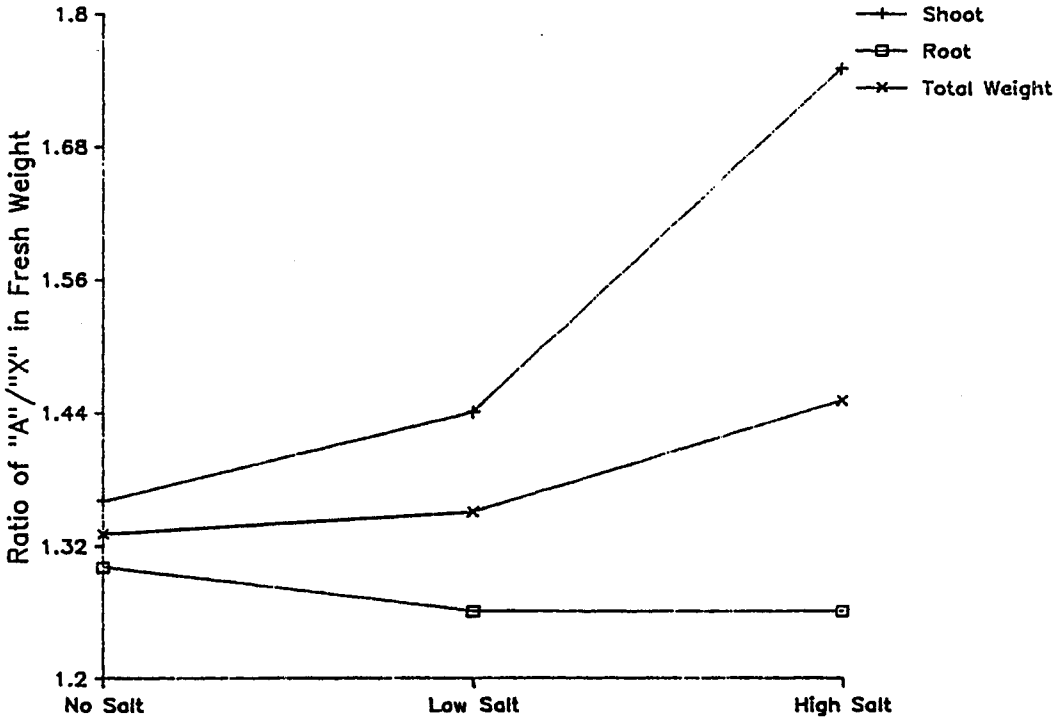


Fig 2. The schematic representation of ratio of the fresh weight of the "A" genotype to the "X" genotype.

Table 4. The ratio of "A" genotype (salt tolerant) to "X" genotype (salt sensitive) in the three different treatments.

Treatment		No Salt	Low Salt	High Salt
Shoot (gm/plant)	"A" Genotype	21.0	13.1	7.0
	"X" Genotype	15.5	9.1	4.0
	"A"/"X"	1.35	1.44	1.75
Root (gm/plant)	"A" Genotype	17.2	12.0	8.2
	"X" Genotype	13.2	9.5	6.5
	"A"/"X"	1.30	1.26	1.26
Total	"A"/"X"	1.33	1.35	1.45

Interestingly the ratio of the root biomass does not appear to change from the low salt to the high salt treatments (Fig 2 and Table 4).

In Vitro Translational Activity of mRNA

It is of interest to note that a higher yield of RNA was isolated from the root and shoot tissue under control condition vs low salt (Table 5). Over all tissues, an average of 307 μg of RNA was obtained from each gram of the fresh tissue. The poly(A)$^+$ RNA accounted for about five per cent of the total RNA (Table 5).

The translational activities of the mRNA from both genotypes under salt stressed and unstressed conditions were compared in a reticulocyte cell-free system. The translation efficiencies (CPM ^{35}S-methionine incorporation per μg poly (A$^+$) RNA) were very different during in vitro translation. The highest translation efficiency of mRNA per μg poly (A$^+$) RNA was from unstressed "A" (salt tolerant) genotype root tissues. The lowest was from unstressed "X" (salt sensitive) genotype shoot tissues (Table 6). Both "X" and "A" mRNA from the unstressed plants showed the same trend in translation

Table 5. The effects of extracting RNA from both genotypes.

"A" Genotype	Shoot		Root	
	No Salt	Low Salt	No Salt	Low Salt
Total RNA (μg)	7896	5232	6632	5072
Poly(A) RNA(μg)	522	241	174	113
mRNA/RNA (%)	6.6	4.6	2.6	2.2
RNA/Tissue*(μg/g)	395	262	332	254
mRNA Conc.* (μg/μl)	2.1	1.0	0.9	0.5
"X" Genotype	Shoot		Root	
	No Salt	Low Salt	No Salt	Low Salt
Total RNA (μg)	6992	7512	5720	4200
Poly(A) RNA(μg)	449	558	312	272
mRNA/RNA (%)	6.4	7.4	5.5	6.5
RNA/Tissue*(μg/g)	340	376	288	210
mRNA Conc.* (μg/μl)	3.3	3.8	2.7	1.9

*Used in IVT experiments.

+Twenty gm of a fresh tissue was used to extract RNA and two samples (unstressed tissue and low salt stressed tissue) were extracted in a parallel experiment for each genotype.

Table 6. In vitro translational activity (CPM/μg poly (A^+) RNA) of the mRNAs from the root and shoot tissues of two alfalfa genotypes.

Genotype		Root		Shoot	
		No Salt	Low Salt	No Salt	Low Salt
"X" (CPM/μg)	1st	11,885	17,905	8,031	18,125
	2nd	14,703	15,219	6,158	18,356
	3rd	41,756	33,923	15,296	34,035
	Mean	22,781	22,349	9,829	23,505
"A" (CPM/μg)	1st	27,194	22,390	20,577	22,328
	2nd	12,796	7,083	16,407	18,212
	3rd	92,053	36,516	20,391	46,027
	Mean	44,015	21,997	19,125	28,856

efficiency, i.e. IVT efficiency in the shoot message was much lower than in the root message (Figure 3 and 4). During salinity (low salt level), the efficiency of the "X" (salt sensitive) shoot message increased greatly, from 9829 CPM to 23505 CPM, but the efficiency of root message translation remained the same as the control. The "A" (salt tolerant) shoot IVT efficiency increased with salinity while the efficiency of the "A" root message translation decreased from 44015 CPM to 21997 CPM.

One Dimensional Electrophoresis

An autoradiogram of the in vitro translation products from root and shoot mRNA's of both genotypes under control and low salt concentrations is shown in Fig. 5. A laser densitometer was used to scan the X-ray films. The laser scan did not reveal dramatic differences between stressed and unstressed tissues within genotypes (Fig. 6, 7 and Appendix Fig. 1, and 2).

One-dimensional gels did not show new bands associated with saline growth, however the radioactive intensity of several bands between comparable lanes was greatly different.

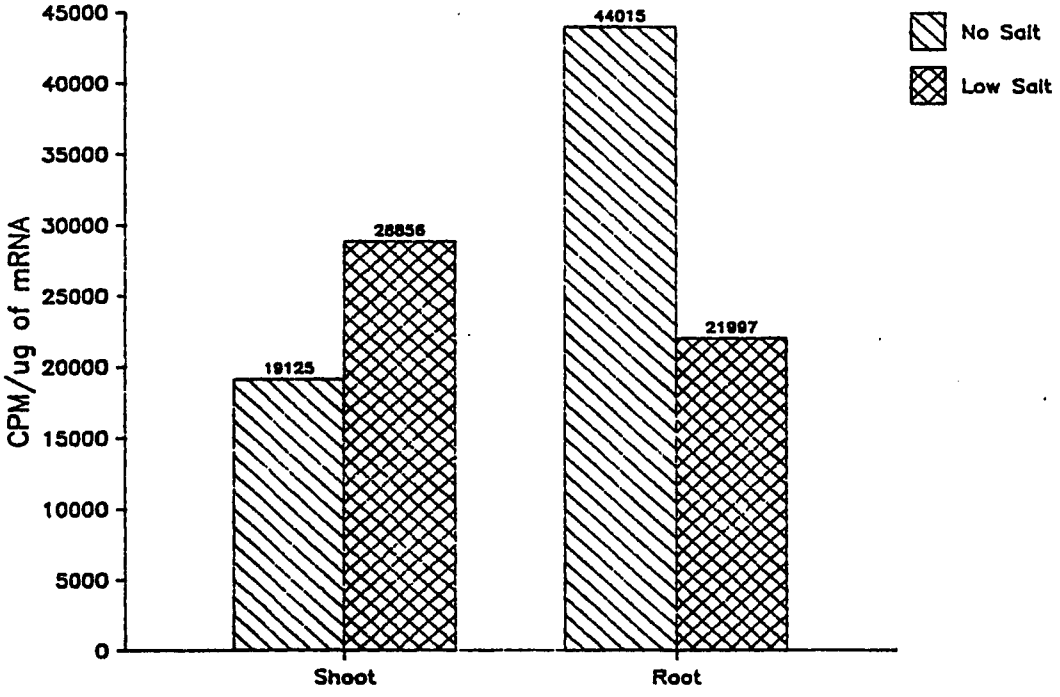


Fig 3. In vitro translational activity of the "A" genotype.

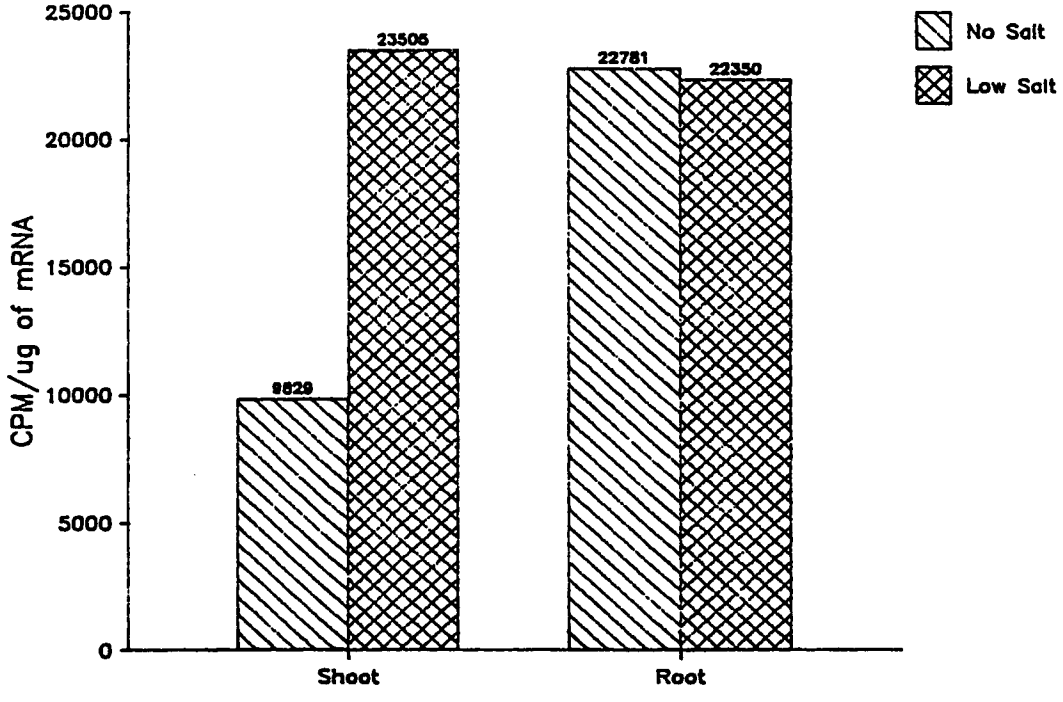


Fig 4. In vitro translational activity of the "X" genotype.

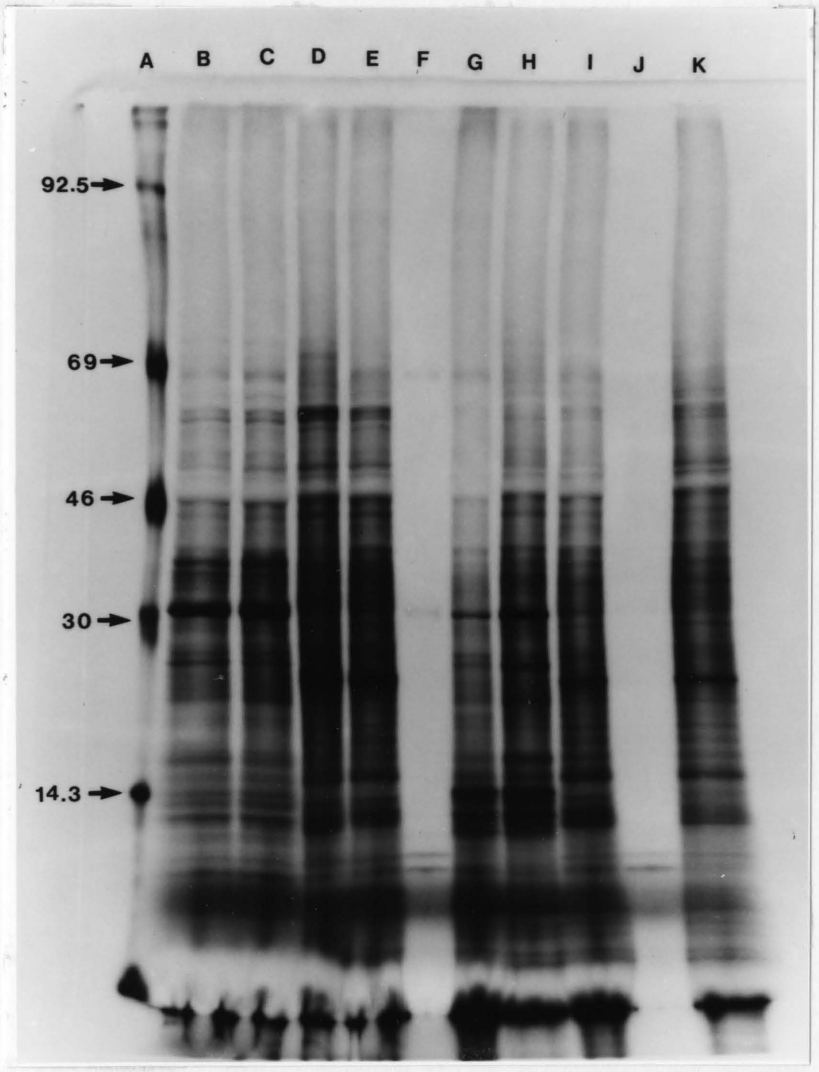


Fig 5. An autoradiogram of the in vitro translation products with mRNA isolated from the roots and shoots of both genotypes. The proteins were labeled with ^{35}S-methionine in rabbit reticulocyte lysate and separated by one dimensional SDS-PAGE gradient gel (10% to 20% polyacrylamide). Lane A is molecular weight markers; Lane B and C are 2.0 μg mRNA from the "A" shoot, no salt stress(B), and low salt(C); Lane D and E - 2.0 μg mRNA from the "A" root, no salt(D) and low salt(E); Lane G and H - 2.0 μg mRNA from the "X" shoot , no salt(G) and low salt(H); Lane I and K - 2.0 μg of mRNA from the "X" root, no salt(I) and low salt(K); Lane F and J are control (without mRNA).

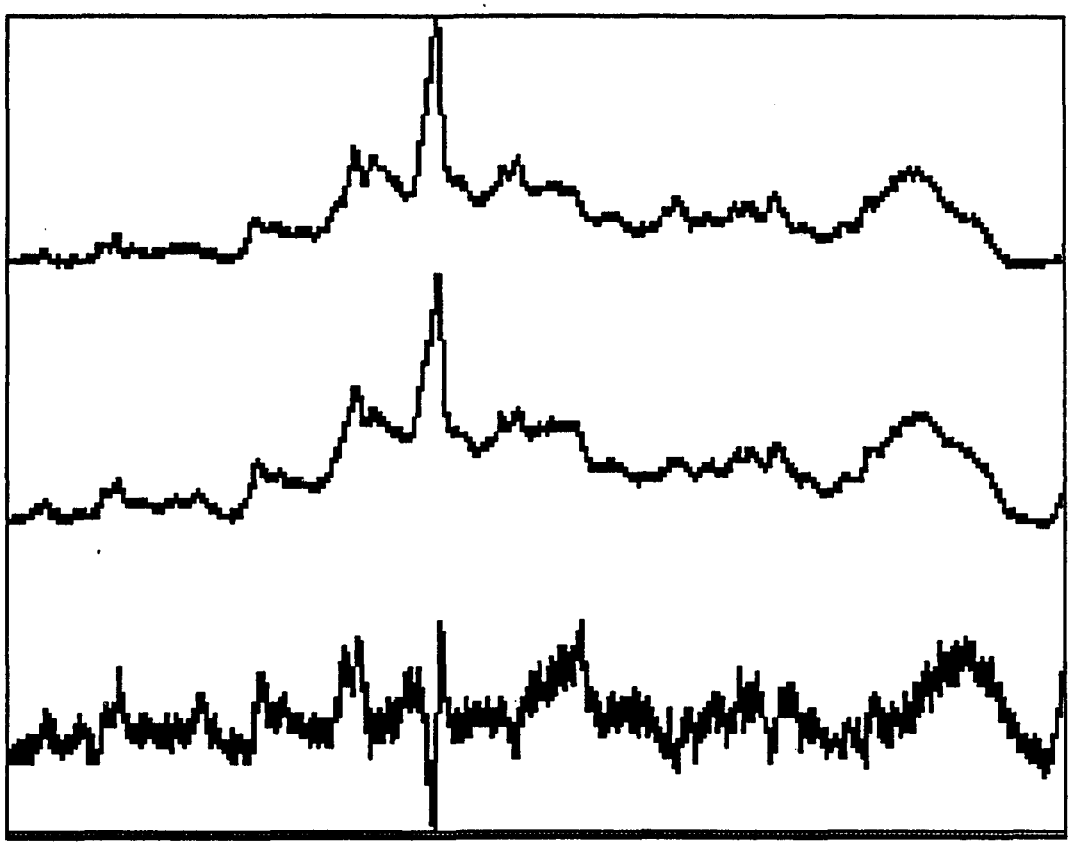


Fig 6. Laser densitometer difference scan of the autoradiogram from the "A" shoot mRNA in Fig. 5. The upper curve shows the radioactive intensity of protein bands from the control condition. The middle curve from the low salt stress. The bottom curve indicates the result of the middle curve (salt stress) minus the upper curve (no salt stress).

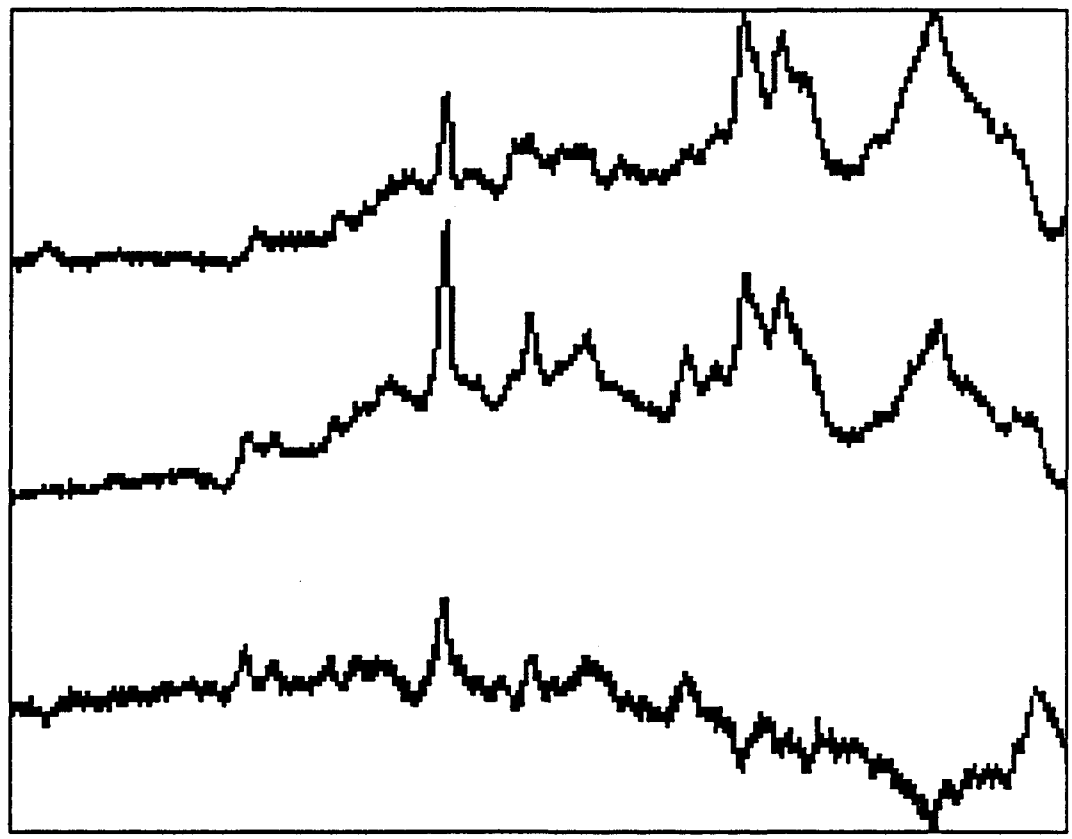


Fig. 7. Laser densitometer difference scan of the autoradiogram from the "X" shoot mRNA in Fig. 5. The upper curve shows the lane G (control) and the middle curve from the lane H (salt stress). The bottom curve is the result of the middle curve minus the upper curve.

The bottom curve in Fig. 6 shows a difference scan resulting from the subtraction of the "A" (salt tolerant) stressed protein pattern from the unstressed control. A similar analysis for "X" shoot mRNA protein patterns is shown in Fig. 7. There appear to be more changes in the "A" (salt tolerant) genotype shoot mRNA translation products in response to salinity condition than in the "X" (salt sensitive) genotype. However no large consistent changes in the protein patterns were observed in the 1D gels.

The intensity of the protein patterns encoded by the "A" (salt tolerant) root mRNA under the salt stress increased in the region of low molecular weight (Appendix Fig. 1). The "X" (salt sensitive) root mRNAs showed the opposite result (Appendix Fig. 2). In other words, the genes responsible for low molecular weight proteins appear to be expressed much more strongly in the "A" genotype shoot tissues under salinity.

Two Dimensional Electrophoresis

Differences in gene expression during unstressed and low salt stressed growth were resolved by two dimensional electrophoresis of IVT products according to charge and

apparent size. Autoradiograms of the two dimensional gels are shown in Fig. 8, 9, and 10.

Fig. 8 shows the protein products of the in vitro translation of "A" (salt tolerant) genotype message. The messages were isolated from the shoot and root tissues, respectively. The polypeptides on the left (A: shoot tissues and C: root tissues) were from the mRNA of the unstressed plants. The open arrows indicate those polypeptides present only under the unstressed condition. In other words, they were repressed under salt stress. We found four proteins in the shoot tissue and three in the root tissue which were only present under control conditions. The right two figures (B and D) show the protein products of the mRNAs under low salt stress. The closed arrows show those polypeptides visible only under stress. We identified four new proteins from the low salt stress root tissues. No salt-induced proteins were observed in the shoot tissues.

In the "X" (salt sensitive) genotype (Fig. 9), there is one protein in the shoot tissues and three in the root tissues induced by the low salt treatment (picture B and D in Fig. 9). These are indicated by the closed arrows. One protein was repressed in both salt stressed root and shoot (the open arrows in Fig. 9, A and C).

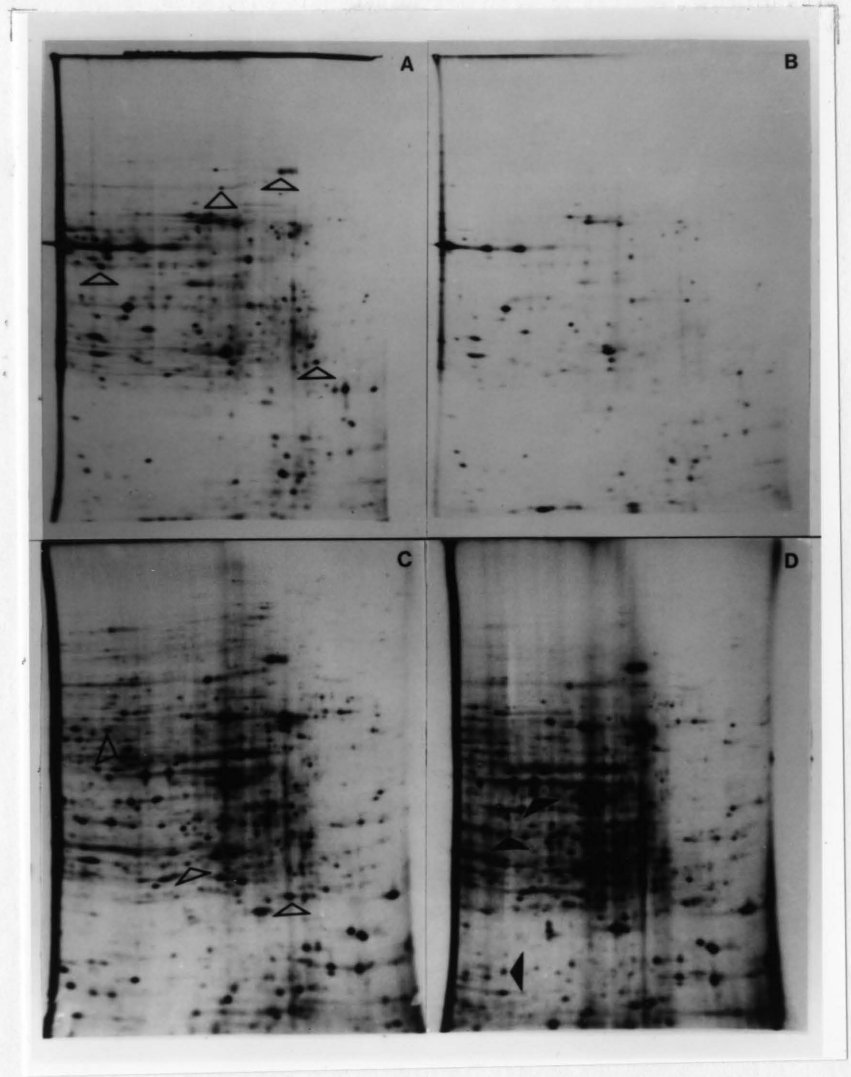


Fig 8. In vitro translation protein products from the "A" genotype mRNAs resolved by two-dimensional PAGE. The A (control) and B (low salt stress) figures show the protein pattern from the shoot mRNAs. The C (control) and D (low salt stress) figures from the root mRNAs. The open arrows indicate polypeptides present only under the no salt stress and the closed arrows show those present only under the salt stress.

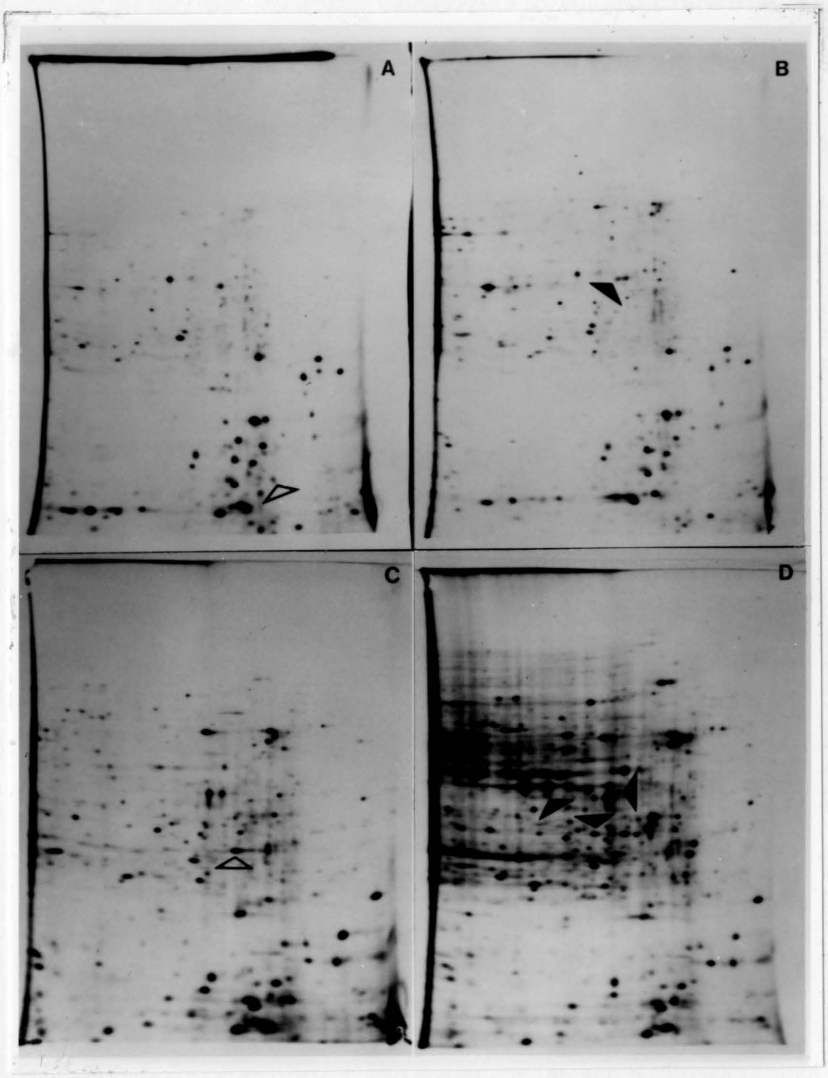


Fig 9. In vitro translation protein products from the "X" genotype mRNAs resolved by two-dimensional PAGE. The A (control) and B (low salt stress) figures show the protein pattern from the shoot mRNAs. The C (control) and D (low salt stress) figure s from the root mRNAs. The open arrows indicate polypeptides present only under the no salt stress and the closed arrows show those present only under the salt stress.

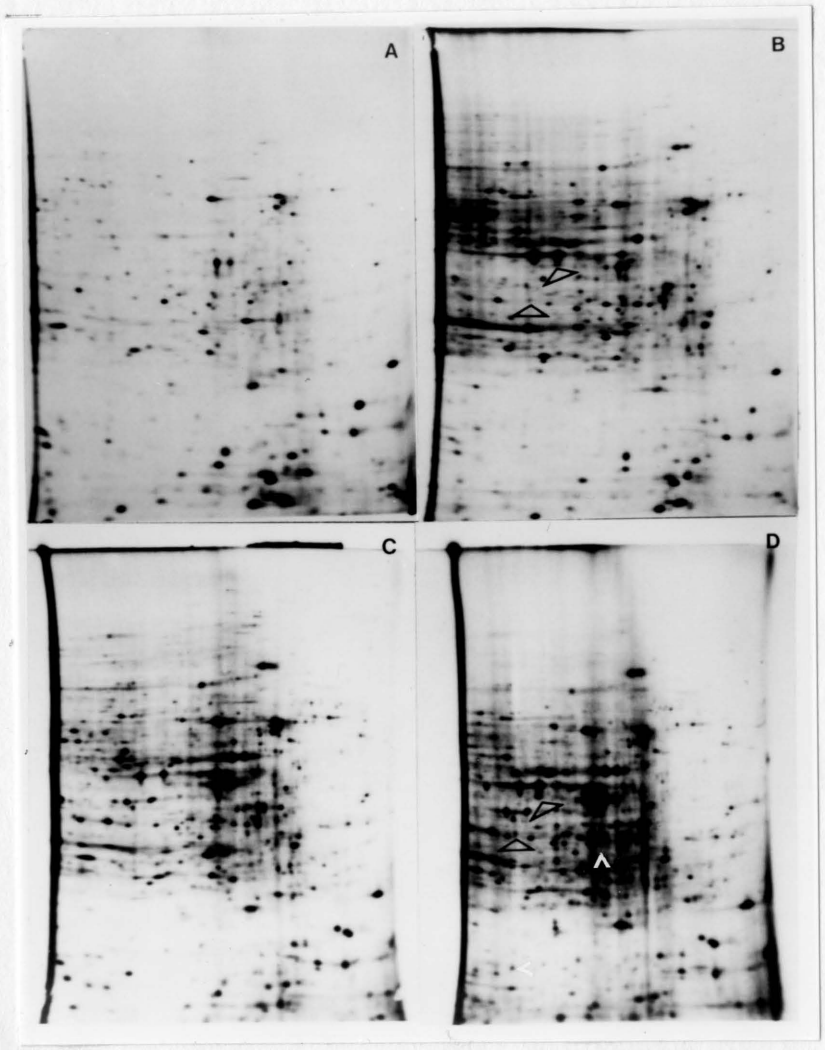


Fig 10. Comparison of the in vitro translation protein products from the root mRNAs of both genotypes resolved by two-dimensional PAGE. Figure A - "X" genotype, no salt stress; B - "X" genotype, low salt stress; C - "A" genotype, no salt stress and D - "A" genotype, low salt stress. The Open arrows show the same spots of the "X" genotype with which the "A" genotype only present under the salt stress. The white arrows indicate salt stress proteins unique to the "A" (salt tolerant) genotype.

We also compared the patterns of the IVT products from the salt stressed root and shoot message of both genotypes. Variation in the pattern of IVT products from shoot message was too high to allow meaningful comparison across genotypes. The pattern of IVT products from root message was similar across genotypes. Fig 10. shows that two out of four salt induced proteins found in the "A" (salt tolerant) genotype may be the same as two proteins induced by salt in the "X" (salt sensitive) genotype during the salinity. The assignment of identity to individual proteins is based on a visual analysis of the positions of the protein spots on the gel relative to other proteins in the adjacent region of the gel and cannot be unequivocal. The upper of the two proteins (Fig. 10 B) appeared only under salinity stress. The lower one appeared under the control and salt stressed condition. The "A" (salt tolerant) genotype apparently produced both stress proteins observed in "X". This genotype also produced two apparently unique proteins shown by the white arrows (Fig. 10 C and D).

Without additional analysis, including internal MW and pI markers and computer-assisted video or laser scanning, it is not possible to determine unequivocally if the two salt-induced proteins produced by the salt-tolerant genotype are uniquely and reproducibly present as a function of salt

stress.

DISCUSSION

In order to study the changes in gene expression that occur in two different genotypes of alfalfa, salt sensitive and salt tolerant, we grew the plants in a growth chamber in hydroponic culture under a level of salt stress that reduced growth rate but did not cause gross modification to the phenotype. Our working hypothesis was that changes in gene expression under these conditions reflected adaptive growth as opposed to stress shock. Salt-tolerance mechanism(s) in alfalfa will probably involve many induced genes. We believe our approach has the highest probability of identifying gene expression phenomenon involved in "stress-adaptive growth maintenance" under saline conditions.

Although the stress tolerant genotype used in this study was selected for germination and seedling growth in saline water, it also showed salt tolerance whole plant vegetative growth under our conditions. Both salt-tolerant and salt-sensitive genotypes showed reduced biomass accumulation with increased salt level. However, they differed in the accumulation of photosynthesis product under salinity. Differences were greater at high salinity than at low salinity or control conditions.

The "A" (salt tolerant) genotype did better (ratio of total biomass = 133%) under control conditions. The difference was about the same under low salt (A/X = 135%) but increased to 145% under high salt (Table 4). Under our experimental conditions this difference was due entirely to shoot biomass accumulation (Fig. 2).

One dimensional protein analysis of in vitro translation products did not reveal clear or dramatic changes in gene expression under salinity. However, since the salt-tolerant plants did show an enhanced ability to accumulate biomass under salt stress, we considered it probable that changes in gene expression were occurring. Therefore, we moved to 2D gel electrophoresis to enhance the resolution of the analysis.

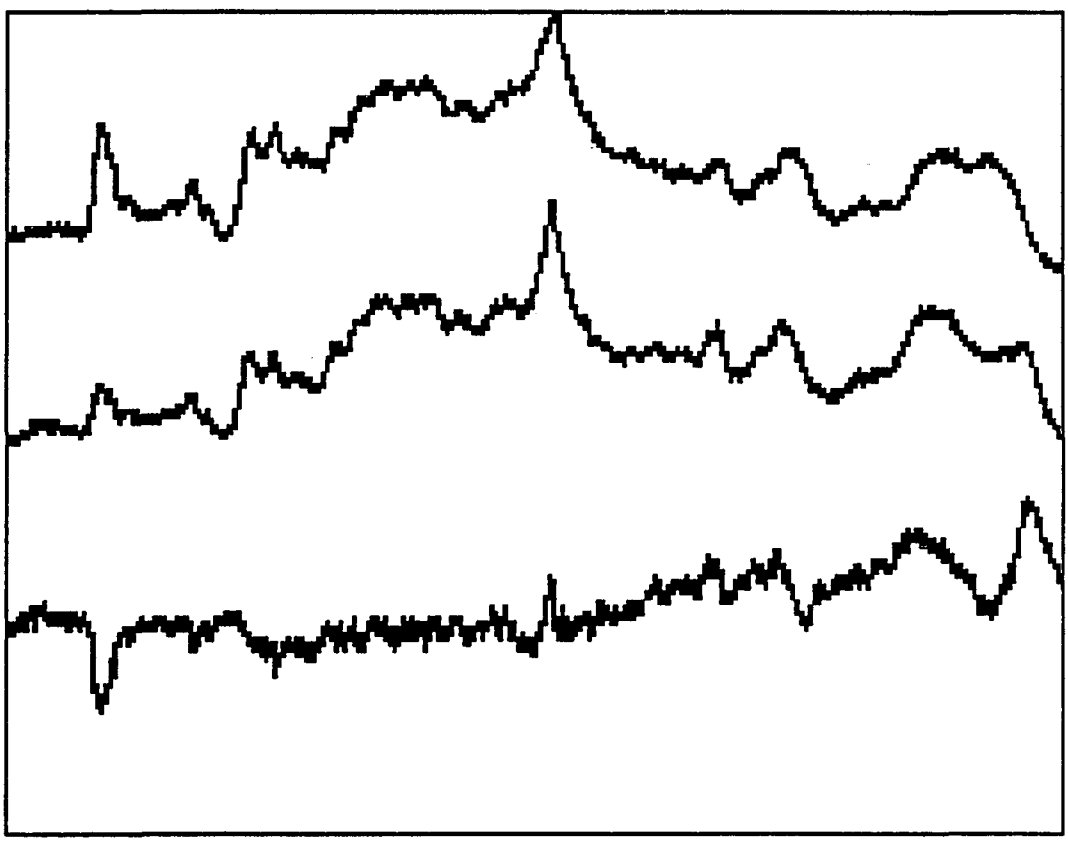
We did observe changes in gene expression, via resolution of the IVT protein products by the IEF/SDS-PAGE two dimensional gel system. Under salinity, a few new proteins, presumably the result of new messenger RNAs, were observed not only in the salt-tolerant alfalfa, but also in the salt-sensitive. In the meantime, some proteins disappear in the both genotypes. It is obvious that gene expression was affected the salt stress and that regulation in different genotypes differed. Gene expression, induced and repressed, was tissue specific for both genotypes. In the "A" (salt

tolerant) genotype, four new proteins (mRNAs) appeared and three disappeared in the root tissue. No proteins (mRNAs) appeared and four proteins (mRNAs) were repressed in the shoot tissue under the salt stress (Fig 8). In the "X" (salt sensitive) genotype, the same general effect occurred (Fig. 9). The "A" (salt tolerant) genotype was selected from the salt sensitive parent population for germination and seedling growth under salinity. In this experiment, the selected genotype ("A") did show greater salt tolerance than "X". The "A" genotype produced two new proteins in the root tissue that are only present under salt stress. The mRNAs encoding these proteins are strong candidates for salt tolerance gene products. Two points support this conclusion. 1) Based on detailed visual analysis, we were able to match up several hundred other protein spots on the two gels shown in Fig 8 C and D. This provides several hundred internal controls and indicates observed differences are the real result of gene expression rather than artifacts of mRNA isolation or in vitro translation. 2) Because we used a low stress system, plant growth was only slightly reduced and the phenotype was normal. Under this conditions we expected that stress adaptive metabolism would be superimposed onto a relatively normal metabolic background. The 2D gels confirm this hypothesis since the protein patterns for the control and low stress

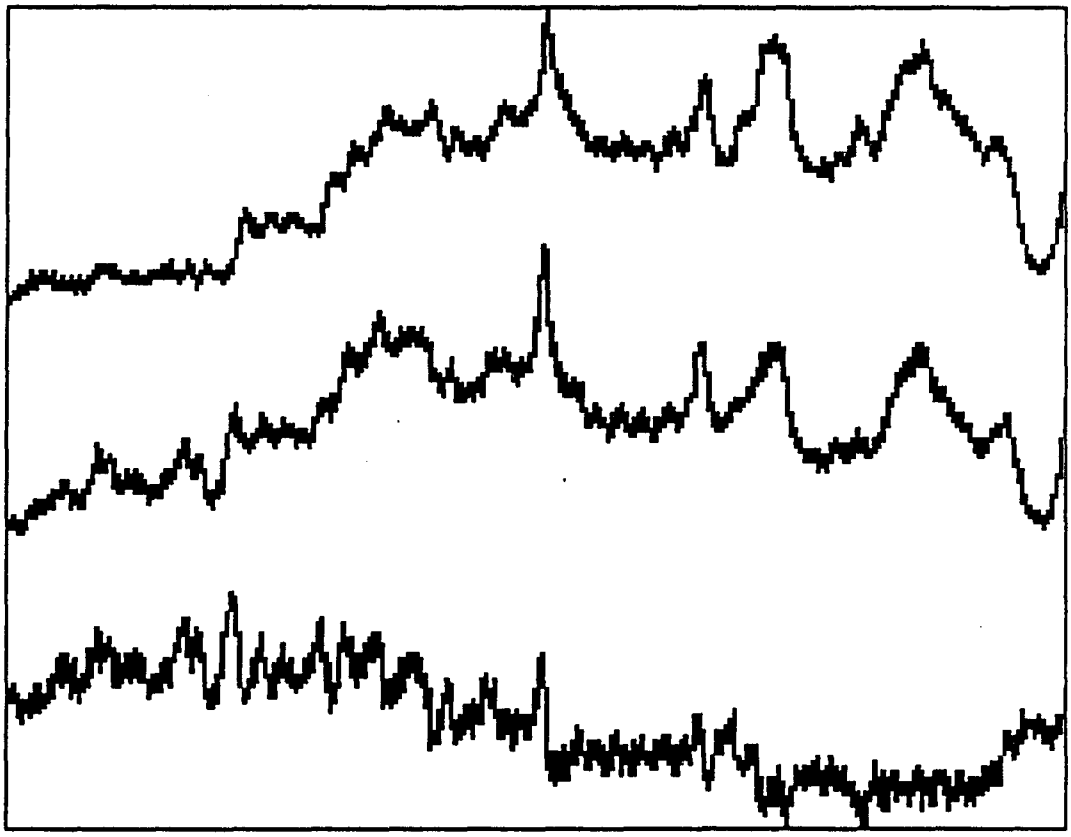
plants are almost identical. Additional analysis, including internal MW and pI markers and computer assisted video or laser gel scanning will be required to determine if the two salt-induced proteins produced by the salt-tolerant genotype are uniquely and reproducibly present as a function of salt stress. It is technically possible to microsequence these proteins and obtain the cDNA clones of their mRNA's. These clones could be used to verify enhanced production of message during stress adaptive growth.

The amount of IVT products of some mRNAs differ between the unstressed and low salt stressed tissues. This may imply that plants modify or switch their metabolic pathways to adapt to the new saline environment. It is likely that the observed proteins (mRNAs) were involved in salt tolerance metabolism. Further research will be required to study the temporal regulation of stress-induced and stress-repressed transcripts in alfalfa.

APPENDIX FIGURES



Appendix Fig. 1. Laser densitometer difference scans of the autoradiogram from "A" root mRNA in Fig. 5. The upper curve shows the radioactive intensity of every protein bands under unstressed condition. The middle curve under the low salt stressed condition. The bottom curve is result of the middle curve minus the upper curve.



Appendix Fig. 2. Laser densitometer difference scans of the autoradiogram from "X" root mRNA in Fig. 5. The upper curve is from the lane I, and the middle curve from the lane K. The bottom is a curve of the middle curve minus the upper curve.

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