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Characterization of salt tolerant alfalfa (*Medicago sativa* L.) plants regenerated from salt tolerant cell lines

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ABSTRACT

Salt tolerant cell lines have been selected from Medicago sativa, by a single step selection process on tissue culture medium containing 1% NaCl. Plants regenerated from these lines show improved salt tolerance compared to parent plants. The regenerated plants are vigorous, have flowered and are self fertile. The cellular salt tolerance characteristic can be passaged through the regenerated plants, since callus cultures initiated from immature ovaries of the salt tolerant regenerated plants are salt tolerant without additional selection on 1% NaCl. Several of these "second generation" callus cultures have been regenerated to produce vigorous plants which maintain the salt tolerance characteristic. The tolerance phenotype appears dominant in seeds obtained from self fertilization of the tolerant plants. The regenerated salt tolerant plants are therefore a valuable source as genotypes in plant breeding for salt tolerance and isolation, identification and manipulation of genes which confer salt tolerance in alfalfa.

KEY WORDS: Alfalfa - Salt tolerance - Regeneration - Callus culture.

ABBREVIATIONS: SH: Schenk and Hildebrandt medium; 2,4-D: 2,4-dichlorophenoxyacetic acid.

INTRODUCTION

Salt tolerance in plants is a topic of vital agronomic interest in most parts of the world not only because of limited water supplies but also because of increasing salinization of cultivated areas requiring irrigation. Traditional plant breeding methods requiring long term selection and testing have produced an improved germplasm capable of germinating at high levels of salinity (Dobrenz et al. 1983), but are slow to yield substantial improvements in salt tolerance and growth of crop plants. This requires the development of alternate genetic strategies for improving the salinity tolerance of crop plant species in order to provide yield increases in marginal lands and to protect plants exposed to transient osmotic stress.

Plant cell culture and regeneration of plants from potential cell mutants has led to the expectation that these techniques could be used to generate and study genetic diversity of useful mutant traits from plant cell cultures (Meredith 1984). Stress tolerance selection in plant cell culture (Tal 1983; Handa et al. 1984 a,b), have suggested that salt and water stress tolerance may be characteristics which are linked in selection processes at the cellular level and therefore might be manipulated in culture. Salt tolerant cell lines of Medicago sativa have been selected in several laboratories (Croughan et al. 1978; Smith and McComb 1983; Bingham and McCoy 1986; Winicov et al. 1989) yielding alfalfa cells tolerant to 1% (0.171 M) NaCl. Selected and nonselected alfalfa cells show no differences in intracellular Na^+ and Cl^- concentrations when grown in 1% NaCl, indicating that tolerance is not achieved by salt exclusion (Stavarek and Rains 1984b), but rather depends on cellular adaptation at the molecular level. This latter conclusion has been indirectly substantiated by our experiments which show similarly increased levels of histone hyperacetylation in response to salt by both salt tolerant and salt sensitive alfalfa cells, suggesting a similar response in both cell types to changed nuclear ionic environment (Waterborg et al. 1989). Physiological adaptation at the cellular level has been proposed as the mechanism that allows plant adaptation to arid or saline environments in the facultative halophyte Mesembryanthemum crystallinum (Cushman et al. 1989). Therefore it is a reasonable hypothesis that increased gene expression for those physiological systems that are affected by salt stress in glycophytic plants, such as alfalfa, may confer increased tolerance to saline environments through cellular salt tolerance.

Past attempts at the direct and successful isolation of vigorous salt tolerant plants via cell culture have been inconclusive. In several cases, cellular salt tolerance has not always correlated with whole plant salt tolerance (Bingham and McCoy 1986; McCoy 1987a). A major roadblock in using this technique has been the finding that, although salt tolerant cell lines have been selected in a variety of plant species (Stavarek and Rains 1984a), only a limited number of plants could be regenerated from these lines. Selected cell lines of tobacco have yielded one NaCl tolerant plant with preliminary reports of regenerated salt tolerant plants obtained from oats and rice (Nabors et al. 1980, 1982; Nabors and Dykes 1985); however subsequent information on the propagation and continued maintenance of the salt tolerant phenotype in these plants has been lacking. Cellular regeneration in the past has yielded growth impaired plants (Stavarek and Rains 1984b), or plants that were not salt tolerant (Smith and McComb 1983; Chandler and Vasil 1984). Two salt tolerant alfalfa plants were regenerated from salt tolerant callus of M. sativa (McCoy 1987b), but the plants grew poorly in comparison with the parental type, and the only plant that flowered was both male and female sterile, precluding further genetic analysis. It appears that a balance of several factors is needed for conferring and maintaining salt tolerance at the whole plant level. During the cellular selection process, chromosomal segments may be lost or interchanged as observed **in vitro** (McCoy et al. 1982) and it has been suggested that the action of transposable elements may be particularly active **in vitro** (Groose and Bingham 1986).

In order to assess if temporal genomic changes in culture can affect regeneration or cellular salt tolerance expression in differentiation, a rapid and highly specific screening and selection procedure was developed for production of plants with the salt tolerant phenotype from cultured cell variants. This paper describes the rapid single step selection of salt tolerant alfalfa cell lines and the regeneration of several of these lines to produce plants with a stable salt tolerant phenotype that are vigorous, have flowered and are self fertile. The apparently dominant salt tolerant phenotype is transmissible through seed.

MATERIALS AND METHODS

Cell culture and selection of tolerant lines. Cell cultures were initiated from immature ovaries (McCoy and Walker, 1984) in Schenk and Hildebrandt (1972) growth medium (SH) supplemented with 2 mg/L kinetin plus 2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid). Callus

was subcultured at four-week intervals on either the control SH medium or SH medium containing 1% (171 mM) NaCl. Plants were regenerated on SH medium without hormones. Callus cultures and regenerating plants were grown at 24+1° C under continuous light (approximately 30 umol quanta m^{-2} s^{-1}) in 60% chamber humidity.

Selection for salt tolerant cell lines was carried out on newly initiated callus cultures, no more than two months in culture. About 200 mg callus pieces were placed on 100 x 20 mm culture plates containing 50 ml of SH medium with 1% NaCl. After four to eight weeks, viable foci could be detected on several of the callus explants. On the average it has been possible to obtain one focus from approximately 5-10x10^6 cells. Such foci were further propagated on SH medium containing 1% NaCl and cell lines showing equivalent growth on SH medium with and without 1% NaCl (Winicov et al., 1989) were retained for regeneration.

Plant regeneration and testing for salt tolerance. Plants were regenerated from salt tolerant callus starting after three subcultures on 1% NaCl containing SH medium. Callus from control cultures of comparable age was also treated similarly to regenerate control plants. Callus for regeneration was transferred in fragments of about 300 mg directly to SH medium without hormones and incubated as described above. Usually roots and shoots were detected on a portion of the calli after four weeks. At this time the plantlets were transferred to 125 ml Erlenmyer flasks containing 50 ml SH medium without hormones and were allowed to develop for an additional four weeks. Subsequently individual plantlets were transferred to "Jiffy peat" and were grown in a closed container with high humidity for 3 to 4 weeks, after which they were transferred to the greenhouse. The small plants were hardened to the atmosphere for one week on the mist bench, transferred to regular potting medium and were grown under greenhouse conditions. When the plants flowered, fertility was determined by self fertilization of the regenerated plants and the set seed was collected after five weeks.

Retention of cellular salt tolerance was tested by re-initiation of callus cultures from immature ovaries of the regenerated plants or cotyledons on normal SH medium and testing the ability of the callus to grow on 1% NaCl without prior selection on salt containing medium. The cultures were visually or quantitatively rated as salt tolerant or salt susceptible.

For measurement of salt tolerance of the regenerated plants, rooted cuttings were established in Containers in perlite and watered daily with a regimen of water to flush out any accumulation of salts, followed by thorough watering with 1/4 strength Hoaglands solution (Hoagland and Arnon, 1938). After four weeks, the plants were cut back and divided into three groups with at least five replica cuttings of each individual regenerated plant in each group. Group I (control, or 0% NaCl), continued to be treated with the regimen of water and 1/4 strength Hoaglands as described above. Group II regimen included 0.5% NaCl in the Hoaglands solution and Group III included 1% NaCl in the Hoaglands solution. Tolerance was expressed as number of survivors per number of replica plants in each group. Plant growth was quantitated by harvesting the shoots of surviving plants at the end of each experiment and calculating the average total shoot fresh weight per plant in each group. This value represents the net increase in mass during the test period under the given salt conditions. Controls in these experiments included rooted cuttings of the parent plants and regenerated control plants derived from the salt sensitive callus.

RESULTS AND DISCUSSION

Plants regenerated from alfalfa cultures. Plants were regenerated from alfalfa callus cultures initiated from immature ovaries. Salt tolerant cell lines were selected and the described regeneration protocols yielded the fifteen plants listed in Table 1. The fifteen plants included two plants
regenerated from salt sensitive control callus and thirteen plants regenerated from salt tolerant cell lines. Additional plants (IW-41A,B through IW-47A,B) were regenerated from callus cultures initiated from the original group of regenerated plants. As shown in Table 1, the thirteen plants regenerated from salt tolerant calli came from three individual ovary cultures (A, B and C) and represent regeneration from callus cultures after 3 to 8 months growth on 1% NaCl. In general, plants regenerated from 8 months culture on 1% NaCl (IW-31 and IW-32) were less vigorous than plants regenerated only after 3 months growth on 1% NaCl, indicating that changes in gene expression accumulated in culture were important in growth.

Table 1. Plants Regenerated from Salt Selected Alfalfa Cultures

Regenerated Plant No.	Callus source(a)	No.Months on NaCl(b)	Flowered	Self-Fertile	Callus-Salt Tolerant(c)
IW-2	A	3	+	N/D	+
IW-6	A	3	+	+	+
IW-8	A	3	+	+	N/D
IW-9	A	3	+	+	+
IW-4A	A	4	+	+	+
IW-5	A	5	+	+	+
IW-31	A	8	+	N/D	N/D
IW-32	A	8	N/D	N/D	N/D
IW-3	B	3	+	N/D	+
IW-4B	B	5	+	+	+
IW-7	B	5	+	+	N/D
IW-10	B	5	+	+	+
IW-11	C	0	+	N/D	N/D
IW-12	C	0	+	+	N/D
IW-13	C	3	+	+	+
IW-41 A,B	IW-4B	0	+	+	N/D
IW-45 A-D	IW-4B	0	+	+	N/D
IW-43 A-D	IW-13	0	+	+	N/D
IW-51 A,B	IW-13	0	+	+	N/D
IW-47 A,B	IW-6	0	+	+	N/D

(a) Callus cultures were initiated from ovaries from control M. sativa or tolerant plants. A, B or C designates ovary of plant origin.
(b) Total number of months (selection + growth) on SH medium + 1% NaCl before start of regeneration.
(c) Callus derived from immature ovaries from the plant was salt tolerant without prior selection.
N/D = not determined

Individual plants regenerated from different callus at the same culture stage showed differences in growth characteristics, for example IW-3 was much less vigorous than IW-2 or IW-6, suggesting that individual cells that give rise to a plant, determine the composite growth phenotype. This is further supported by the observation that IW-3 was also less vigorous than IW-4B and IW-7, which were regenerated from the same salt tolerant callus culture, but at a later passage on NaCl (Table 1). The individual characteristics of the regenerated plants shown in Figure 1, summarize survival and growth rates of cuttings from individual plants in the presence and absence of salt. These data indicate that although survival and growth rates differed among the salt tolerant plants, all regenerants showed greater survival and growth rate than the parental R4 variety

Most of the regenerated plants flowered and yielded salt tolerant callus from immature ovary culture without additional selection on NaCl (Table 1). These results strongly suggest that the cellular phenotype of salt tolerance in culture is stable not only to propagation in absence of NaCl **in vitro** (Winicov et al. 1989), but is retained throughout more than a year of propagation at the whole plant level. Almost all of the regenerated plants are self fertile (Table 1), thus both male and female fertility is retained in these plants. The viable seed yield from self fertilization was comparable between the parent salt sensitive plants (22-20 seeds/ten pods) and six of the regenerated salt tolerant plants (23-16 seeds/ten pods).

Salt tolerance of the regenerated plants. Salt tolerance of the regenerated plants was examined by growth of replicate cuttings in perlite on dilute Hoaglands solution and 0%, 0.5% or 1.0% NaCl for a period of four weeks. Figure 1 summarizes the data of growth characteristics of several plants on one such test regimen under greenhouse conditions. The results were qualitatively similar from several experiments showing increased salt tolerance by the variant regenerated plants as compared to control plants. At the time of the experiment shown in Figure 1, the regenerated plants had grown under greenhouse conditions for more than a year and had been propagated by cuttings. Diploid (HG2) or the parental tetraploid (R4) (Figure 1) control M. sativa, as well as plants IW-11 and IW-12 (data not shown) regenerated from salt sensitive callus were killed by growth in 1% NaCl. In contrast, most of the plants derived from salt tolerant callus showed increased resistance to NaCl as measured by survival in 0.5 or 1% NaCl containing medium. The only plants that were as sensitive to NaCl as the control plants were IW-31 and

IW-32 (data not shown), which were regenerated after 8 months on 1% NaCl suggesting that the shorter length of time in culture with salt may contribute to regenerability of vigorous salt tolerant plants.

While growth in 1% NaCl decreased the average shoot fresh weight of most variant plants, growth in 0.5% NaCl affected the average shoot fresh weight very little, indicating that the salt tolerant variants were able to grow equally well in perlite with or without 0.5% NaCl (Figure 1). Occasional plantlets appeared to grow better in the presence of 0.5% NaCl, but larger numbers of plants are required in order to make statistically reliable conclusions on this point. The individual characteristics of each variant plant show that they differ in the level of growth inhibition by 0.5% NaCl from 0 to 29% and at 1% NaCl the levels of growth inhibition range from 58 to 100%. These results suggest that selection of individual variants at the whole plant level is an essential step in deriving optimally vigorous salt tolerant plants via regeneration from tissue culture as described in this report.

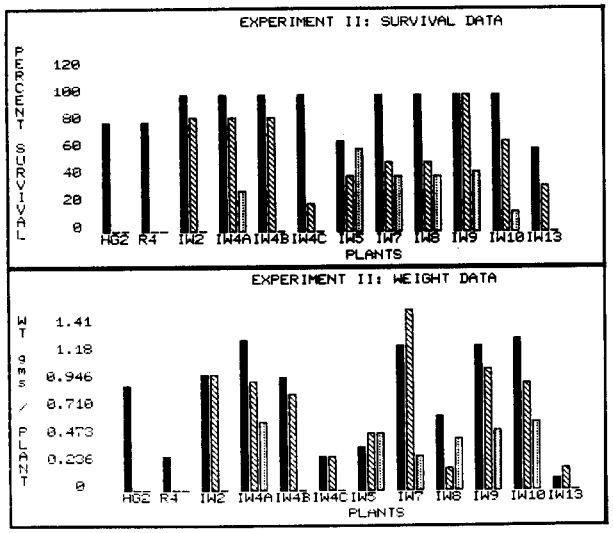

Figure 1. Salt tolerance of control and regenerated plants. Replicate cuttings of the regenerated and control plants were tested for salt tolerance as described in Materials and Methods. [O] control, or 0% NaCl; [O], 0.5% NaCl and [O] 1.0% NaCl. Tolerance was expressed as percent survival. Plant growth was quantitated by harvesting the shoots of surviving plants. Controls included rooted cuttings of the diploid HG2 and the tetraploid R4 plants.

Salt uptake in the regenerated plants. We determined the levels of Na^+ and K^+ by atomic absorption measurements to test if the tolerance phenotype was caused by a Na^+ exclusion mechanism at the plant level. The data summarized in Table 2 show increased accumulation of Na^+ in shoots of alfalfa with increased concentrations of NaCl in the growth medium correlated with a relative decrease in shoot K^+. However, no major differences could be detected in Na^+ accumulation or the Na^+/K^+ ratio between salt sensitive control plants and the regenerated salt tolerant variants. These results are consistent with the observations in alfalfa callus culture (Croughan et al. 1978) that detected no differences in NaCl uptake between salt tolerant and salt sensitive alfalfa lines. The results also indicate that the tolerance phenotype depends on the maintenance of the necessary physiological processes in presence of increased salt concentrations in the leaf of the regenerated salt tolerant plants.

Stability of the salt tolerance phenotype. Retention of the tolerance phenotype at the cellular level (Table 1) from plants propagated by cuttings for more than a year suggests that the selection process has led us to select potential mutant cell lines and plants. This possibility was tested further by regenerating a number of plants from cell cultures initiated from the first group of tolerant plants that were propagated in culture for several months, but were not exposed to additional growth or selection with NaCl. These "second generation" plants showed retention of their salt tolerance characteristics (data not shown). Plants that were derived from three different initial salt tolerant isolates, showed that they retained the salt tolerance phenotype as measured by survival and growth (shoot increase in mass) in presence of NaCl under conditions where the control plants (parental R4 and Arrow, a commercial variety) were severely inhibited. While the tolerant plants in each group are siblings, regenerated from the same culture, they exhibit individual growth characteristics as was observed with the initial group of regenerated plants. The "second generation' plants are vigorous and remain fertile (Table 1). These results emphasize the stability of the acquired tolerance phenotype and support this method for selection of plant variants representing multigenic traits (Winicov et al. 1989).

Table 2. Na⁺and K⁺levels in control and variant plants grown in dilute Hoaglands solution + NaCl.

Plants	% NaCl	mg/g dry wt. Na^+	mg/g dry wt. K^+
Control	0	1.8 ± 0.5	39.4 ± 4.3
	0.5	30.3 ± 10.4	26.2 ± 8.8
	1.0	43.1 ± 3.1	18.6 ± 3.4
NaCl Tol.	0	2.2 ± 0.6	36.5 ± 4.3
	0.5	29.2 ± 8.2	21.5 ± 4.1
	1.0	40.8 ± 10.5	19.3 ± 2.6

Atomic absorption measurements were carried out in Perkin Elmer Atomic Absorption spectrophotometer. Each value represents the average of three replicate determinations per plant. Four control and nine regenerated tolerant plants were tested after one week of growth at the indicated salt concentration.

Table 3. Callus Tolerance to NaCl by Cell Lines from F-2 Progeny.

Plant	Total number F-2 lines	Tolerant/Sensitive	% Tolerant
Parent	19	0/19	0
Regenerated:			
IW-4A	2	1/1	50
IW-7	4	2/2	50
IW-5	12	6/6	50
IW-6	12	7/5	58
IW-9	12	9/3	75
All Regenerated	42	25/17	60

Parent R-4 plants and plants regenerated from salt tolerant cultures (Table 1) were self pollinated and seed collected. Callus cultures were initiated from cotyledons from individual seeds on control SH medium. Each culture was tested for salt tolerance by placing equal size callus pieces on control and 1% NaCl containing medium and determining callus weight after four weeks growth. Cell lines that grew 50 - 100% as well on 1% NaCl as on control medium were scored as tolerant. The results represent data from duplicate experiments.

Table 4. Growth Characteristics of F-2 Seedlings.

Plant	(A) g/plant	(B) g/plant
Parent		
Control 1	1.29 ± 0.15	2.77 ± 0.48
Control 2	1.05 ± 0.34	2.30 ± 1.32
Regenerated:		
IW-4A	0.70 ± 0.20	1.35 ± 0.26
IW-4B	1.06 ± 0.18	1.31 ± 0.91
IW-5	0.77 ± 0.41	1.75 ± 1.18
IW-6	1.14 ± 0.34	2.87 ± 0.47
IW-9	1.56 ± 0.54	3.54 ± 1.02

Selfed seed was collected as described in Table 3. The seedlings were germinated in sterile distilled water, planted in perlite and watered daily with dilute Hoaglands solution. A: shoot growth in 4 wks. B: shoot growth in next 4 wks. Mean weight in grams per plant ± S.D.

Table 5. Seedling tolerance to NaCl by F-2 Progeny.

Plants	Total No.	Growth on 0.5% NaCl >100%	100-80%	80-60%	<60%	Growth on 1% NaCl >60%	60-40%	<40%
Parent	21	0	2	11	8	1	7	13
Regenerated:								
IW-5	18	4	3	7	4	5	3	10
IW-6	11	1	4	3	3	3	6	2
IW-9	11	1	7	3	0	1	8	3
Total	40	6	14	13	7	9	17	15
Distribution observed:								
% regenerated		15	35	32	18	22	41	37
% parent		0	10	52	38	5	33	62

Seedlings were initiated as described in Table 4 and allowed to establish growth in perlite for 3 weeks in a growth chamber with 12 hr. daylight, 27° C and 80% humidity. Seedling response to NaCl was measured by comparing shoot fresh weights after four weeks growth on dilute Hoagland's solution ± 0.5% NaCl. After harvest of the shoots, the salt concentration was increased to 1% and the second growth comparison was done after an additional four weeks growth ± 1% NaCl.

To test the heritability of the putative mutation for salt tolerance, experiments were carried out to measure salt tolerance of the F-2 generation seeds obtained by selfing either the parent salt sensitive plants, or several of the regenerated salt tolerant plants. Table 3 shows the results of callus salt tolerance to NaCl by 42 cell lines initiated from the F-2 progeny. The results clearly show that overall cellular salt tolerance is maintained in 60% of the progeny from five different salt tolerant plants, indicating the presence of a dominant mutation in our regenerated plants.

Further tests were carried out to determine the F-2 progeny salt tolerance at the plant level. We have determined that the F2 seed obtained by self pollination of IW-5 and IW-6 germinates relatively well on 0.5% NaCl (78% and 53% respectively), while similarly selfed seed of the parent plant R4 shows a low level of germination on 0.5% NaCl (21%).

The growth characteristics of the F-2 seedlings from five regenerated plants is shown in Table 4 for two successive periods of growth. It is clear that most of the F-2 seedlings from the salt tolerant plants show the same vigor as those from the parent salt sensitive plants. The F-2 seedlings from IW-5, IW-6 and IW-9, which most resembled the parental type, were selected to further test the heritability of the salt tolerance trait by measuring individual seedling growth in dilute Hoaglands solution ± 0.5 or 1% NaCl. These results are summarized in Table 5 and again show that more than 50% of the selfed progeny appear tolerant. The observed number of tolerant calli (Table 3) and plants (Table 5) appear to be somewhat smaller than the expected 75% for a single dominant trait. This may be due to: a) a necessary interaction of the segregating locus with other independently segregating loci, or b) the natural distribution of individual plant growth responses of an autotetraploid (Table 4) influences the growth measurements ± NaCl. Nevertheless, these results demonstrate that the salt tolerance characteristic is indeed stable and is maintained through seed propagation.

Heritable somaclonal variation has been observed in wheat (Larkin et al. 1984), with both homozygous and heterozygous mutations obtained in the primary regenerated plants. This report describes a method for the salt selection of somaclonal variants that behave like mutants for salt tolerance in culture, and are able to regenerate into salt tolerant plants with heritable increased tolerance to growth in 0.5 to 1% NaCl. These results demonstrate that the salt tolerance characteristics expressed in cell culture can contribute to increased salt tolerance of the whole plant.

Alfalfa is an autotetraploid (see rev., McCoy and Walker 1984) and the tetra-allelic state of plant varieties has been associated with higher yields. The regenerated plants described here are therefore a valuable resource as genotypes in plant breeding for salt tolerance and as experimental plants for isolation, identification and manipulation of genes contributing to salt tolerance in alfalfa and other crop plants. The selection technique described in this paper will be especially useful for generating salt tolerant plants from callus cultures in naturally diploid plants such as rice, that can be regenerated from cell culture and are more amenable to genetic analysis.

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