Plant Physiol. (1989) 91, 855–861
0032-0889/89/91/0855/07/$01.00/0

Received for publication October 31, 1988
and in revised form May 23, 1989

Stable NaCl Tolerance of Tobacco Cells Is Associated with Enhanced Accumulation of Osmotin[1]

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ABSTRACT

Osmotin is a major protein which accumulates in tobacco cells (*Nicotiana tabacum* L. var Wisconsin 38) adapted to low water potentials. Quantitation of osmotin levels by immunoblots indicated that cells adapted to 428 millimolar NaCl contained 4 to 30 times the level of osmotin found in unadapted cells, depending on the stage of growth. Unadapted cells accumulated low levels of osmotin with apparent isoelectric points, (pI) of 7.8 and >8.2. Upon transfer of NaCl-adapted cells to medium without NaCl and subsequent growth for many cell generations, the amount of osmotin declined gradually to a level intermediate between that found in adapted and unadapted cells. NaCl-adapted cells grown in the absence of NaCl accumulated both pI forms; however, the form accumulated by cells adapted to NaCl (pI > 8.2) was most abundant. Adapted cells grown in the absence of NaCl exhibited absolute growth rates and NaCl tolerance levels which were intermediate to those of NaCl-adapted and unadapted cells. The association between osmotin accumulation and stable NaCl tolerance indicates that cells with a stable genetic change affecting the accumulation of osmotin are selected during prolonged exposure to high levels of NaCl. This stable alteration in gene expression probably affects salt tolerance.

NaCl stress evokes changes in the levels of several plant gene products (8, 12, 14, 21, 24–28). Since none of these gene products have been shown to be unequivocally involved in physiological adaptation to NaCl stress, additional information on the regulation of their expression is needed (11). Adaptation of tobacco to high levels of NaCl induces the accumulation of several proteins, particularly one with an apparent mol wt of 26 kD termed osmotin (6). Osmotin is a cationic protein occurring in tobacco cells in water soluble (osmotin-I) and insoluble (osmotin-II) forms. Osmotin accumulates in vacuolar inclusion bodies of cells which have adapted to NaCl (23). It is localized also in the cytoplasm at much lower levels (23) and is loosely associated with isolated plasma membrane and tonoplast vesicles (18). At least two polypeptides from tobacco with similar charges and mol wt cross-react with the polyclonal rabbit IgG[2] raised against purified osmotin-I. The major form of osmotin synthesized by tobacco cells adapted to NaCl has an apparent pI greater than 8.2. Unadapted cells synthesize mainly a cross-reacting protein with a lower apparent pI of 7.8 (25) but accumulate low levels of both forms. The synthesis of osmotin in tobacco cells and cells of proso millet, field bindweed, cotton, carrot, and potato is induced by ABA (25), a treatment that accelerates adaptation of tobacco cells to stress induced by ionic solutes (15) and hastens osmotic adjustment in response to water deficits imposed by ionic and nonionic solutes (16).

The biological function of osmotin is not yet known. However, analysis of the amino acid composition and sequence of osmotin indicates substantial homology to the sweet protein thaumatin, the bifunctional maize α-amylase/proteinase inhibitor, and a pathogenesis related protein induced by tobacco mosaic virus infections (22, 23). These latter three proteins are hypothesized to be involved in defense reactions of plants to pathogens (22).

In this report, we show that NaCl-adapted cells grown in the absence of NaCl are able to grow and adapt to salt more rapidly than cells never exposed to salt. These cells continue to overaccumulate predominantly the higher pI form of osmotin. These results further support the hypothesis that enhanced tolerance of NaCl-adapted cells is at least in part related to accumulation of osmotin. These data also support the hypothesis that selection for salt-tolerant cells has occurred during prolonged growth in medium containing high NaCl concentrations.

MATERIALS AND METHODS

Culture Maintenance and NaCl Tolerance

Cell suspensions of *Nicotiana tabacum* L. var Wisconsin 38 were initiated and maintained as described by Hasegawa *et al.* (10), except that casein hydrolysate was omitted from the medium. Fresh and dry weight measurements, maintenance of stock cultures for experiments, and preparation of media were as described earlier (15). Isolation of cell clones was described earlier (5, 9). Briefly, cells adapted to 428 mM NaCl were screened through stainless steel and nylon meshes with increasingly smaller pores, as small as 43 μm, which

[1] Supported by Purdue University Agricultural Experiment Station Program Funds, U.S. Department of Agriculture grant No. 86-CRCR-1-12064 and U.S. Department of Energy grant No. DE1309. Journal Paper No. 11963, Purdue University Agricultural Experiment Station.

[2] Abbreviations: IgG, Immunoglobulin G; GAR-HRP, goat-anti-rabbit horse radish peroxidase; IEF, isoelectric focusing; NEPHGE, non-equilibrium pH gel electrophoresis; pI, isoelectric point; TI, tolerance index.

allowed separation of cell clumps no larger than four cells each. These cell clumps were plated onto solid nutrient medium containing 428 mM NaCl and grown into separate callus colonies large enough to establish cell suspensions of each colony, termed a clone. The cell suspension clones were grown in the absence of NaCl for three passages. Unadapted cells were treated similarly except that NaCl was not included during the isolation of cell clones. Tolerance to NaCl was determined by conducting dose-response experiments using cells taken from a large stock culture at different times over the entire course of the culture growth cycle as described by Binzel *et al.* (1). This procedure was necessary because of the major changes in the dose-response of cells taken at different growth stages. Tolerance was expressed as a TI in order to summarize the large amount of data collected from these dose response experiments. The TI was calculated as follows:

$$TI = 100 + \sum^{n} \left(x \left[\frac{WG_x}{WG_0} \right] 100 \right)$$

where $x = 0, 2, 4, 6, 8, 10, 12$ g/L NaCl, WG_0 = fresh weight gain on 0 g/L NaCl, WG_x = fresh weight gain on x g/L NaCl, and where $n = 7$, the number of summations of $x(WG_x/WG_0)100$. The cells in a dose response experiment used to calculate TI were harvested after the cells grown without NaCl had reached stationary phase. This index emphasizes the cells ability to grow at higher NaCl concentrations, since the weighting factor (x) more heavily weighs the relative difference in growth compared to unstressed cells (WG_x/WG_0) as a function of the NaCl concentration. This weighting factor was applied since cell growth differences tended to be greater at the higher NaCl levels.

One- and Two-Dimensional Slab Gel Electrophoresis

One- and two-dimensional SDS-PAGE of acetone powdered cells was performed essentially as described by Singh *et al.* (24) except that 10 or 12% polyacrylamide running gels were used rather than gradient gels and proteins in the first dimension were separated by IEF or NEPHGE (19). The apparent pH gradients of IEF gels were determined by sectioning the gels into 1 cm sections, immersing the sections in 0.5 mL of degassed distilled H_2O for 6 h under partial vacuum in a desiccator, and then measuring the pH of the H_2O. The assignment of PIs of the osmotin isoforms was done by IEF (25). Osmotin isoforms on NEPHGE SDS-PAGE were matched to the isoforms on IEF SDS-PAGE on the basis of their relative migration in the first dimension and their M_rs in the second dimension. This matching can be justified if one considers that NEPHGE is simply IEF interrupted before the proteins have approached their pI (19).

Immunoblot of Gels and Slot Blot Procedures

For immunoblots of gels, protein concentrations in extracts of acetone powdered cells (24) were assayed by the procedure of Bradford (4) with the following modifications: Twenty μL of extract was added to 0.4 mL of 100 mM KPi buffer (pH 7.2) contained in a 1.5 mL Eppendorf tube to precipitate the SDS in the extraction buffer. After vortexing and a 10 min incubation period, the sample was centrifuged (16,000*g*) for 3 min. Protein concentrations in samples and standards prepared similarly with 20 μL of extraction buffer were assayed from 0.25 mL of the supernatant added to 2.75 mL of 5-fold diluted Bio-Rad protein assay dye reagent concentrate (catalog No. 550-0006). Depending on the range of protein concentrations of the extracts, equal amounts of protein (20–80 μg) were loaded onto each lane of a particular gel. The running gel contained 10 or 12% polyacrylamide. Most gels were run in duplicate. After electrophoresis, one of the duplicate gels was stained with Coomassie brilliant blue R-250 and examined to determine if equal amounts of protein were loaded into each lane. The other gel was equilibrated in transfer buffer containing 48 mM Tris, 39 mM glycine, and 1.3 mM SDS (pH 9.2) and 20% MeOH (v/v) for 30 min with one exchange of transfer buffer after 15 min. The protein was transferred to nitrocellulose paper (BA85 0.45 μM average pore diameter, Schleicher & Schuell, Keene, NH) using a semidry electroblotter (SemiDry Electroblotter, Janssen Life Science Products) for 1 h applying 1 mA/cm^2. Compared to a tank buffer system, the semidry transfer device allowed much more even, rapid and efficient transfer of osmotin, as well as other proteins resolved on SDS-PAGE gels. Gels were stained with Coomassie brilliant blue R-250 after transfer and typically revealed virtually no residual protein. Uniform binding over the nitrocellulose was evaluated in preliminary experiments by staining of the transferred proteins with amido black. The uniformity of the transfer on each blot was evaluated by examining the background staining after the color development reaction, which is not readily apparent in photographs of blots. Flaws in the transfers such as air bubbles and uneven transfer were readily apparent. Blots displaying major flaws in the areas where osmotin bound were not used further. Osmotin bound to the nitrocellulose was visualized using a Bio-Rad Immun-Blot GAR-HRP kit (catalog No. 170-6500). The partially purified polyclonal rabbit anti-osmotin IgG (24, 25) was diluted 1000-fold. All antibodies used in this study were raised against purified osmotin-I, an aqueous soluble form (23, 24). These antibodies cross-react with osmotin-II, a detergent soluble form (23). Some of the immunoblots of one-dimensional gels were scanned as described below. Immunoblots of gels allowed determination of the presence or absence of several isoforms and possible nonspecific binding of the anti-osmotin IgG. In certain instances noted in the figure legends, the areas derived from scanning individual blots were shown to be within the linear range of the amount of antigen-antibody complex formed when increasing amounts of antigen were used.

For slot blot analysis, acetone powders of cell samples were extracted by sonication for 30 s in buffer (50 μL/mg) containing 10 mM sodium phosphate (pH 7.0), 0.9% NaCl, 0.2% $NaDodSO_4$, 0.5% Triton X-100, and 1 mM PMSF, followed by heating in boiling H_2O for 3 min. The extracts were centrifuged at 16,000 *g* for 3 min, and up to 500 μL of each of the supernatants were purified on Sephadex G-25 spin columns (1 × 4 cm). The protein concentrations of the eluates were determined as outlined above by determining the protein contents of pellets after precipitation of the protein in methanol with 100 mM ammonium acetate at −15°C for 1 h and

centrifugation in a microfuge (16,000 *g*). Fifty μL samples of the eluates from the spin columns each were loaded onto nitrocellulose paper in a slot of a Minifold II slot blotter (Schleicher & Schuell, Keene, NH) and blotted onto the paper with gentle suction for 15 min. The nitrocellulose paper was subsequently dried for 1 h. Two slot blot procedures were used. In the first procedure the paper was transferred to blocking buffer (extraction buffer with 0.2% nonfat dry milk) and gently agitated for 1 h. One-hundred mL volumes were used in this and subsequent steps. The paper was then transferred to blocking buffer containing ^{125}I-labeled antibodies for 6 to 18 h. The IgG was diluted 500-fold from the serum concentration. The paper then was washed five times in blocking buffer and dried. Radioactivity from the blot was detected by autoradiography. Developed x-ray films (Kodak XAR 5) of the blots were scanned using a Beckman DU-8 spectrophotometer in the densitometric mode. Protein was quantitated by estimating the total protein in each aliquot and comparing the calculated areas of sample bands to areas of bands from standards prepared from purified osmotin. The unknown samples were assayed in the linear range of detection by making dilution series. This procedure was found to be the best for quantitation of all soluble osmotin isoforms. However, problems with high background and insolubility of the purified osmotin and the ^{125}I-labeled antibodies after freezing were periodically encountered so another immunodetection assay was applied. In the second slot blot procedure, Bio-Rad GAR-HRP was used to visualize the primary rabbit IgG bound to osmotin. After color development and drying, the blots were scanned directly. The relative quantity of osmotin was estimated by comparing the areas of the sample bands to the areas of standards within a linear response range prepared from the protein extracted from a standard batch of cells grown in 428 mM NaCl. This standard osmotin extract was also used to determine the relative amounts of osmotin in total cell proteins separated by SDS-PAGE and visualized by immunoblotting.

RESULTS

Stability of NaCl Tolerance in NaCl-Adapted Cells

Dose response experiments on cell clones revealed that gradual exposure of tobacco cells to higher levels of NaCl (428 mM) over many cell generations resulted in both selection of stable tolerant cell types and physiological adaptation of all cell types (5). Cells grown in medium without added NaCl but derived from NaCl-adapted cells continued to display higher tolerance to NaCl compared to unadapted cells. This was indicated by their higher tolerance indices at comparable physiological and temporal stages of the growth cycle and their higher average tolerance over the entire growth cycle (Fig. 1). For two separate experiments, the mean fresh weight gain of the adapted cells off NaCl for 20 generations and then transferred to medium containing 10 g/L, was 2.4 g/25 mL; the mean fresh weight gain of unadapted cells transferred to medium containing 10 g/L was 0.97 g/25 mL. For the same two separate experiments, the mean fresh weight gain of adapted cells off NaCl for 20 generations and then transferred to medium containing 12 g/L was 1.6 g/25 mL; the mean fresh weight gain of unadapted cells transferred to medium containing 12 g/L was 0.5 g/25 mL. Thus, as we have indicated in a preliminary report (6), the enhanced tolerance of adapted cells is partially stable for up to 20 generations in the absence of NaCl.

Accumulation of Osmotin is Stabilized in NaCl-Adapted Cells Grown in the Absence of NaCl

Immunoblots of one-dimensional SDS gels revealed one or two major bands which formed immunocomplexes with anti-osmotin-I IgG, with M_rs of about 26 and 27 kD. The accumulation of osmotin in cells harvested in the stationary phase of the growth cycle was greatly enhanced in cells adapted to 428 mM NaCl for many (>400) cell generations (22; Figs. 2 and 3) and in cells adapted to the low water potential imposed by 30% (w/v) PEG in the nutrient medium (Fig. 2). Cells which had been adapted to 428 mM NaCl were returned to medium without NaCl. Such cells harvested at the end of the growth cycle after each of the first four passages (about five generations for each passage) and after 25 passages (over 100 generations) in medium without NaCl maintained higher levels of osmotin compared to cells never exposed to NaCl as visualized from the immunoblots (Fig. 2). It was clear that the levels of osmotin declined relative to NaCl-grown cells, but even after more than 100 cell generations in the absence of NaCl, these cells accumulated higher levels of osmotin at the end of the growth cycle than cells never exposed to NaCl (Fig. 3, A, B, C, and D). The slot blot procedure using ^{125}I-labeled IgG (Fig. 3B) and the procedure of immunoblotting gels (Fig. 3, C and D) indicated that cultured tobacco cells in normal medium accumulated more osmotin as the cells approached the stationary phase of the growth cycle, a pattern of accumulation consistent with the incorporation of ^{35}S into newly synthesized protein in such cells (24). The band of unadapted cells in the stationary phase of growth could be visualized in gels stained with Coomassie brilliant blue R-250 in contrast to previous results where osmotin accumulation was below the level of detection by this staining method (24).

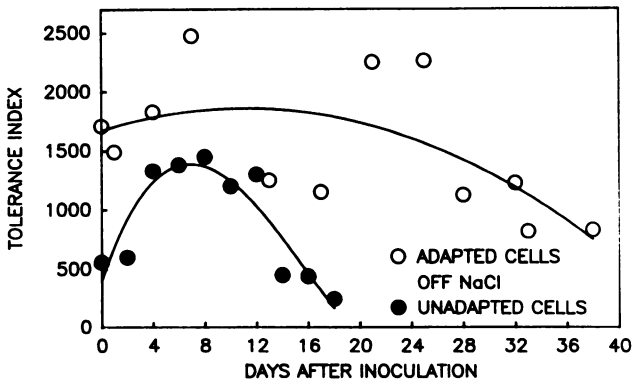

Figure 1. NaCl tolerance of unadapted (●) cells and cells which were adapted to 428 mM NaCl and returned to medium without NaCl for 20 generations (○). Data are from a single experiment from among three experiments. The mean tolerance index of unadapted cells is 921; for cells adapted to 428 mM NaCl returned to medium without salt, it is 1319.

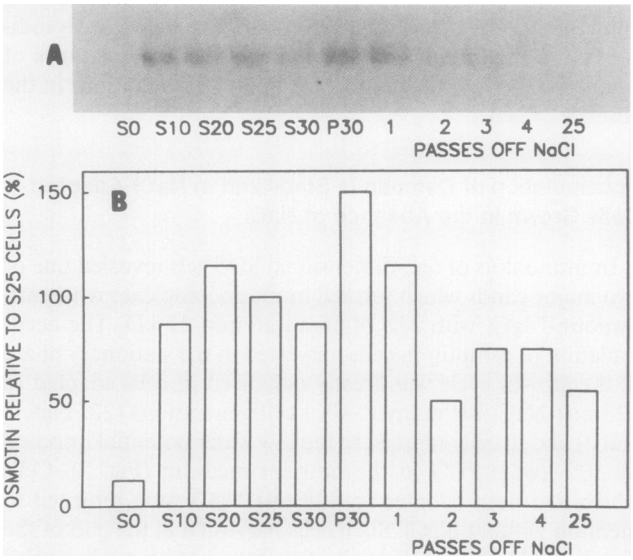

Figure 2. A, Osmotin accumulation in tobacco cells adapted to 0 (S0), 171 (S10), 342 (S20), 428 (S25), 513 (S30) mM NaCl, 30% polyethylene glycol (P30), and cells adapted to 428 mM NaCl grown in medium without NaCl for 1, 2, 3, 4, and 25 passages analyzed by immunoblotting with GAR-HRP. Cells were sampled in the stationary phase of the growth cycle and 50 μg of protein was added to lanes except to the sample labeled 25, which had a lower amount of protein according to the staining intensity of the duplicate gel. B, Relative amounts of osmotin in each sample as determined by densitometry of the blot and normalizing amounts of osmotin by use of a standard curve as in Figure 4C.

This appeared to be the result of long-term growth of the cells in culture since newly initiated cell cultures accumulated much less osmotin that the older cell cultures (data not shown). The slot blot procedure used to detect osmotin with GAR-HRP (Fig. 3A) appeared to be less specific since growth cycle related changes in osmotin accumulation were not clearly resolved. One explanation for this apparent lack of specificity was the presence of background staining. A source of background staining may have been the binding of colored compounds when the cell protein extracts were loaded onto the nitrocellulose paper.

Proteins from 10 randomly selected cell clones derived from the adapted and the unadapted lines were quantitated on immunoblots of SDS-PAGE gels (Fig. 4). The osmotin accumulation was greater in most of the clones derived from the NaCl adapted cells (Fig. 4A) compared to the unadapted cells (Fig. 4B). Both the osmotin peak areas determined by scanning the protein bands and the amounts of osmotin calculated relative to the standard osmotin preparations (Fig. 4B) were on the average significantly greater by 1.4- and 2-fold, respectively. From these data we conclude that most of the cells in the adapted cell population maintained enhanced accumulation of osmotin when in the absence of NaCl stress.

Immunoblots of two-dimensional gels revealed that two major protein spots with an M_r near 26 kD cross-reacted the anti-osmotin-I IgG (Fig. 5). A third minor spot running between the 26 and 27 kD spots was seen on some gels. These basic proteins did not focus well using our protocols for the

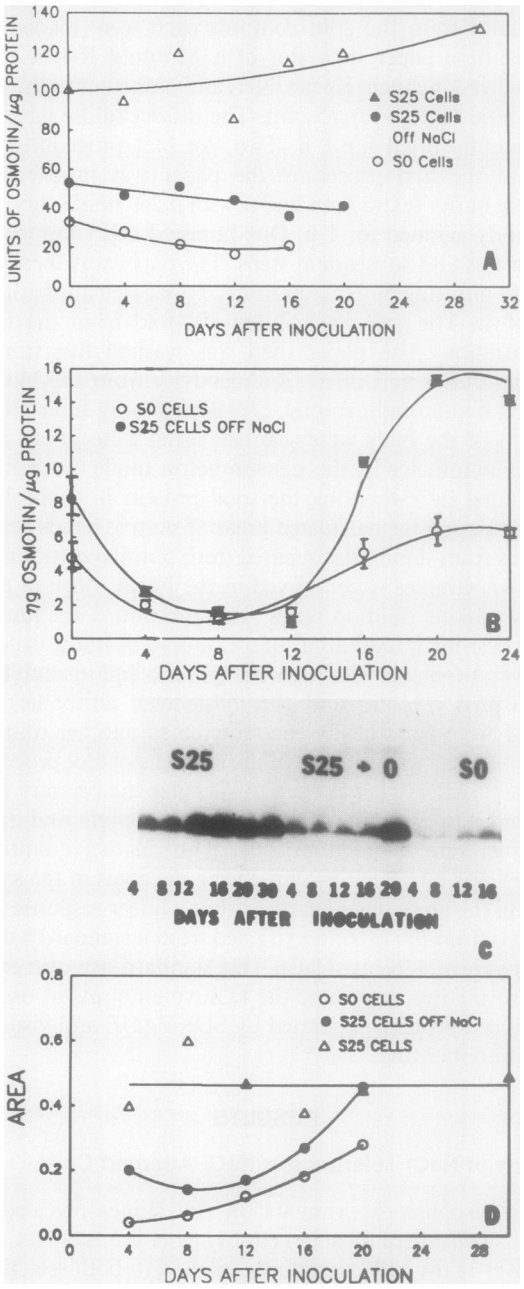

Figure 3. Accumulation of osmotin in S0-cells and S25-cells maintained without NaCl for 10 cell passages, *i.e.* 40 to 45 mass cell doublings (S25-cells off NaCl) and S25-cells maintained in 428 mM NaCl for at least 400 cell doublings. A, Quantitation of osmotin by slot blot procedure with GAR-HRP. B, Quantitation of osmotin by slot blot procedure with ^{125}I-labeled IgG. Osmotin quantity in S25-cells by slot blot procedure using ^{125}I-labeled IgG was 38 ηg osmotin/μg protein. Bars in B represent standard errors of the mean from three replicates. On some of the symbols the bars are not evident because they are smaller than the symbols. Measurements shown in A and B are within the linear range of the antibody-antigen reaction. C, Photograph of immunoblot of gel probed with antiosmotin IgG visualized with GAR-HRP. D, Quantitation by scanning of immunoblot shown in C. The areas of the scanned immunoblot (D) were not shown to be in the linear area of detection, since no standards were run in this experiment.

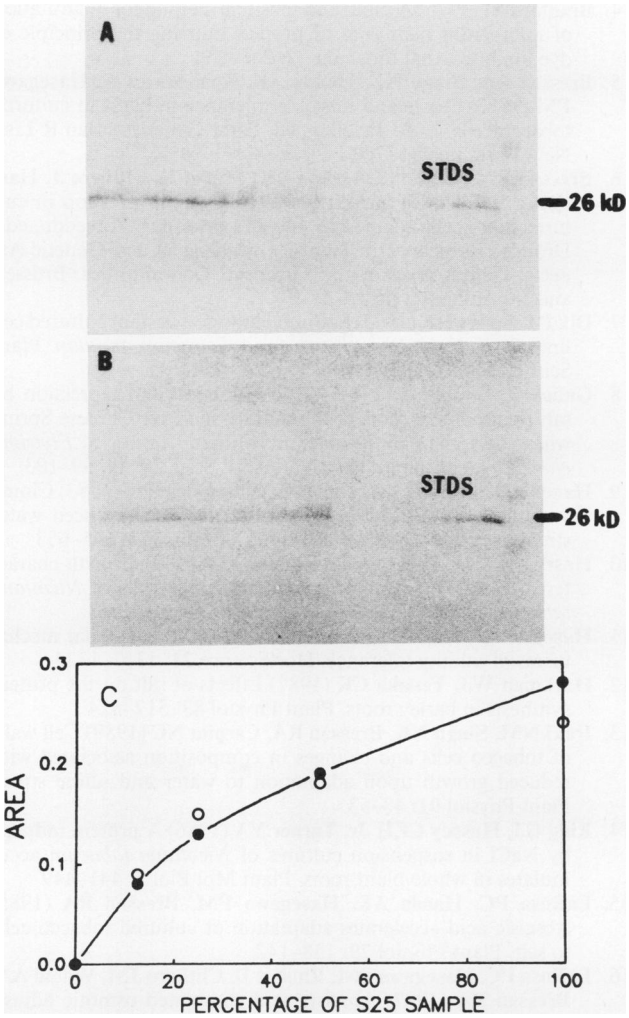

Figure 4. Accumulation of osmotin in cell clones derived from adapted (A) and unadapted cells (B) grown in the absence of NaCl. GAR-HRP was used to visualize osmotin rabbit IgG immunocomplexes. Clone samples are on the left sides of the blots different amounts of protein extracted from cells grown in 428 mM NaCl (STDS) are on the right. (C) Standard curve of osmotin using different amounts of the protein extracted from a standard batch of adapted cells grown in medium with 428 mM NaCl showing that the areas derived by scanning of the unknown samples were in the linear range. The average relative amounts of osmotin of the cell clones derived from adapted cells was 52 ± 2% (SE). The average relative amounts of osmotin of the cell clones derived from the unadapted cells was 25 ± 1% (SE). The average amounts of osmotin in the two types of cell clones were different based on the *F* test at the 95% level.

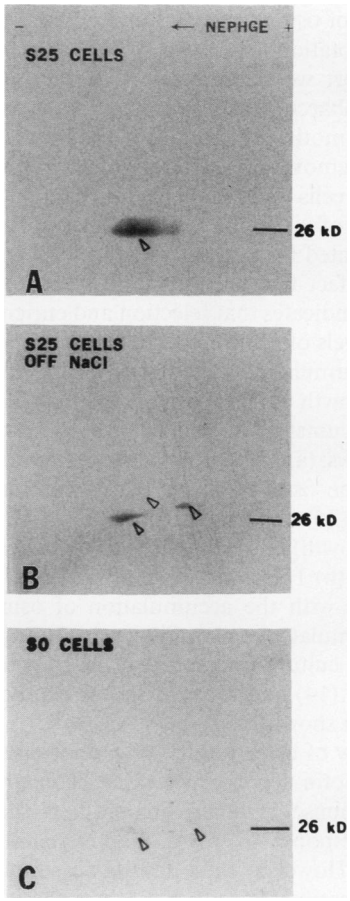

Figure 5. Immunoblots of two-dimensional slab gels (NEPHGE) of proteins of S0-cells (A), S25-cells off NaCl (B), S25-cells (C). Protein samples were 80 μg each from stationary phase cells when the accumulation of osmotin would be expected to be maximum.

IEF gels and the more basic form did not completely enter the gel (24, 25, data not shown). The two major proteins had apparent pIs of 7.8 and >8.2 and M_rs of 27 and 26 kD, respectively (Fig. 5). The assignment of the pIs was made by comparing the pattern of osmotin spots on the NEPHGE gels to those of focused gels (29; immunoblots of IEF gels not shown). Cells adapted to 428 mM NaCl accumulated almost entirely the pI >8.2 form (Fig. 5A), although adapted cells clearly synthesize both forms (25). Unadapted cells accumulated both forms (Fig. 5C), but only the pI 7.8 form could be detected by $^{35}SO^{2-}_4$ labeling (25). These results could be explained if the pI >8.2 form is more rapidly synthesized in adapted cells or if it is rapidly converted from the pI 7.8 form in adapted cells. Quantitation of osmotin by immunoblotting of one-dimensional gels and slot blot analysis did not distinguish between these various forms.

DISCUSSION

NaCl tolerance of cultured cells from various species is stable through many generations in the absence of NaCl (5–7, 20, 29, 30). The biochemical and genetic basis for the expression of the NaCl tolerant phenotype in cell cultures is not well understood. However, changes in some physiological characteristics including growth (1, 10, 17), water relations, solute accumulation (2, 30), ion compartmentation (3), cell wall properties (13), and changes in the expression of specific gene products (14, 24); (reviewed by Hasegawa *et al.*, 11) have been demonstrated to be associated with the adapted state. We assume that the permanent alteration in the expression of one or more genes is responsible for conferring the stable tolerance phenotype of NaCl-adapted cells. We have indicated in a preliminary report that a permanent alteration in the

accumulation of osmotin in cultured tobacco cells occurs as a result of adaptation to salt (6).

In this report we demonstrate that the enhanced NaCl tolerance of tobacco cells is associated with enhanced accumulation of osmotin protein through more than 40 cell generations after removal of NaCl from the medium. In addition, it appears that cells with stable tolerance to NaCl continue to produce more of a specific isoform of osmotin which is the form accumulated by adapted cells growing in the presence of NaCl. The fact that accumulation of osmotin is a stable characteristic indicates that selection and enrichment for cells with higher levels of osmotin occurs in the presence of NaCl. Thus, the accumulation of osmotin is linked to increased survival or growth in the presence of high levels of NaCl. Additional circumstantial evidence which supports this concept is as follows: (a) Treatment of the cells with ABA induces synthesis of the osmotin form that accumulates in NaCl-adapted cells (25) and is correlated with ABA accelerated adaptation (growth) of tobacco cells to a newly imposed NaCl stress (15, 16). (b) The resumption of growth of NaCl stressed cells coincides with the accumulation of osmotin (25). (c) Osmotin accumulates in plant tissues including tomato roots in hydroponic culture in response to a 10 d treatment with 171 mM NaCl (14) and tobacco shoots exposed to 500 mM NaCl (data not shown).

Even in view of these results we cannot dismiss the possibility that osmotin accumulation is a secondary process that occurs in response to some unidentified change, perhaps involving a response to ABA, that is stabilized in NaCl-adapted cells. However, these results do supply justification for further experiments to test the hypothesis that osmotin accumulation plays some role in NaCl tolerance. If osmotin is involved with tolerance it is probably only one of many factors. The physiological characteristics associated with NaCl adaptation probably are the result of many mechanisms which involve many gene products. The question is open as to whether osmotin-like proteins are those induced by salinity in other plant species (8, 12, 21). The permanent alteration in the accumulation of osmotin indicates that a permanent change in some gene regulating osmotin has occurred. The osmotin gene has not been grossly rearranged or amplified after NaCl adaptation (27). However, osmotin accumulation could be affected by a minor undetected change in the osmotin gene or by changes in other regulatory genes and *trans/cis*-acting factors.

ACKNOWLEDGMENT

The excellent technical assistance in the isolation of cell clones and NaCl dose response experiments by Jean Clithero is appreciated.

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