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Expression of Osmotin-Like Genes in the Halophyte *Atriplex nummularia* L.[1]

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ABSTRACT

A peptide (molecular mass 50 kilodaltons) that is immunologically related to tobacco osmotin was detected in cells of the halophyte *Atriplex nummularia*. This protein was constitutively expressed in both unadapted and NaCl-adapted cells. A predominant osmotin-like peptide (molecular mass 24 kilodaltons) was also found in culture media after cell growth. Two unique *A. nummularia* cDNA clones, pA8 and pA9, encoding osmotin-like proteins have been isolated. The pA8 and pA9 inserts are 952 and 792 base pairs and encode peptides of 222 and 224 amino acids, respectively. The peptide deduced from pA8 has a molecular mass of 23,808 daltons and theoretical isoelectric point of 8.31, whereas the peptide derived from pA9 has a molecular mass of 23,827 daltons and an isoelectric point of 6.88. Unique transcripts were detected by the inserts of the cDNA clones, two (1.2 and 1.0 kilobases) by pA8 and one (0.9 kilobase) by pA9. The pA8 transcripts were constitutively accumulated in unadapted and NaCl-adapted cells, whereas the mRNA levels were up-regulated by abscisic acid treatment. The level of pA9 mRNA was induced by NaCl treatment and increased in cells as a function of NaCl adaptation. Southern analysis of the genomic DNA indicated the presence of osmotin-like multigene families in *A. nummularia*.

Atriplex nummularia L. is a euhalophyte that exhibits optimum growth in the presence of 100 mM NaCl and is able to withstand up to 400 mM NaCl without showing growth reduction (9). Isolated *A. nummularia* cells growing in liquid suspension are substantially more capable of surviving NaCl treatments then are cells of the glycophyte tobacco (3). These results indicate that cellular based mechanisms are significant components of the salt tolerance of this halophyte even though other mechanisms undoubtedly contribute to the adaptation capacity of the plant.

The cells of many glycophytes have been adapted *in vitro* to grow in the presence of high levels of salt (26). Apparently, at the cellular level, the occurrence of genetic elements unique to halophytes is not necessary to maintain cell viability and growth in a saline environment, particularly if the rate of adaptation is stringently controlled. This does not mean that unique halophyte genes are not substantial determinants of mechanisms of salt tolerance. However, it is likely that at least part of the genetic basis for the greater salt adaptability of halophytes is the altered capacity to regulate as well as to coordinate the regulation of genes that contribute to the adaptability of glycophytes.

Several genes have been shown to be induced by hyperosmotic environments and by the hormone ABA (5, 8, 11, 15, 18, 25). One family of osmotically regulated genes encodes osmotin (16, 22, 24–26), which has been categorized as a family 5 PR[2] protein (14). The quantity of osmotin increases with the level of osmotic adaptation, and stable NaCl tolerance is associated with increased accumulation of osmotin (13). Osmotin accumulates predominantly in vacuolar inclusion bodies but can also be detected in the cytosol, on the plasma membrane and the tonoplast, and in the extracellular matrix and external medium of NaCl-adapted tobacco cells (22) (Fig. 2).

In NaCl-adapted *Nicotiana tabacum* L. var W38 cells, at least two isoforms of osmotin can be detected based on minor differences in molecular mass on SDS gels and differences in pI (13). One osmotin isoform is synthesized from a transcript that encodes a 26.38-kD preprotein that contains a 2.5-kD signal sequence (25). Osmotin has substantial sequence identity with thaumatin, the bifunctional maize α-amylase/proteinase inhibitor, and PR proteins (16, 25). Peptides antigenically analogous to tobacco osmotin have been detected in several other plant species (24).

Here, we report that proteins immunologically related to osmotin are found in both glycophytes and halophytes, that unique isoforms are synthesized in the halophyte *A. nummularia*, and that osmotin halophytic genes have different regulatory properties from those found in the glycophyte tobacco.

MATERIALS AND METHODS

Cell Cultures

Atriplex nummularia L., *Distichlis spicata*, and *Nicotiana tabacum* L. var Wisconsin 38 (tobacco) unadapted cells and

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[2] Abbreviations: PR, pathogenesis related; pI, isoelectric point; SSC, standard sodium citrate, 1× = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0.

cells adapted to grow in the presence of 342 mM, and 428 mM NaCl in the case of tobacco, were maintained as described previously (2, 3, 29). The salt-adapted cells were maintained in media with NaCl for at least 100 generations before the initiation of experiments. Cells in the stationary phase of growth were inoculated at a fresh weight density of 0.2 g/mL of culture medium. For induction experiments, concentrated autoclave-sterilized NaCl (4 M) or filter-sterilized ABA (10^{-2} M) solutions were added to medium containing unadapted cells 3 d after inoculation. Cells were harvested 24 h after addition of NaCl or ABA.

Protein Extraction

Cells and media were separated by filtration using Whatman No. 1 filter paper. Cellular proteins were precipitated with cold acetone (23). Culture medium (100 mL) was dialyzed against (−20°C) 4 L of distilled water, which was changed three times. The dialyzed media were centrifuged at 10,000*g* for 20 min, and the supernatant and pellet were lyophilized. Proteins were extracted as previously described (23), except that 5 mM DTT was used instead of β-mercaptoethanol in the SDS-PAGE buffer. Some cellular proteins were reduced with 60 mM DTT and carboxyamidated by addition of 240 mM iodoacetamide in SDS-PAGE buffer in an attempt to cleave disulfide linkages (19).

Gel Electrophoresis and Immunoblotting

One-dimensional PAGE of cell or media protein samples was performed essentially as described previously (13, 23). Protein quantity was determined in samples after precipitation of SDS (13). Proteins were separated on 10 to 16% gradient or 12% denaturing polyacrylamide gels.

For immunoblots, the separated proteins were electroblotted onto nitrocellulose. Osmotin cross-reactive proteins were detected with chicken anti-osmotin IgY at a dilution of 1:1000 using rabbit anti-chicken antibody conjugated to alkaline phosphatase (Jackson Immunochemicals) as the second antibody. Molecular masses were determined by comparison to Bio-Rad low molecular mass standards.

Construction of *A. nummularia* cDNA Library and Clone Isolation and Characterization

An *A. nummularia* λZAPII (Stratagene) cDNA library consisting of 8×10^5 recombinants was constructed from 5 μg of polyadenylated mRNA isolated from cells adapted to 342 mM NaCl. Approximately 2×10^5 recombinants were screened using tobacco osmotin cDNA insert as a probe (25). The hybridizations were performed in 6× SSC, 5× Denhardt's solution (21), 0.1% SDS, and 100 μg/mL of denatured herring sperm DNA for 18 h at 42°C. After hybridization, the filters were washed sequentially with 6× SSC + 0.1% SDS, 4× SSC + 0.1% SDS, and finally 2× SSC + 0.1% SDS for 30 min each at room temperature. λZAP clones showing strong hybridization with osmotin cDNA were isolated, screened to plaque purity, and converted to pBluescript by coinfection with R408 helper phage.

The sequence of both strands of the cDNA inserts was determined by the Sanger dideoxy nucleotide chain termination method using the Sequenase kit (U.S. Biochemical). Restriction fragments of the cDNA inserts were subcloned into pBluescript or pGEM-7Zf(−) (Promega) vectors. Typically double-stranded DNA was used as the template for sequencing; however, in some instances, single-stranded DNA templates were required. Single-stranded DNA was rescued by coinfection of XL1Blue cells containing pBluescript phagemids with VCSM13 helper phage (10). Sequence comparisons were made utilizing the Genetics Computer Group software package-1989 (Biotechnology Center, University of Wisconsin).

Isolation of RNA and DNA

Total RNA was isolated by the acid guanidinium thyocyanate phenol-chloroform extraction procedure (4). It was further purified by precipitation with 2 M LiCl followed by ethanol precipitation. Polyadenylated RNA was isolated by affinity chromatography using oligo(dT)-cellulose.

To extract DNA, ground frozen tissue was lysed by shaking gently with buffer (100 mM LiCl, 50 mM EDTA, 10 mM Tris-HCl [pH 7.5], and 0.5% SDS; 4 mL/g fresh weight). The lysed tissues were then gently extracted with an equal volume of a 1:1 mixture of phenol equilibrated with buffer (as above) and chloroform:isoamyl alcohol (24:1) for 15 min. The aqueous phase was collected by centrifugation and precipitated with a half-volume of 7.5 M ammonium acetate overnight at 4°C. DNA was precipitated from the ammonium acetate supernatant with an equal volume of isopropanol at 4°C. The DNA was further purified by RNase treatment and cetyltrimethylammonium bromide extraction (1). Nucleic acids were quantified spectrophotometrically.

Northern and Southern Blots

Total RNA (15 μg) was separated on formaldehyde agarose gels and blotted onto nitrocellulose by capillary transfer. Transcript size was estimated by comparing with a 0.24- to 9.5-kb RNA ladder (BRL). For Southern blots, 20 μg of DNA was treated with different restriction endonucleases, separated on 0.8% agarose gels, and transferred to nitrocellulose. Lengths of DNA fragments were determined by comparisons to *Hin*dIII/*Eco*RI-cut λDNA. ^{32}P-labeled probes were prepared using a random primer labeling kit (Boehringer Mannheim) with [α-^{32}P]dCTP. The solutions and conditions for hybridization were as described by Singh *et al.* (25). Filters were washed at 30°C for 30 min each in 1×, 0.2×, and then 0.1× SSC, each with 0.1% SDS, and finally in 0.1× SSC + 0.1% SDS at 65°C for 30 min.

RESULTS

Adaptation to osmotic stress involves many physiological and biochemical changes. These changes are undoubtedly the result of the altered expression of several genes (7, 23). During the adaptation of *A. nummularia* cells to NaCl, we observed that there were changes in the total SDS-soluble polypeptide pattern (Fig. 1A) indicating that altered gene expression is associated with adaptation of halophytes also.

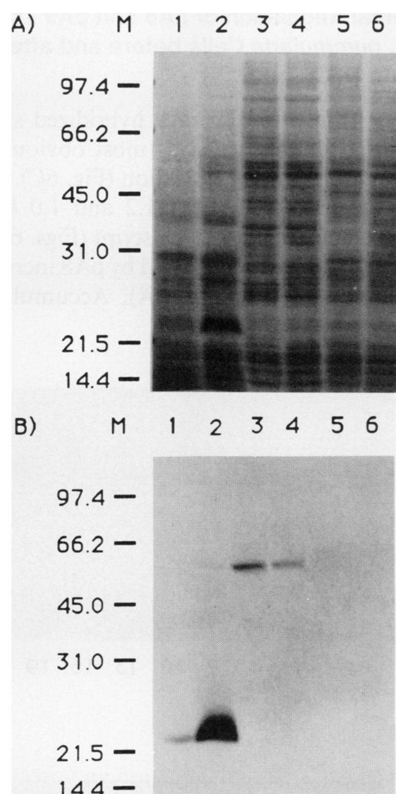

Figure 1. SDS-PAGE gradient (10–16%) gel (A) and immunoblot (B) of cellular proteins (40 μg per lane). Proteins were obtained from unadapted and NaCl-adapted cells of tobacco (lanes 1 and 2), *A. nummularia* (lanes 3 and 4), or *D. spicata* (lanes 5 and 6). *A. nummularia* and *D. Spicata* cells adapted to grow in the presence of 342 mM NaCl, and 428 mM NaCl in the case of tobacco. The proteins were isolated 9 d after inoculation into fresh medium, and the immunoblot was reacted with tobacco antiosmotin. M, Molecular mass in kD.

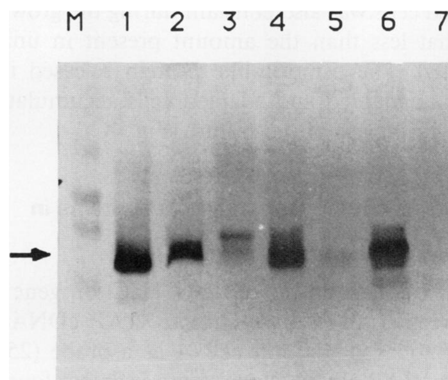

Figure 2. Immunoblot of tobacco and *A. nummularia* media proteins (15 μg per lane). Lane 1, Cellular proteins from NaCl (428 mM) adapted tobacco cells; soluble and precipitable fractions of proteins released into the medium of adapted tobacco cells (lanes 2 and 3, respectively) or *A. nummularia* unadapted (lanes 4 and 5, respectively) or NaCl (342 mM) adapted cells (lanes 6 and 7, respectively). The proteins were isolated 9 d after inoculation into fresh medium, and the immunoblot was reacted with tobacco antiosmotin. The protein standards (lane M) are 106, 80, 49.5, 32.5, 27.5, and 15.8 kD.

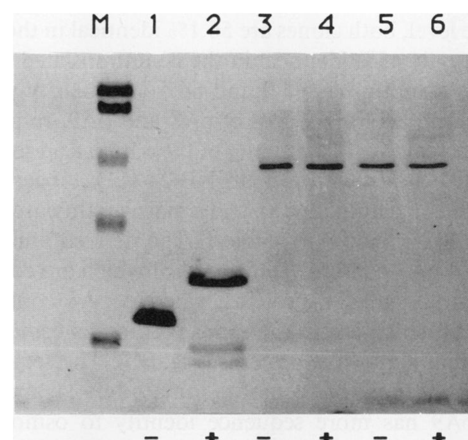

Figure 3. Immunoblot of proteins isolated from tobacco and *A. nummularia* cells. Proteins (15 μg) from NaCl (428 mM) adapted tobacco cells (lanes 1 and 2) and unadapted (lanes 3 and 4) and NaCl (342 mM) adapted (lanes 5 and 6) *A. nummularia* cells without (−) or with carboxyamidation treatment (+) were separated on an SDS-PAGE gel (12%). The proteins were isolated 9 d after inoculation into fresh medium, and the immunoblot was reacted with tobacco antiosmotin. The protein standards (lane M) are 106, 80, 49.5, 33.5, 27.5, and 18.5 kD.

Osmotin is one of the most extensively studied proteins whose expression is enhanced during adaptation to osmotic stress. A peptide immunologically related to tobacco osmotin was detected in *A. nummularia* but not in *D. spicata* cells, although this protein had a molecular mass of about 50 kD, in contrast to about 26 kD for tobacco osmotin (Fig. 1B). The level of the 50-kD osmotin-like protein in *A. nummularia* slightly decreased during adaptation to NaCl in contrast with the increase observed in tobacco. Furthermore, in medium in which the cells were growing, there was a smaller polypeptide, approximately 24 kD, also antigenically related to osmotin (Fig. 2) that may result from processing of the high molecular mass protein during extracellular release.

Because intramolecular disulfide linkages occur in tobacco osmotin (Fig. 3), SDS-PAGE-separated osmotin-like protein from *A. nummularia* was subjected to carboxyamidation (Fig. 3) and performic acid oxidation (data not shown) treatments. The results indicate that dimerization based on disulfide linkages is not the reason why the *A. nummularia* cellular protein has a molecular mass of about twice that of osmotin. If the monomeric unit of the *A. nummularia* protein is similar

to that of tobacco, then other types of linkages must be involved.

The quantity of *A. nummularia* osmotin-like protein in unadapted cells remained constant throughout the growth cycle (Fig. 4A). The accumulation of osmotin-like protein in NaCl-adapted cells was also constant during the growth cycle but somewhat less than the amount present in unadapted cells (Fig. 4B). The osmotin-like protein released into the medium by unadapted and adapted cells accumulated proportionately to the age of the culture (Fig. 4C).

cDNA Clones Encoding Osmotin-Like Proteins in *A. nummularia*

To further understand the effect of NaCl on gene expression, we screened an *A. nummularia* λZAP cDNA library with labeled tobacco osmotin cDNA as a probe (25). Nine independent λZAP cDNA clones were isolated, four cDNA clones with the longest inserts were *in vivo* excised, and determination of the partial 5′ and 3′ sequences of these inserts indicated that they were different. The complete nucleotide sequence of these inserts was determined by sequencing both strands.

The cDNA clones were designated pA8 and pA9 and contained inserts of 952 and 792 base pairs, respectively (Fig. 5, A and B). The longest open reading frame of pA8 encodes a polypeptide of 222 amino acids, 23,808 D, and a theoretical pI of 8.31; pA9 encodes a 224-amino acid peptide of 23,827 D and pI of 6.88 (Fig. 5, A and B). The deduced amino acid sequence of pA8 is 59.3% similar to that of pA9. At the nucleotide level, both clones are 56.1% identical in the coding region but only 45% identical in the 3′-untranslated region.

Tobacco osmotin has 63.0 and 66.5% similarity with the derived amino acid sequences of pA8 and pA9, respectively (25). The percentage of similarity of the amino acid sequences of these two clones with tomato NP-24 (11), tobacco PR-R proteins (6, 17), thaumatin (8), and a maize α-amylase/trypsin inhibitor (20) is shown in Table I. The derived amino acid sequence of pA9 contains 16 Cys, 14 of which are conserved in osmotin and related proteins, whereas pA8 contains 13 Cys, nine of which are in the same position as osmotin and the other thaumatin-like proteins (Fig. 5C). The sequence of pA8 is equally similar to osmotin, tomato NP-24, and PR-R, whereas pA9 has more sequence identity to osmotin and maize α amylase/trypsin inhibitor than to PR-R (Table I). Based on these sequence comparisons and northern hybridization of *Atriplex* mRNA with tobacco probes, the *Atriplex* genes represent family members that are as different from osmotin and PR-R as these two genes are different from each other.

Based on the deduced amino acid sequence, it is not possible to conclude whether the proteins encoded by the *A. nummularia* cDNA clones contain leader sequences. However, the N-terminal sequences derived from pA8 and pA9 contain 11 and 7 amino acids, respectively, that are conserved in the leader sequence of osmotin (25). In this case, the first amino acid of the mature proteins would be Thr instead of Ala, as it is for the different thaumatin-like proteins (Fig. 5C).

Developmental Regulation of pA8 and pA9 Transcript Levels in *A. nummularia* Cells before and after Adaptation to NaCl

The inserts from pA8 and pA9 hybridized specifically to different transcripts, and this was most obvious when both probes were used in the hybridization (Fig. 6C). pA8 hybridized to two transcripts of about 1.2 and 1.0 kb, and pA9 hybridized to a smaller 0.9-kb transcript (Figs. 6 and 7). The accumulation of mRNAs recognized by pA8 increased slightly after adaptation to NaCl (Fig. 6A). Accumulation of the

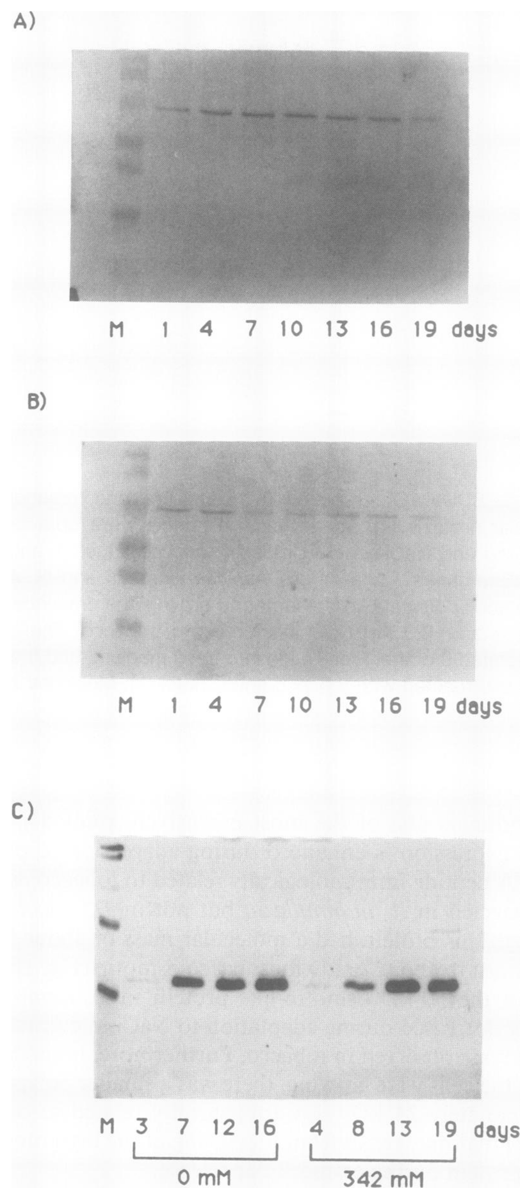

Figure 4. Developmental accumulation of osmotin-like proteins in *A. nummularia* cells and media. Immunoblots of 15 µg of proteins isolated from unadapted cells (A), NaCl (342 mM) adapted cells (B), and media of unadapted (0 mM) and NaCl (342 mM) adapted cells (C) on the day indicated after inoculation into fresh medium. Proteins were separated on SDS-PAGE gels (12%), and immunoblots were reacted with tobacco antiosmotin. The protein standards are 106, 80, 49.5, 32.5, 27.5, and 18.5 kD.

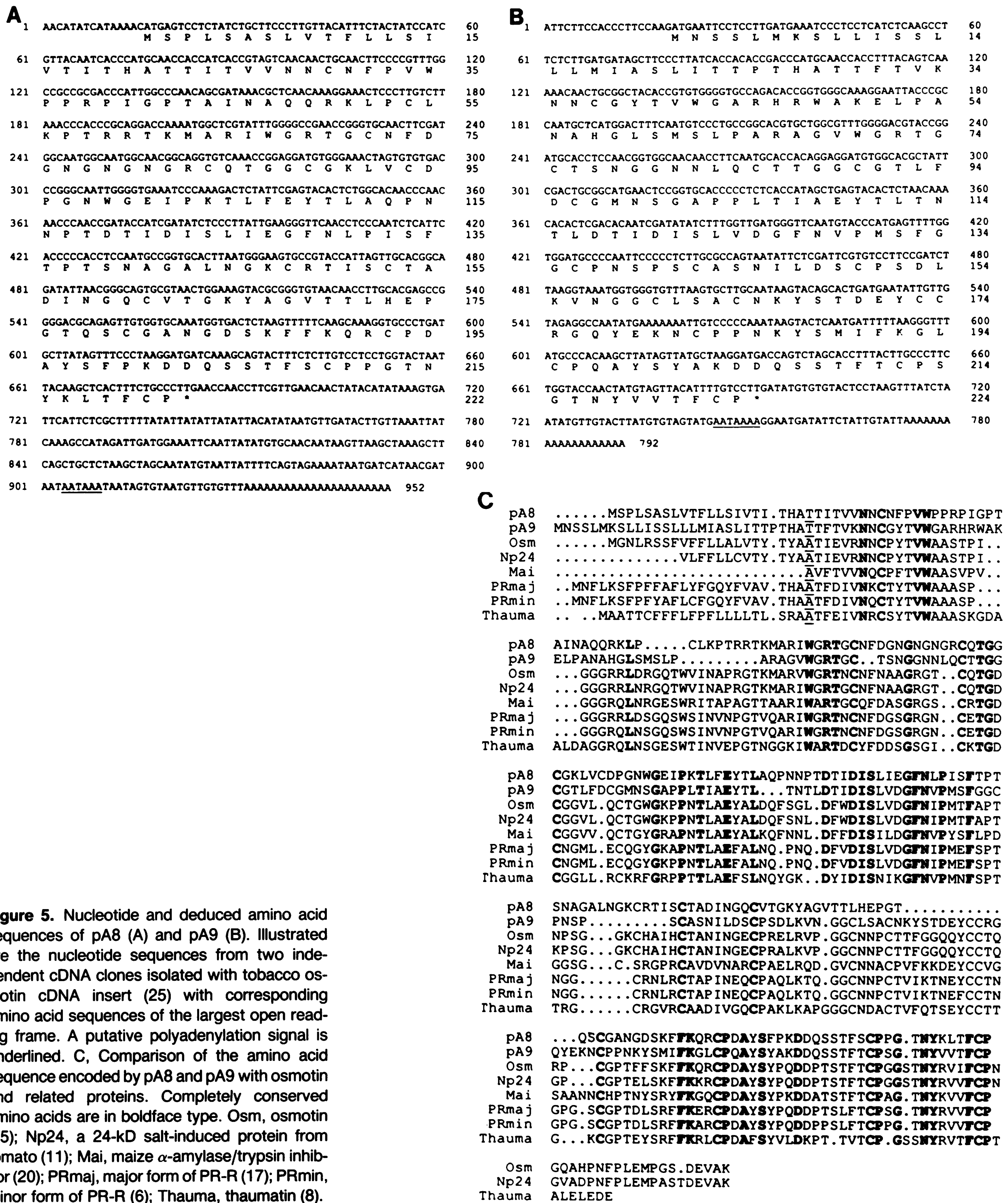

Figure 5. Nucleotide and deduced amino acid sequences of pA8 (A) and pA9 (B). Illustrated are the nucleotide sequences from two independent cDNA clones isolated with tobacco osmotin cDNA insert (25) with corresponding amino acid sequences of the largest open reading frame. A putative polyadenylation signal is underlined. C, Comparison of the amino acid sequence encoded by pA8 and pA9 with osmotin and related proteins. Completely conserved amino acids are in boldface type. Osm, osmotin (25); Np24, a 24-kD salt-induced protein from tomato (11); Mai, maize α-amylase/trypsin inhibitor (20); PRmaj, major form of PR-R (17); PRmin, minor form of PR-R (6); Thauma, thaumatin (8).

Table I. *Amino Acid Comparisons of Osmotin and Osmotin-Like Proteins*

Percentage of similarity of the deduced amino acid sequences of pA8 and pA9 with tobacco osmotin (25), tomato NP-24 (11), tobacco PR-R major form (17), tobacco PR-R minor form (6), thaumatin (8), and maize α-amylase/trypsin inhibitor (20).

	Osmotin	NP-24	PR-R Major	PR-R Minor	Thaumatin	α-Amylase/Trypsin Inhibitor
pA8	63.0	63.0	64.3	64.6	61.1	57.4
pA9	66.5	62.1	59.8	59.6	59.0	64.4

transcript that was detected by pA9, however, was increased substantially after adaptation to NaCl (Fig. 6B), and message was detected in unadapted cells only by prolonged exposure of the X-ray film (Fig. 7C).

The pattern of pA8 mRNA accumulation in unadapted cells was very much related to the culture growth cycle stage (Fig. 7A). Accumulation of the message increased upon inoculation of cells into fresh medium, reached a peak at log phase (4 d postinoculation), and decreased as the cells reached stationary phase about 13 d after commencement of the growth cycle. The pA9 transcript appeared to reach maximal levels somewhat later than PA8 transcripts but also declined later during the growth cycle in unadapted cells (Fig. 7C).

Both pA8 and pA9 mRNA accumulation followed similar patterns during the growth of NaCl-adapted cells (Fig. 7, B and D). The levels of transcripts were elevated 24 h after inoculation, reached a maximum during log phase (7 d postinoculation), and then decreased as the cells approached stationary phase on day 16. Although we did not attempt to quantify the difference, the amount of pA8 message was always greater than pA9 (Fig. 6, A *versus* B; Fig. 7, B *versus* D), but the level of induction after adaptation to NaCl was substantially higher for pA9 (Fig. 6B; Fig. 7, C *versus* E) compared to pA8 transcript (Fig. 6A; Fig. 7, A *versus* B).

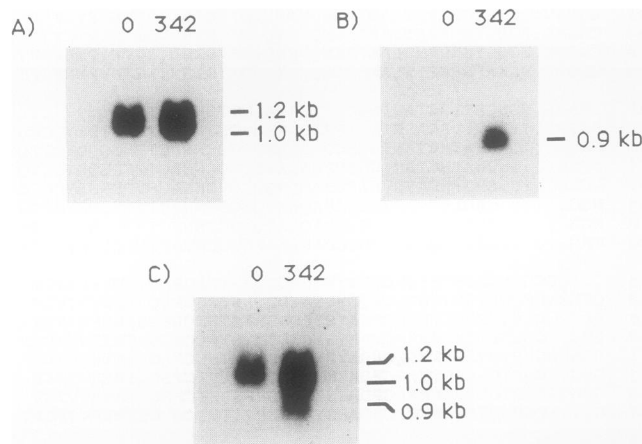

Figure 6. Accumulation of osmotin-like mRNA in unadapted (0) and NaCl (342 mM) adapted (342) *A. nummularia* cells. The blots of 15 μg of total RNA isolated from cells 4 (0 mM NaCl) and 8 d (342 mM NaCl) after initiation of the growth cycle were hybridized with labeled inserts from pA8 (A), pA9 (B), or both (C). Estimated transcript sizes are indicated on the right.

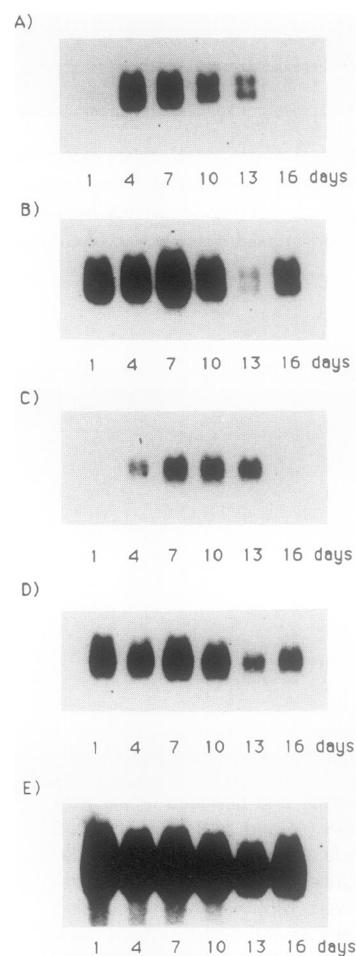

Figure 7. Accumulation of osmotin-like mRNA in *A. nummularia* cells during cell growth. Total RNA was isolated from unadapted and NaCl (342 mM) adapted cells on the days indicated after inoculation into fresh medium. RNA from unadapted cells probed with pA8 (A) or pA9 (C) labeled inserts and RNA from NaCl-adapted cells hybridized with pA8 (B) or pA9 (D and E) inserts. pA9 detected a transcript in RNA from unadapted cells (C) only after a longer exposure of the film as compared to the signal of NaCl-adapted cells (E). A, B, and D, 5-h exposure; C and E, 2-d exposure.

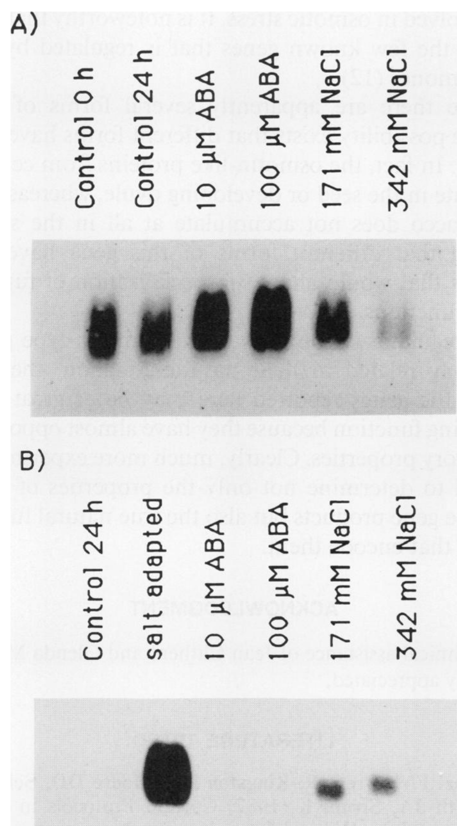

Figure 8. Induction of osmotin-like mRNA accumulation in *A. nummularia* cells by NaCl or ABA. Northern blots of total RNA (20 μg) from unadapted cells were probed with pA8 (A) or pA9 (B) labeled insert. Three days after inoculation into fresh medium, cells were treated with either NaCl or ABA or an equal volume of water in the control and then harvested 24 h later for RNA isolation. RNA from NaCl (342 mM) adapted cells was included in B as a control.

NaCl and ABA Regulation of Osmotin-Like Transcript Accumulation in *A. nummularia*

It appeared from previous results (Fig. 6B) that pA9 mRNA accumulation was regulated by NaCl because this message was highly enhanced in NaCl-adapted cells. Induction of pA9 mRNA accumulation by NaCl occurred within 24 h after treatment of unadapted cells with 171 or 342 mM NaCl (Fig. 8B), although the level of NaCl-induced message was not equivalent to that observed in cells adapted to NaCl. The level of pA8 transcript increased slightly after adaptation to NaCl and could not be induced by NaCl shock of unadapted cells but actually appeared to decrease as a result of NaCl shock (Fig. 8A). However, the hormone ABA, which also induces osmotin mRNA accumulation in tobacco, induced accumulation of only the pA8 transcript and not the salt-inducible pA9.

Analysis of Osmotin-Like Genes in *A. nummularia*

Identification of pA8 and pA9 transcripts provided evidence that there are at least two unique osmotin-like genes in *A. nummularia*. The results of genomic Southern blotting indicated that the inserts from pA8 and pA9 appeared to recognize two different gene families, despite the fact that these two cDNAs are structurally similar (Fig. 9). Analysis of the number and size of the restriction fragments that were detected after treatment with different enzymes and the fact that both cDNAs contain one internal *Hin*dIII site (835 in pA8; 609 base pairs in pA9) indicated that there may be three pA8 and two pA9 genes. Because pA8 and pA9 inserts hybridized to six and four *Hin*dIII-digested restriction fragments, respectively, there appear to be at least three genes representing pA8 and at least two genes for pA9 present in the genome.

DISCUSSION

We did not detect a large number of changes in accumulated proteins of *A. nummularia* cells after NaCl adaptation. This could be because halophytes constitutively express many genes that are required for salt adaptation. However, it is more likely due to the inability of this procedure to detect changes in the expression of low-abundance proteins.

Antiosmotin from tobacco reacts specifically to a 50-kD polypeptide in *A. nummularia* cells. Because osmotin transcripts detected in *A. nummularia* are long enough to encode only about half of a 50-kD protein, and tobacco osmotin is about 26 kD, the protein in *A. nummularia* might be a dimer. Also, an immunologically detectable 24-kD protein was found

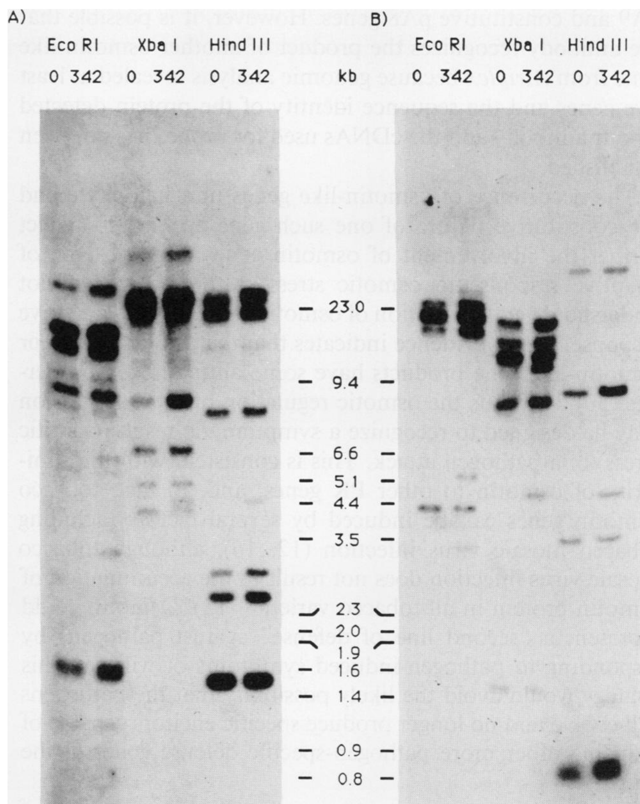

Figure 9. Southern analysis of genomic DNA from cells of *A. nummularia*. DNA was isolated from unadapted (0 mM) or NaCl (342 mM) adapted cells (342) and digested with *Eco*RI, *Xba*I, or *Hin*dIII as indicated. Southern blots were probed with pA8 (A) or pA9 (B) labeled insert.

in *Atriplex* cell culture medium. It is possible that this protein is a secreted form that is either the result of processing of the dimer before release or is protected from dimerization during transport to the extracellular matrix. It is of interest to note that a larger, approximately 50-kD form of osmotin also can be detected by immunoblotting in cell extracts of tobacco (Fig. 1B) and that a 24-kD osmotin is found in tobacco cell medium (Fig. 2). The proteins from *Atriplex* that were detected using tobacco antiosmotin can be considered osmotin like; however, their sequence or function relationship to tobacco osmotin is as yet unknown.

At least two different osmotin-like gene families, containing perhaps three and two genes each, can be identified in *A. nummularia* based on Southern blots with cDNA probes (pA8 and pA9 inserts). We could identify two pA8 transcripts and one pA9 transcript in *A. nummularia* cells growing *in vitro.*

Regulation of the genes identified by pA8 and pA9 cDNAs were substantially different. The accumulation of transcripts detected by pA8 did not respond to the initial change in the osmotic environment, whereas pA9 mRNA increased after NaCl shock and increased substantially after adaptation. It is interesting also that the pA8 transcript that is unresponsive to NaCl is induced by ABA and the salt-induced pA9 transcript is not responsive to ABA.

Because the protein in *Atriplex* that is detected by the tobacco antiosmotin is constitutively expressed (Fig. 4), this antibody probably recognizes products of both the induced pA9 and constitutive pA8 genes. However, it is possible that the antibody recognizes the product of another osmotin-like gene from *Atriplex* because genomic analysis revealed at least five genes and the sequence identity of the protein detected by our antibody and the cDNAs used for probes has not been established.

The occurrence of osmotin-like genes in a halophyte and the constitutive nature of one such gene appears to reflect further the involvement of osmotin genes in some type of adaptive response to osmotic stress. Although we do not understand yet the function of osmotin in the overall adaptive response, recent evidence indicates that the osmotin genes or osmotin-like gene products have some antipathogen properties (30), and thus the osmotic regulation of their expression may be designed to recognize a symptomatic result (osmotic stress) of a pathogen attack. This is consistent with the similarity of osmotin to other PR genes, and, in fact, tobacco osmotin genes can be induced by several factors including tobacco mosaic virus infection (12, 16), although tobacco mosaic virus infection does not result in the accumulation of osmotin protein in all tobacco varieties (12). Osmotin could represent a "second line of defense" against pathogens by responding to pathogen-induced symptoms of wilting. This strategy would avoid the likely possibility that the pathogens will evolve and no longer produce specific elicitors capable of inducing other more pathogen-specific defense genes of the host.

The overall expression patterns of many PR genes and of genes related to osmotic stress indicate that ethylene is the plant hormone most often involved with induction of genes thought to participate in defenses against pathogens, whereas ABA is the hormone that has been found to induce most genes involved in osmotic stress. It is noteworthy that osmotin is one of the few known genes that is regulated by both of these hormones (12).

Because there are apparently several forms of osmotin genes, the possibility exists that different forms have separate functions. In fact, the osmotin-like proteins from cereals (28) accumulate in the seed or developing ovule, whereas osmotin from tobacco does not accumulate at all in the seed (12), indicating that different forms of this gene have specific properties that would allow subspecialization of function or separate functions.

If the regulatory properties of the osmotin-type genes are in any way related to their natural function, the *Atriplex* forms of the genes reported here may be of great value in determining function because they have almost opposite types of regulatory properties. Clearly, much more experimentation is needed to determine not only the properties of these osmotin-like gene products but also the true natural function of the genes that encode them.

ACKNOWLEDGMENT

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LITERATURE CITED

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