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Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein

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Abstract

The cDNA clone (pNP24) coding for a protein induced by exogenous NaCl has been isolated from a tomato root cDNA library with the use of an inosine containing synthetic oligomer. The authenticity of the clone has been established by comparing the sequence of the clone to the NH_2-terminal sequence of the protein which has been purified to homogeneity by HPLC. The nucleotide sequence of pNP24 reveals a 5′ signal sequence, an open reading frame of 718 nucleotides, a 3′ AT rich untranslated region containing a probable polyadenylation signal sequence, and a poly A stretch. The mature polypeptide sequence as deduced from the nucleotide sequence reveals a protein with a molecular weight of 24226. This protein has been named NP24. It is slightly basic and has an unusually high number of cysteines (15). Northern blot analyses reveal that the abundance of mRNA for NP24 is at least 100-fold greater in tomato suspension cells in log phase grown in medium with NaCl than in cells grown in the control medium. The mRNA for NP24 is below the level of detection in roots of young control tomato plants until several weeks after germination but it is induced earlier and to higher levels in roots stressed by 0.171 M NaCl. Thus salt stress accelerates the accumulation of message in tomato roots. A comparison of the steady state levels of mRNA for NP24 to the accumulation of NP24 by immuno analyses indicates that the accumulation of this protein is determined by its mRNA level. The protein is not secreted and is localized within the cytoplasm or the soluble fraction of the nucleus, vacuole, or microbodies. NP24 has a high degree of homology (58%) with thaumatin, a protein which has considerable value as an artificial sweetener.

Introduction

The most prominent protein change occurring when tomato and tobacco suspension cells are adapted for growth in NaCl is an accumulation of a protein previously estimated to be about 26000 daltons [10, 15, 26, 27]. This protein, NP24, also accumulates after the cells are stressed with other agents such as PEG and KCl which lower the water potential but not with nonosmotic stresses such as $CdCl_2$ or heat shock [15]. This protein is the most abundant protein in the salt stressed suspension cells as determined by Coomassie Blue stained PAGE. Utilizing antiserum to the purified NP24 protein, we previously characterized the expression of the protein and found that its accumulation in suspension cultures is dependent on the growth phase of the cultures. In these suspension cells it begins to accumulate as the cells reach log phase. NP24 is found in control tobacco and tomato suspension cells but at a considerably lower concentration than in the NaCl stressed cells [15].

NP24 is present in whole tomato plants grown un-

der normal conditions but it accumulates to a higher level when the plants are stressed with NaCl. It accumulates primarily in the root. An immunologically related protein of the same molecular weight is found in genera as diverse as *Medicago* and *Phaseolus* [15]. The role of this protein in the growth of cells and plants exposed to exogenous NaCl as well as other osmotic stresses is still unknown. To further characterize NP24 we have 1) examined its subcellular localization, 2) isolated and characterized the cDNA clone for the gene with the use of synthetic oligomers and 3) analyzed its expression at the mRNA level by Northern analyses.

Materials and methods

Plant materials and growth conditions

Suspension cultures of tomato (*Lycopersicon esculentum* VFNT) and tobacco (*Nicotiana tabacum* W38) were maintained as previously described [15]. Tomato plants (*Lycopersicon esculentum* UC82B) were grown in an aerated hydroponic system maintained in a Conviron growth chamber (Conviron Products of America, Pembira, ND). Plants were grown under a regime of 26 °C days, 21 °C nights, and a 16 h photoperiod. Seeds were germinated in moist Grodan (Agro Dynamics, Brooklyn, NY) plugs. Once plants had reached the 4–5 true leaf stage a NaCl solution was added directly to the hydroponic solution (Peters Hydro-sol 5-11-26) to a final concentration of 0.171 M NaCl. This was approximately two weeks after germination. Conductivity and pH of the medium were monitored and water levels maintained throughout the experiment. NaCl treated plants and controls were harvested 8 h following the addition of NaCl and on subsequent days. Roots were excised from stems, immediately immersed in liquid N_2, and stored at −70 °C until RNA was extracted.

Preparation of protoplasts and gradients

Protoplasts were isolated from log phase cultures of control tobacco suspension cells and from cells grown in medium with 0.171 M NaCl. Protoplast isolation procedures were as previously described [31] with the following modifications. The enzyme solution consisted of MS (Murashige and Skoog) salts, 2% cellulase, 0.1% macerozyme, 0.001 M dextran sulfate, 0.03 M MES buffer, and 0.6 M mannitol adjusted to pH 5.7. Salt stressed cells were incubated in a similar solution which contained 0.8 M mannitol and 0.09 M sucrose.

Protoplasts were filtered through nylon mesh to remove debris before pelleting at 100× *g* for 5 min. Control protoplasts were resuspended in 0.6 M sucrose solution containing MS salts, overlayed with 0.6 M mannitol solution containing MS salts and centrifuged at 100× *g* for 5 min. The intact protoplasts were collected from the sucrose-mannitol interface with a pasteur pipette, washed with isolation buffer, and pelleted at 300× *g* for 3 min. Protoplasts from salt stressed cells were recovered and resuspended in 0.4 M sucrose and 0.8 M mannitol and floated on 0.8 M mannitol by centrifugation at 100× *g* for 5 min. Yields of protoplasts were greater than 5×10^6 protoplasts/g fresh weight (gfw) cells. Protoplasts were resuspended in 0.3 M sorbitol, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10 mM Tris-HCl pH 8.0, and were gently lyzed by passage through a pasteur pipette. Aliquots of the ruptured protoplasts were layered onto linear sucrose gradients (30% – 60% w/w sucrose) buffered with 10 mM Tris-HCl pH 8.0, containing 1 mM EDTA and 10 mM 2-mercaptoethanol.

Gradients were centrifuged for 30 min at a maximum speed of 27000 rpm in a SW28 rotor and were fractionated into 1 ml fractions. Gradient fractions and ruptured protoplasts were analyzed spectrophotometrically for PEP carboxylase [30] and fumarase [13] activities. RuBP carboxylase activity was determined according to Wishnick and Lane [34] after a 10 min activation at 30 °C in the assay buffer without RuBP. Chlorophyll was determined by the method of Arnon [1]. Protein was determined by the technique of Bradford [4]. Cell wall and membrane fragments and soluble protein fractions from both salt adapted and control cells were prepared from cells (1–3 gfw) homogenized in liquid N_2 as previously described [20]. Homogenates were centrifuged at 100000× *g* for 1 h and the supernatant

was retained. The pellet was resuspended in homogenization buffer (10 mM Tris-HCl pH 7.0, 10 mM EDTA, 1% polyvinylpyrrolidone, 1 mM DTT), washed by centrifugation at 100000× *g* and resuspended in homogenization buffer. Both the supernatant and the washed pellet were analyzed for the presence of NP24.

Protein purification

Log phase tomato suspension cells grown in the presence of 0.34 M NaCl were harvested after subculture by filtration onto coarse filter paper (Whatman 3MM). After being frozen in liquid N_2, the cells were ground to a fine powder with a mortar and pestle. An equal volume of homogenization buffer was added per volume of cells and the cells were ground again as described above. A second volume of buffer was added and the cells were homogenized with a Tekmar Tissumizer (Tekmar Co., Cincinnati, Ohio) at a speed of 90 rpm for 1 min. Particulate matter was removed by filtration through miracloth followed by centrifugation in a Beckman SW28 rotor at 27000 rpm for 1 h. All subsequent steps were done at 4 °C. Proteins in the supernatant fluid were precipitated by the addition of ¼ volume of saturated $(NH_4)_2SO_4$ and removed by centrifugation in a Beckman JA 14 rotor at 9000 rpm for 10 min. A second ¼ volume of $(NH_4)_2SO_4$ was added and the precipitate collected as before. The pellet was resuspended in 10 mM Tris-HCl pH 6.8 and 1 mM EDTA and dialyzed exhaustively against the same buffer. Protein from the second $(NH_4)_2SO_4$ cut was injected onto a cation exchange column (BioGel TSK-SP-5PW, 75 × 75 mm) with a Negrette' and Zambra injector with a fixed loop size. A 50 min linear gradient was accomplished with buffer A (20 mM phosphate, pH 5.5) and buffer B (20 mM phosphate, pH 5.5, 0.5 M NaCl). The gradient was held at 100% buffer B for 10 min before returning to the starting condition.

The column was eluted with buffer solutions at a rate of 1 ml/min and effluent was monitored with a Micromeristic 787 variable uv-vis detector set at 280 nm. The appropriate protein peak was collected and an aliquot analyzed by SDS polyacrylamide gel electrophoresis (PAGE) [16], silver stained [19], and determined to be homogeneous.

NH_2-terminal amino acid sequencing

Automated Edman degradations [9] were performed by William Gray (University of Utah) on 0.3 mg of HPLC purified protein with a Beckman 890D sequenator using 0.25 M Quadrol. The program analysis was essentially the 1 M Quadrol program supplied by the manufacturer. Prerun polybrene (3 mg) was utilized as a carrier [29]. The resulting PTH derivatives were analyzed by HPLC [33]. The analysis identified the NH_2-terminal residues 1 to 80. The repetitive yield for $Ala_1 - Ala_{56}$ was 95%.

SDS-PAGE and immuno (Western) blot analyses

Plant materials were prepared for electrophoresis by freezing in liquid N_2 and grinding in a mortar and pestle with an equal volume of SDS sample buffer [16] followed by boiling for 15 min and removal of insoluble material by centrifugation. PAGE was carried out according to the procedure of Laemmli [16] with 10% acrylamide and 1% (w/v) SDS. Immuno analyses were carried out as described [3] with BA-85 nitrocellulose paper (Schleicher and Schuell) and a BioRad Trans Blot cell. The preparation of the antiserum has been described previously [15]. Protein concentrations were determined by Bradford assay [4].

cDNA cloning

A cDNA library was prepared from poly $(A)^+$ RNA isolated from tomato roots using the method of Chuck Gasser (Monsanto, personal communication). After first strand synthesis with reverse transcriptase (Life Sciences), the reaction was extracted with phenol and the DNA purified on a Biogel P60 column. After second strand synthesis and methylation, Eco RI linkers (New England Biolabs) were added. The cDNA was purified from excess linkers and

size selected by separation through 0.8% Sea Plaque agarose (FMC). The cDNAs were cloned into the EcoRI site of λgt10.

Screening of λgt10 cDNA library

Oligonucleotides were synthesized by the phosphoramidite method with a 380A Applied Biosystems DNA synthesizer. The oligomer which we utilized, a 29-mer, had two mixed T/C bases and utilized inosine [21, 28] at the other six positions of codon degeneracy (nucleotides 251–289, Fig. 2). Oligonucleotides were labeled without further purification at the 5′-end with gamma-^{32}P ATP and T4 polynucleotide kinase (BRL) [18] and used to probe the phage cDNA library by *in situ* plaque hybridization [2]. Hybridizations were done at 35 °C with the 29-mer probe. Plaques which gave positive signals in the first high density screening were purified through three successive rounds of screening at densities which yielded isolated plaques. Approximately 300000 plaques were screened. Inserts of interest were subcloned into pUC19.

DNA sequence analysis

Nucleotide sequence data were generated with the dideoxy chain terminating method of Sanger *et al.* [25]. The sequence information was obtained directly from the plasmid pNP24 by using the supercoil sequencing method of Chen and Seeburg [5]. To avoid the time-consuming generation of overlapping subclones for sequencing, synthetic deoxynucleotide primers were extensively used. The insert (cloned into pUC19) was sequenced from both ends using the universal (PL) and reverse (NEN) primers to give about 300 bases from each end. Primers homologous to the 3′-end of the newly obtained sequence were then synthesized and used to extend the sequence for another 300 bases on each end. A total of 7 synthetic primers was required to cover the insert on both strands. Sequence reactions using the "mixed" sequence 29-mer required an increase in primer from 5 pmol to 50 pmol. DNA sequences were analyzed with the Beckman Microgenie program (Beckman Instruments, Inc., Palo Alto, CA).

Determination of gene copy number

Genomic DNA from greenhouse grown tomato plants was prepared [11] and digested with Hind III and Bgl II according to the manufacturer's instructions (BRL). DNA was electrophoresed through a 0.8% agarose gel and transferred to MSI paper (Fisher). Prehybridization was done in 5× SSC, 2× Denhardt's, 0.25% SDS, 10 mM EDTA, and 200 μg/ml denatured calf thymus DNA [18]; Hybridization was carried out in the same buffer with ^{32}P-labeled nick translated pNP24 overnight at 65 °C [24]. Washing conditions were 2× SSC, 0.1% SDS at room temperature for 1 h.

Northern hybridization of RNA from tissue culture and whole plants

Polyadenylated RNAs from both tomato tissue cultures and whole plants were prepared by homogenizing the material in 50 mM Tris-HCl pH 8.8, 150 mM NaCl, 5 mM EDTA, and 5% SDS, followed by several phenol and phenol-chloroform-isoamylalcohol extractions [18], and ethanol precipitation. The RNA fraction was enriched for polyadenylated mRNA by chromatography over oligo dT cellulose. Ten μg of the oligo dT selected RNAs were run through a 2% agarose gel containing 3% formaldehyde. The gels were blotted onto MSI paper (Fisher) according to the manufacturer's directions and hybridized with nick translated pNP24. The prehybridization and hybridization mixes were as previously described [23] except that formamide was increased to 50%.

Results

Isolation of the cDNA clone

The polypeptide, NP24, was purified to homogeneity by HPLC and its NH_2-terminal amino acid se-

quence was determined (not shown). A synthetic oligomer was synthesized to correspond to a region of the NH_2-terminal amino acid sequence with limited codon degeneracy. A library of tomato root cDNA cloned into λgt10 was screened with this inosine containing 29 base oligomer (Fig. 1). Twelve positive signals were obtained in the primary screen of 3×10^5 recombinant phage with the synthetic oligomer. Two of these were consistently positive upon retesting. The inserts from both of these recombinant phage were subcloned into pUC19 and sequenced. Initial sequencing of the smaller clone showed that it was identical to a section of the other clone and it was not characterized any further. The sequenced clone will be referred to as pNP24. Comparison of the NH_2-terminal amino acid data (not shown) and the DNA sequence data showed conclusively that this was indeed a cDNA clone from the gene family of the salt stress induced protein. Of the 80 NH_2-terminal amino acids determined by Edman degradation, there were discrepancies with this cDNA clone in 6 amino acids which could arise from a minimal divergence of 7 nucleotides (data not shown).

Sequence of the pNP24 insert

The use of seven synthetic oligonucleotides resulted in the complete sequence of the pNP24 insert from both strands. The nucleotide sequence through the end of the 3′ poly(A)$^+$ stretch is shown in Fig. 2. These 934 nucleotides include an open reading frame of 718 nucleotides coding for a mature protein of 226 amino acids with a molecular weight of 24226. Following the open reading frame is a TGA stop codon, a 3′ AT rich untranslated region (198 nucleotides in length) and a poly (A)$^+$ tail. There is an ATAAAA 15 bases upstream of the poly (A)$^+$ stretch which differs slightly from the common polyadenylation signal sequence of AATAAA which is usually found 6–26 bases upstream from the first A [22, 35]. The primary structure of the polypeptide as deduced from the cDNA nucleotide sequence is also shown in Fig. 2. The Ala (nucleotide 41) corresponds to the first amino acid in the NH_2-terminal sequence of the mature polypeptide (not shown). A partial 5′ signal sequence is also shown. The absence of an initiating ATG indicates that the insert does not contain the complete signal sequence. The mature polypeptide is slightly basic with a ratio of 15:22 acidic to basic residues. It is unusual in the high number (15) of cysteines present and particularly the number (7) of cysteines either followed or preceded by a proline.

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      Gln  Cys  Thr  Gly  Trp  Gly  Lys  Pro  Pro  Asn
A.    CAG TGT ACC GGA TGG GGC AAA CCC CCA AA
B.    GT T/C ACI TGI CCI ACC CCI TT T/C GGI GGI TT
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Fig. 1. The amino acid sequence and the corresponding nucleotide sequence of the synthetic inosine containing oligomer (B) used to identify the cDNA clone for NP24. Inosine was used at six of the degenerate positions and at the other two unknown positions mixed bases were used. The actual nucleotide sequence of this region determined after confirmation of the correct clone is shown in (A). I refers to inosine.

DNA hybridization

Figure 3 illustrates the hybridization of ^{32}P labeled pNP24 to tomato genomic DNA. Several large discrete restriction fragments hybridize to pNP24 when genomic DNA is digested by 2 restriction enzymes that do not cleave within the coding sequence. These results and sequence data from several genomic clones (data not shown) may indicate that NP24 is encoded by one member of a small gene family.

Northern analyses with pNP24

Radiolabeled pNP24 was used as a probe on Northern blots of mRNA from tomato suspension cells grown in control medium and cells grown in medium with 0.34 M NaCl. The strong hybridization signal indicates that pNP24 mRNA is present in the salt stressed cells while faint hybridization to the RNA from cells grown in the control medium indicates the relative lack of pNP24 mRNA in the control cells (Fig. 4).

Poly(A)$^+$ RNA from tomato roots was similarly analyzed for hybridization to pNP24. The level of NP24 mRNA is below the level of detection in young

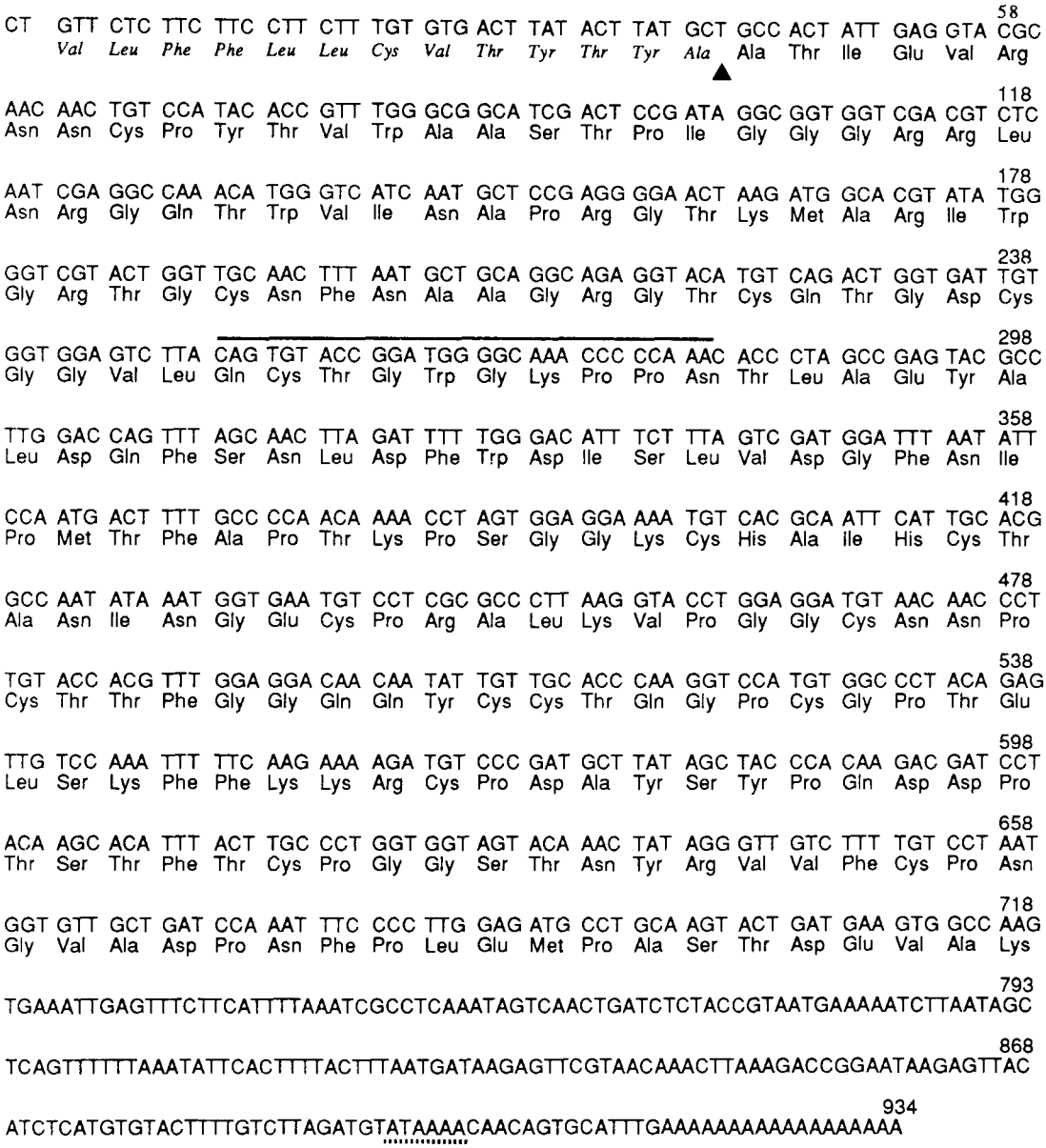

Fig. 2. The sequence of the insert of pNP24 and the deduced amino acid sequence. The overline denotes the nucleotide region corresponding to the oligomer used to select the clone; the arrowhead denotes the site of cleavage of the signal peptide; the dotted line shows a probable poly(A)$^+$ signal sequence.

control plants at day 1 (approximately 2 wks after germination) but the message is evident by day 7 (Fig. 5A). However, by 8 h (day 0.3) after the addition of 0.171 M NaCl the mRNA is evident in the salt treated plants. This mRNA is still present in the salt treated plants 7 and 11 days after the addition of NaCl (Fig. 5A). A corresponding immuno analysis with root samples taken at the same time points (Fig. 5B) indicates that protein accumulation in the roots closely reflects the presence of the mRNA. NP24 protein is present in control roots by day 7 but not at the level present in the salt stressed material.

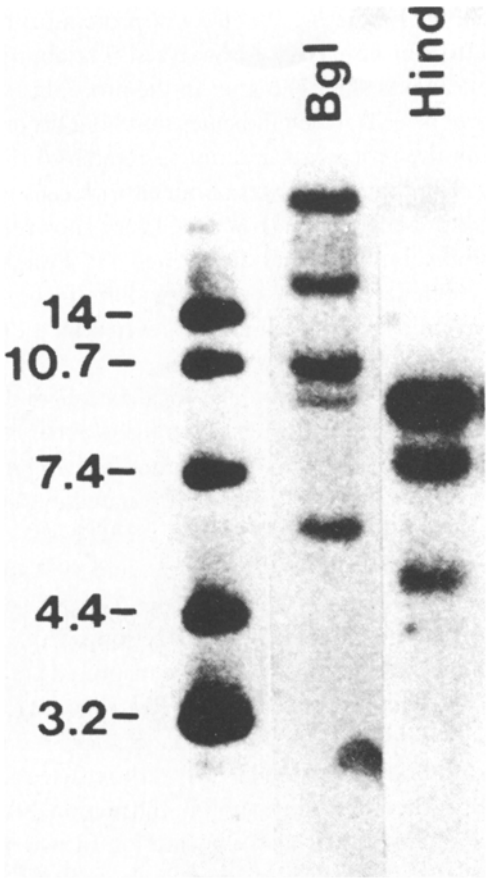

Fig. 3. Southern blot of tomato DNA digested with Hind III and Bgl II probed with pNP24. Molecular weight markers are shown and the size denoted in kilobases.

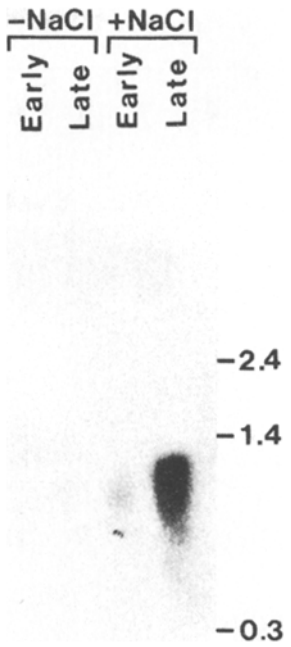

Fig. 4. Norther blot of poly(A)$^+$ RNA from tomato suspension cells at early and late log phase grown in normal medium (−NaCl) and in medium with 0.34 M NaCl (+NaCl) hybridized with radiolabeled pNP24. All lanes were loaded with 10 μg of RNA. Molecular weights (in kilobases) are shown on the right.

The mean fresh weight of the plants at the beginning of the experiment (day 0) averages 4 g. By days 7–11 the mean fresh weight of the stressed plants was 36% of the controls. Control plants remained healthy and vibrant looking and showed no symptoms of water stress. In contrast, the salt treated plants lost turgor upon addition of the salt solution but had recovered by 8 h. The electrical conductivity and the pH of the control solution remained stable throughout day 11.

Comparison of the amino acid sequence to known proteins

Comparison of the deduced protein sequence, by computer analysis with the Microgenie Data Base, revealed an extensive homology with only thaumatin, a group of sweet proteins that occurs in the arils of the tropical plant *Thaumatoccus daniellii* Benth, a west African shrub. At least five different forms of thaumatin (I, II, III [32] and b, c [8, 12]) can be isolated, all of which are almost 100000 times sweeter than sucrose on a molar basis [12]. The most abundant forms are thaumatin I and II, two polypeptides of 207 amino acids each that differ at only 5 positions [17]. The signal sequences of both NP24 and thaumatin are cleaved between an Ala/Ala junction. The homology of NP24 with thaumatin II between the cleavage site (Ala) and amino acid 204 (Pro) where the homology ends is 58% (Fig. 6). It has been speculated that domains II and III (Fig. 6; domain II, amino acids 126–177; domain III, amino acids 56–77) of thaumatin II are responsible for binding to the membrane-bound sweet receptors [7]. The

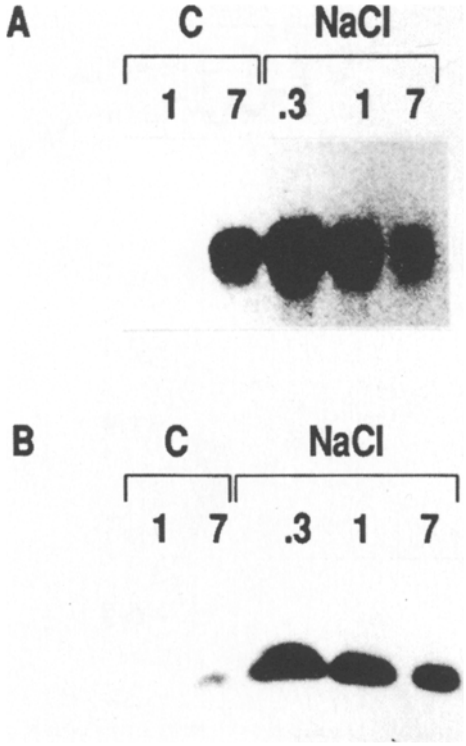

Fig. 5. (A) Northern blot of poly(A)$^+$ RNA from roots of hydroponically grown tomato plants at day 1 (approximately two weeks after germination) and day 7 in control (C) hydro-sol and roots of plants grown in hydro-sol with 0.171 M (NaCl) at 0.3, 1, and 7 days after the addition of NaCl, hybridized with pNP24. All lanes were loaded with 10 μg of RNA. (B) Accumulation of NP24 protein at the same time points described above as determined by immuno analysis.

NP24 amino acid sequence is 61% conserved within domain II and 68% conserved within domain III of thaumatin. There are 15 cysteines in thaumatin I and II all of which are involved in disulfide bridges [7]. All 15 positions are also cysteines in NP24. In addition there are 4 cysteine-prolines (5′ – 3′) in thaumatin I and II, an unusual amino acid combination, which are also present in the same positions in NP24.

Localization of the NP24 protein

Immuno analysis was used to determine the relative abundance of NP24 in extracts from cells and protoplasts prepared from tobacco cell suspension culture (Fig. 7, lanes C, P); 50 μg of protein from cells and from protoplasts were analyzed. The abundance of NP24 was slightly higher in the protoplasts relative to the cells which indicates that NP24 is present within the protoplast and not secreted into the cell wall. The same result was obtained with cells grown in the presence of 0.171 M NaCl (not shown). Furthermore, immuno analyses showed NP24 was at undetectable levels in the culture medium of both the control and the salt adapted cells even after a 10-fold concentration (data not shown).

To further investigate the localization of NP24 and, in particular, to demonstrate whether it was localized in the plastids or mitochondria, organelles from lysed protoplasts were fractionated by sucrose density gradient centrifugation. PEP-carboxylase was used as a cytoplasmic marker, and 99% of this enzyme was found in fractions 1 – 3. The peak density of the intact mitochrondrial fraction, as indicated by fumarase activity (74% of the recovered activity) was located at 1.225 g/cm^3 (fraction 31) (not shown). Immuno analysis of the gradient fractions with antibody to purified RuBP carboxylase showed RuBP carboxylase large subunit in fraction 30 (data not shown). This is also the fraction in which the chlorophyll was recovered, which indicates the majority of the plastids were intact, and located at a peak density of 1.224 g/cm^3.

The distribution of NP24, as determined by immuno analysis of the gradient fractions, is shown in Fig. 7 (lanes 1 – 10). The distribution of NP24 mirrors the distribution of PEP-carboxylase, and no NP24 was detectable in any other gradient fraction including those containing the plastids and the mitochondria.

Discussion

In this report we describe the isolation and characterization of a cDNA clone from tomato which encodes for the mature peptide of a salt (NaCl) induced protein. Previously, we have studied this protein primarily by immuno analyses [15]. The cDNA clone was isolated from a tomato root cDNA library by using a synthetic inosine containing oligonucleotide probe. The use of inosine at several

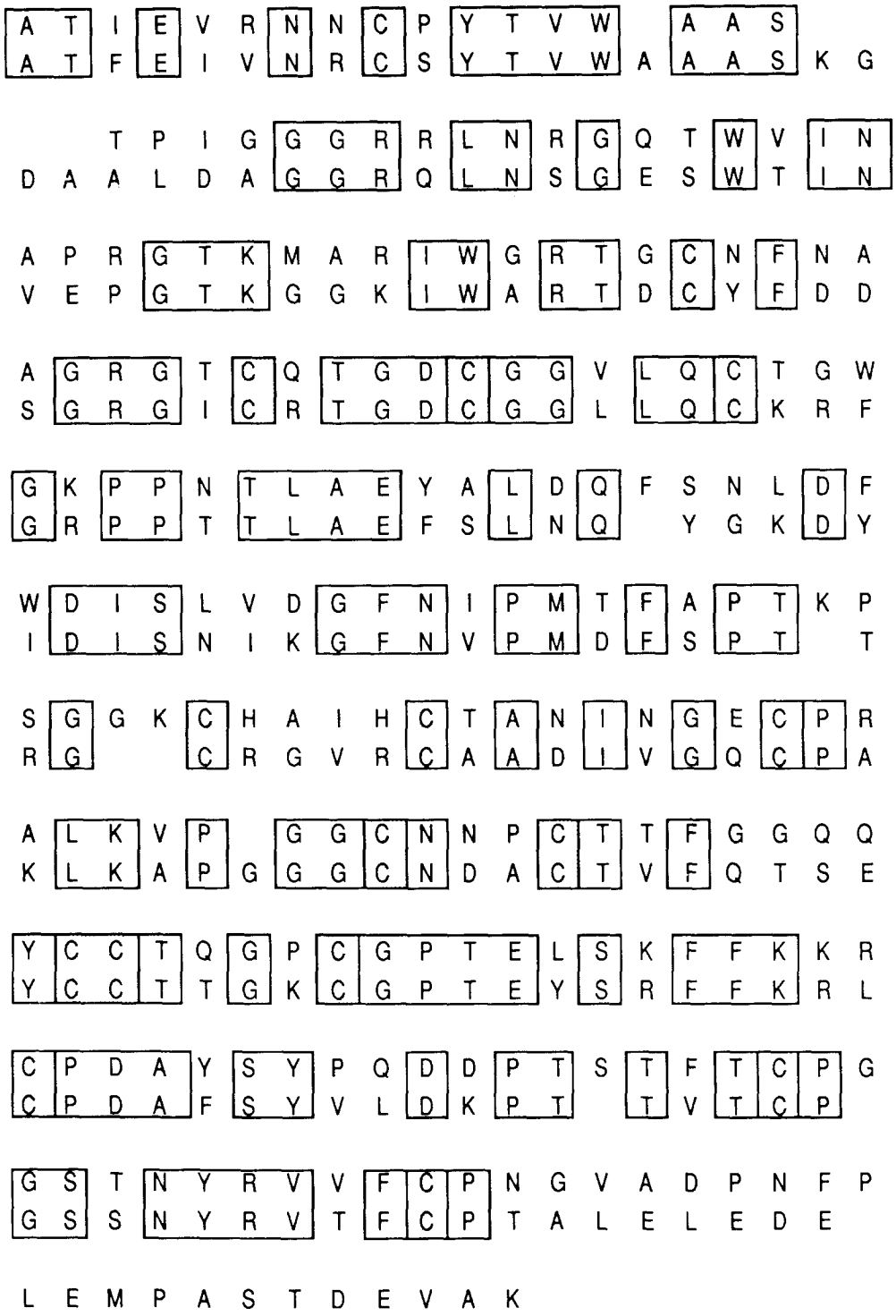

Fig. 6. Comparison of NP24 amino acid sequence (top line) with thaumatin II (bottom line). The homologous regions are boxed. All cysteines are shaded.

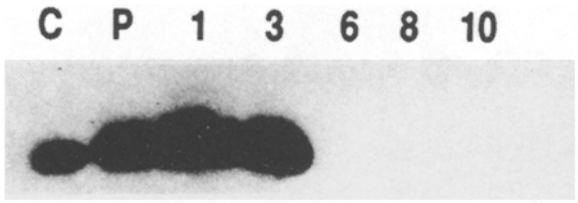

Fig. 7. Presence of NP24 in intact suspension cells (C), protoplasts (P), and gradient fractions (1, 3, 6, 8, 10) as determined by immuno analysis. The remainder of the fractions (through 36) are not shown because no NP24 was apparent in these fractions. Lanes (C) and (P) were loaded with equal protein (50 μg); lanes of the gradient were loaded with equal volume.

of the degenerate positions reduced the need for using mixed bases so there were fewer distinct sequences and a higher proportion of the oligomer could hybridize to the correct clone. The authenticity of the clone was established by comparing the sequence with the 80 NH_2-terminal amino acids from the purified protein. The predicted amino acid sequence derived from the cDNA clone reveals a basic protein of 226 amino acids with a molecular weight of 24226 for the mature polypeptide. The native molecular weight of the protein has not yet been determined. The peptide has an unusually high number of cysteines. The open reading frame 5′ to the NH_2-terminal amino acid of the mature polypeptide presumably codes for a signal peptide. Of the twelve deduced amino acids in the signal peptide 9 are hydrophobic, a characteristic of a signal sequence [20]. The absence of an initiating ATG 5′ to the signal peptide indicates that pNP24 does not contain the complete signal sequence. It is unknown at this time if NP24 is transported into the lumen of the endoplasmic reticulum or through another membrane. Our experiments have demonstrated that it is not transported out of the cell into the medium.

In salt stressed suspension cells, accumulation and relative synthesis of NP24 are low in the early stages of culture and much higher in the log stage of culture. A similar pattern can be seen for control suspension cells, but the total NP24 accumulation is many-fold lower [15]. Northern analyses of NP24 mRNA levels showed that its expression parallels that of the protein.

The mRNA for NP24, although a normal constituent of healthy and vigorously growing tomato plants, is not evident in younger plants. However, it is induced very quickly (within 8 h) after the addition of a salt stress of 0.171 M NaCl. Clearly, the regulation of NP24 is dependent both on osmotic stress and plant development and further investigation is necessary to understand the physiological signals which lead to the accumulation of this protein.

A variety of experimental approaches can be utilized to help determine the function of NP24. Subcellular fractionation coupled with immuno analyses were utilized to gain a preliminary understanding of the subcellular distribution of NP24 within the cell. This very abundant protein is not found in the cell wall, mitochondria, or plastids but is localized in the cytoplasm or within the soluble fractions of other cytoplasmic organelles (e.g. vacuoles, nucleus, microbodies). Additional experiments show NP24 is a soluble protein and is not associated with any of the cellular membranes. These results indicate that NP24 is not a membrane protein involved in altered transport as the osmotic potential of the environment increases. Further experiments will help to determine if it has any involvement, for example, in the balance of the cell's water potential by the vacuole, or whether it is a cytoplasmic structural protein. An elucidation of the function will have to take into account that NP24 is a normal root protein of developing plants but is produced at a much higher concentration when the plants are salt stressed.

Homology is evident between thaumatin proteins and NP24 but the functional relationship is unknown at this time because a function has not been defined for either protein. It is not intrinsically obvious to us what an aril protein from a tropical shrub and a salt induced tomato protein have in common that would enforce the conservation of their sequences. Apparent primary structural homologies between NP24 and thaumatins I and II and the complete conservation of all cysteine residues between thaumatins and NP24 strongly suggest that NP24 may also possess the unique tertiary structure of thaumatins, especially the "sweet" domains of thaumatins.

A thaumatin-like PR (pathogenesis-related) protein in tobacco which is induced with TMV infection

has recently been reported [6, 14]. We have purified the tobacco protein homologous to the tomato NP24 and sequenced the NH_2-terminal end, but this sequence is identical to the tomato NP24 NH_2-terminal and distinct from the PR thaumatin-like protein (unpublished data). We have not determined if NP24 is induced with viral infection. The tobacco thaumatin-like protein is excreted into the intercellular space of the leaf. In contrast, thaumatin is not excreted from the cells but accumulates in vesicle-like organelles [8]. A comparison of the partial signal sequence of pNP24 with the signal sequences of both thaumatin and the tobacco thaumatin-like proteins [14] revealed that the NP24 sequence is more similar to the thaumatin sequence (unpublished data). Although our experiments showed that NP24 was not transported out of the cell, the presence of a signal sequence indicates that NP24 is transported through a membrane. The higher homology with the thaumatin signal peptide together with our localization data indicates that we should consider transport into an intercellular organelle such as the vacuole or microbodies.

Note added in proof

After this manuscript had been submitted for publication, an article by Singh *et al.* appeared (Plant Phys. 85, 529–539; 1987) which utilized *in situ* immunochemical techniques and showed that the tobacco protein homologous to NP24 is concentrated within the vacuoles of suspension cells. This agrees with the localization data presented here.

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