Evidence for an Atrial Natriuretic Peptide–Like Gene in Plants

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The presence of a gene found in the animal kingdom expressing a peptide hormonal system in plants has never been demonstrated. However, there is at least one potential hormonal system in plants (i.e., the atrial natriuretic peptide–like hormonal system) based upon high-performance gel permeation chromatography and radioimmunoassay evidence. In plants, atrial natriuretic–like peptides enhance the flow of water up stems to leaves and flowers. The present investigation was designed to determine within plants the presence of the atrial natriuretic peptide (*ANP*) gene as defined by Southern blot hydridization, indicating the presence of the *ANP* gene sequence, and by Northern blots assessing the ability of this gene to express ANP prohormone mRNA. Southern blots of English ivy (*Hedra helix*) genomic DNA revealed that the *ANP* gene sequence was present in its roots, stems, and leaves. Northern blot analysis of total plant RNA isolated from leaves, roots, and stems of *Hedra helix* revealed a single 0.85-kilobase prohormone *ANP* transcript in stems similar to that detected in rat heart. Semiquantitative analysis suggested that *ANP* gene expression was less in English ivy compared with that of rat heart atria but similar to the amount found in extra atrial rat tissues when corrected for total RNA when quantitated by 2D scanning. The demonstration of the *ANP* gene sequences and expression of the ANP-like gene in plants suggests that plants and animals may have evolved much more similarly than previously thought.

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The presence of a gene found in the animal kingdom expressing a peptide hormonal system in plants has never been demonstrated. However, there is at least one potential peptide hormonal system in plants (i.e., the atrial natriuretic peptide hormonal system) (1, 2). The atrial natriuretic peptide hormonal system consists of four peptide hormones originating from a 126 amino acid (a.a.) atrial natriuretic peptide (ANP) prohormone, synthesized mainly within the heart of vertebrate and invertebrate animals (3–5) (Fig. 1). These four peptide hormones consisting of amino acids 1–30, 31–67, 79–98, and 99–126 of the ANP prohormone have been tentatively named long-acting natriuretic peptide, vessel dilator, kaliuretic peptide, and atrial natriuretic peptide, respectively, for their most prominent effect(s) in humans (6–8) and laboratory rats (9).

There is radioimmunoassay and high-performance gel permeation chromatography evidence that atrial natriuretic–like peptides are present within plants (1, 2). Atrial natriuretic peptides (Fig. 1) enhance the flow of water up stems, a process that is at least partially due to increasing the rate of transpiration (i.e., loss of water from the leaves) (2) *via* opening the stomatal pores in leaves (10). Long-acting natriuretic peptide, vessel dilator, and ANP-like peptides are present in the roots, stems, leaves, and flowers of the more highly developed plants (*Tracheophyta*) (2) and trees (i.e., conifers (11)). These peptides are also present in *Bryophyta* (plants without vascular tissue or roots) and even in *Euglena*, a single-cell, flagellated, chlorophyll-containing plant without leaves, stems, or roots (2). High-performance gel permeation chromatography revealed that long-acting natriuretic peptide, vessel dilator, and ANP-like peptides extracted from plants are very similar to their respective pure synthetic human sequences, with elution profiles and molecular weights of the plant extracts duplicating those of the pure synthetic peptides (2). ANP-like peptide immunoaffinity purified from English ivy (*Hedra helix*) and from rose (*Rosa damascena*) leaf extracts induces opening of the stomata of *Tradescantia sp.* in a concentration-dependent manner (12) similar to that observed using rat ANP to open *Tradescantia sp.* stomata allowing water to transpire out of the plant (10). The present investigation was designed to i) determine whether the gene for the atrial natriuretic peptide prohormone (from which the above atrial peptides are derived) is present in plants as defined by Southern blot hy-

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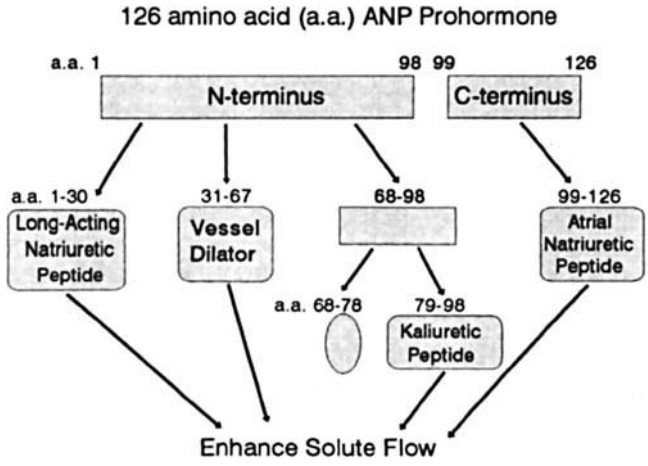

Figure 1. Origination of long-acting natriuretic peptide, vessel dilator, kaliuretic peptide, and atrial natriuretic peptide from the 126 amino acid atrial natriuretic peptide hormone. Long-acting natriuretic peptide consisting of amino acids (a.a.) 1–30, vessel dilator (a.a. 31–67), and kaliuretic peptide (a.a. 79–98) originate from the N-terminus of this prohormone and produce a diuresis in animals (8) while enhancing solute flow in plants (2). Atrial natriuretic peptide (ANP; a.a. 99–126) is the C-terminus of the prohormone.

bridization (indicating the presence of the *ANP* gene sequence) and by the ability to express measured ANP prohormone mRNA (Northern blots); and ii) if present, determine the location of this gene within plants.

Materials and Methods

Genomic DNA Extraction. One hundred milligrams of fresh mature (greater than 2 weeks old) leaves, roots, and stems, respectively, of English ivy (*Hedra helix*) were placed immediately in liquid nitrogen and ground to their respective powders with a mortar and pestle. These plant samples had genomic DNA extracted using a plant Geno-DNA Template (Geno Technology, Inc., St. Louis, MO). The extraction method was as follows: The cell walls were disrupted and solubilized in Template Extraction Buffer for 20 min at room temperature followed by chloroform extraction and centrifugation at 4000 rpm for 10 min. The resulting supernatant was washed with 70% ethanol, and the DNA bound to resin centrifuged at 4000 rpm for 5 min to form a pellet and then washed three times with 80% ethanol. To this pellet, 1 μl RNAase and 50 μl hot (50°-60°C) TE buffer [10 m*M* Tris-HCl (p.H. 8.0), 1 m*M* EDTA] were added, and the mixture was incubated for 10 min at room temperature. After high-speed centrifugation (Eppendorf 5414 High Speed Centrifuge, Hamburg, Germany) for 5 min, the supernatant contained the genomic DNA. The DNA obtained by this technique constantly gave a 260/280 absorbance ratio of 1.6–2.0 indicating high quality intact DNA.

Southern Blot Analysis. Plant genomic DNA was digested with two type II restriction endonucleases (i.e., *Pst* I that recognizes the sequence 5′ . . . C TGCA G . . . 3′ 3′ . . . G ACGT C . . . 5′ and *Taq* I that recognizes the sequence 5′. . . T CG A . . . 3′ 3′. . . A GC T . . . 5′). Both of these restriction enzymes were obtained from Promega Corporation (Madison, WI). Digestion was allowed to proceed overnight in a water bath at these restriction enzyme optimal cutting temperatures of 37°C and 65°C, respectively. Whole-plant genomic DNA and the products of *Pst* I and *Taq* I digestion were separated in 1.0% agarose gels and blotted to nylon membranes (Hybond N^+, Amersham Life Science Inc., Arlington Heights, IL). These membranes were then baked at 80°C for 2 hr. Prehybridization was for 1 hr at 45°C in Hybrisol I (Oncor, Gaithersburg, MD). For detection of ANP prohormone DNA, membranes were hybridized overnight at 42°C in fresh Hybrisol I containing 10^6 cpm/ml of rat ANP complementary cDNA probe. A [α^{32}P] deoxycytidine triphosphate (ICN Pharmaceuticals Inc., Irvine, CA) labeled full-length rat ANP cDNA probe (≈850 base-pair EcoRI/Hind III fragment) was labeled by random priming (Promega Prime-A-Gene, Madison, WI) as previously described (13, 14). The original full-length rat ANP cDNA in the pUC-9 plasmid was a generous gift from Dr. David G. Gardner (15).

After the above overnight hybridization, the membranes were washed for 15 min at room temperature with a solution containing 0.1% sodium dodecyl sulfate (SDS) and 2 × SSC (standard saline citrate), and then twice with 0.1% SDS and 0.5 × SSC for 15 min each at room temperature for a final stringency. Blots were exposed to Kodak X-Omat AR film (Eastman Kodak Corporation, Rochester, NY) for 2 days at −80°C. The base pair (bp) DNA ladder in multiples of 100 bp from 100 to 1500 with an additional fragment at 2072 used in the Southern blot studies was from Gibco BRL Life Technologies (Gaithersburg, MD).

Plant Total RNA. Four grams of roots, leaves, and stems, respectively, of English ivy were placed immediately in liquid N_2 followed by pulverization with a mortar and pestle. The resulting powder was resuspended in 2 ml of chilled solution (pH 7.0) containing 4 *M* guanidinium thiocyanate, 25 m*M* citric acid, and 0.5% N-lauroylsarcosine Na salt and homogenized with a Polytron homogenizer for 15–30 sec. In a series of experiments we have found that 4 g of plant tissue is necessary to isolate 40 μg of total RNA and that 40 μg of total plant RNA is the amount that is necessary to demonstrate ANP prohormone gene expression within plants.

Northern Blot Analysis. Total RNA was isolated from stems, roots, and leaves of *Hedra helix* with RNA- zol B (Tel.:Test, Inc.) as previously described (13, 14, 16, 17). Total RNA (40 μg of plant tissues and 5 μg of rat atria) was separated by electrophoresis on formaldehyde 1.5% agarose gels and transferred to nylon membranes (Hybond N^+, Amersham Life Science, Inc., Arlington Heights, IL). These membranes were then baked at 80°C for 2 hr. Prehybridization was for 1 hr at 45°C in Hybrisol I (Oncor, Gaithersburg, MD). For detection of ANP prohormone mRNA, membranes were hybridized overnight at 42°C in fresh Hybrisol I containing 10^6 cpm/ml of rat ANP complementary (c)DNA probe. A [α-^{32}P] deoxycytidine triphosphate (ICN

Pharmaceuticals Inc., Irvine, CA) labeled full-length rat ANP cDNA probe (≈850 base pair EcoRI/Hind III fragment) was labeled by random priming (Promega Prime-A-Gene, Madison, WI) as previously described (13, 14) The original full-length rat ANP cDNA in the pUC-9 plasmid was a generous gift from Dr. David G. Gardner (15). The integrity of total RNA in the plant and animal extracts was monitored by ethidium bromide staining of Northern blot gels and visualization of ribosomal (r)RNA bands within the gels. For semiquantitative analysis the 18S rat and 18S plant ribosomal RNA were quantitated by 2D scanning, and the bit map was analyzed by Scan Analysis software (Biosoft, Ferguson, MO).

Autoradiographs. Autoradiographs were obtained by exposing the nylon membranes to Kodak X-Omat AR film (Eastman Kodak Corporation, Rochester, NY) for 2 weeks or longer at −70°C with an intensifying screen. Autoradiographs were scanned with LKB 222–020 ultrascan SL laser densitometer (LKB Instruments Inc., Gaithsburg, MD).

Statistical Analysis. All data obtained in this investigation are presented as the means ± SD. Statistical analysis of the differences in measurements between the respective plant tissues and between plant and animal tissues were evaluated by repeated analysis of variance (ANOVA). To be considered statistically significant, we required a probability value to be less than 0.05 ($P < 0.05$, 95% confidence limits).

Results

Southern blot analysis indicated the presence of the ANP-like gene sequence in roots, stems, and leaves of English ivy (*Hedra helix*) (Fig. 2). The intact *ANP* genomic DNA appeared as a band near the 2070 base-pair marker similar to the known base pairs of *ANP* genomic DNA within vertebrates of 2207 bp (18). The *Pst* I restriction endonuclease treatment revealed a band similar to that of intact *ANP* genomic DNA at 2070bp, whereas with *Taq* I all of the intact *ANP* genomic DNA was digested (Fig. 2). Use of restriction endonuleases thus suggested that more of the sequences recognized by *Taq* I (i.e., 5′ . . . T CG A. . .3′ 3′ . . . A GC T . . . 5′) are present in plant *ANP* genomic DNA than the sequence (5′ . . . C TGCA G . . . 3′ 3′ . . . G ACGT C . . . 5′) that is recognized by *Pst* I.

Northern blot analysis using RNA isolated from *Hedra helix* revealed that ANP prohormone mRNA was expressed at detectable levels within the stems but not in the roots or leaves of *Hedra helix* (Fig. 3). Ethidium bromide staining of the Northern blot gels revealed the integrity of the total RNA in plant and animal extracts (Fig. 4). Semiquantitative analysis of 18S rat and 18S plant ribosomal RNA quantitated by 2D scanning suggested that the amount of ANP prohormone mRNA within *Hedra helix* was ≈10-fold less than that found in the atria of the mammalian heart. However, the amount of steady-state ANP prohormone mRNA found in English ivy was very similar to the amount found in extra-atrial tissues in vertebrate (14, 16) and invertebrate animals (13). Thus, the amount of steady-state ANP prohormone mRNA was similar in the English ivy to the amount found in the ventricle of the heart and the gastrointestinal tract and higher than that found in the kidney of the rat (11, 13). This semiquantitative analysis of the amount of ANP prohormone mRNA in plants is an estimate of the amount of ANP prohormone RNA since the exact

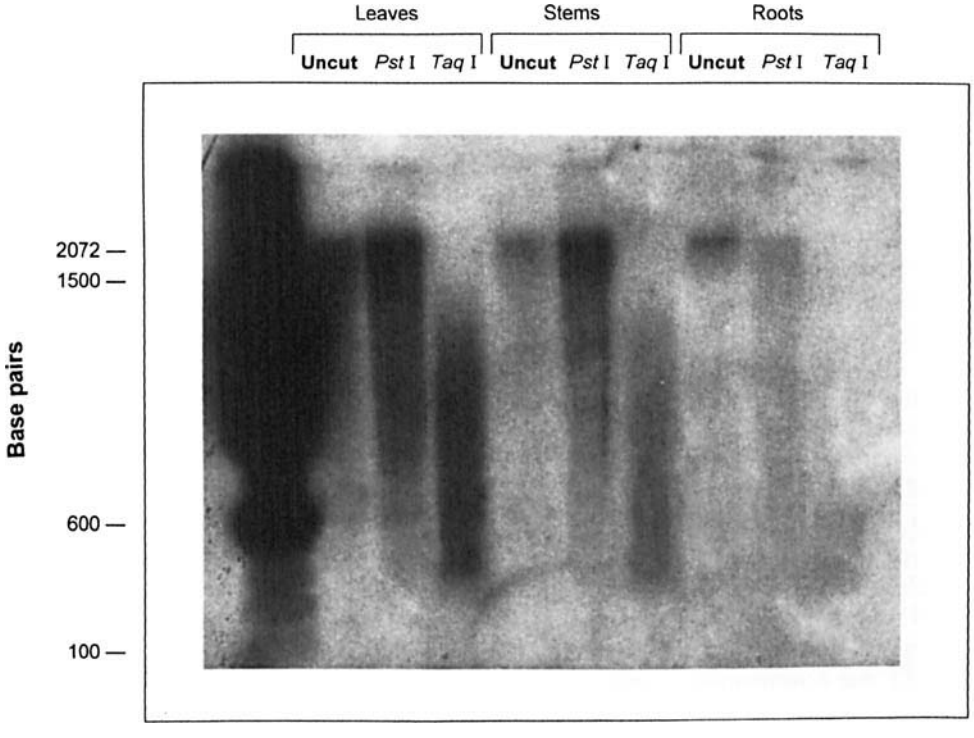

Figure 2. Southern blot analysis of English ivy leaves, stems, roots to detect atrial natriuretic peptide (ANP) genomic DNA and this genomic DNA after digestion with the restriction endonucleases Pst I and Taq I. (Lane 1) DNA ladder (Gibco BRL, Life Technologies, Gaithersburg, MD) in multiples of 100 between 100 and 1500 plus an additional fragment at 2072 base pairs (bp). (Lanes 2–4) English ivy leaves: (Lane 2) uncut ANP genomic DNA; (Lane 3) Pst I; and (Lane 4) *Taq* I-digested DNA. Lanes 5–7 are evaluation of stems from English ivy: (Lane 5) uncut ANP genomic DNA; (Lane 6) *Pst* I; and (Lane 7) *Taq* I-digested ANP genomic DNA. Lanes 8–10 are ANP genomic DNA from English ivy roots: (Lane 8) uncut ANP genomic DNA; (Lane 9) *Pst* 1; and (Lane 10) *Taq* I-digested ANP genomic DNA.

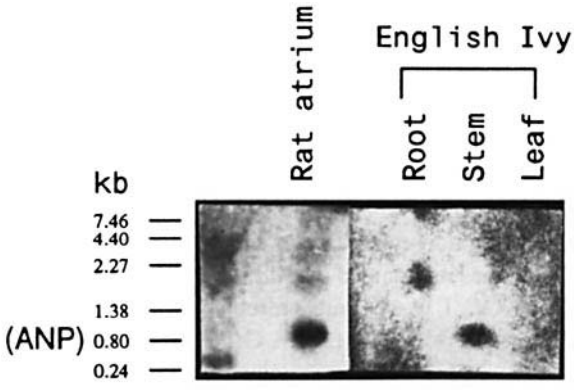

Figure 3. Atrial natriuretic peptide (ANP) – like prohormone gene expression in stems, but not in roots and leaves, of the English ivy compared with ANP-like prohormone gene expression in the atria of rat heart. This evaluation used 5 μg of atria and 40 μg of leaves, stems, and roots for total RNA. ANP prohormone steady-state RNA was 11.67 less ($P < 0.05$) in stems than in rat atria when corrected to total RNA and quantitated by 2D scanning and analyzed by analysis of variance. Each of these evaluations were repeated five times for the plants and animals, respectively. This semiquantitative analysis of the amount of ANP prohormone in mRNA in plants is an estimate of the amount of ANP prohormone messenger RNA since the exact amount of ANP prohormone messenger RNA in plants cannot be determined until the actual sequence of the plant ANP genome is known.

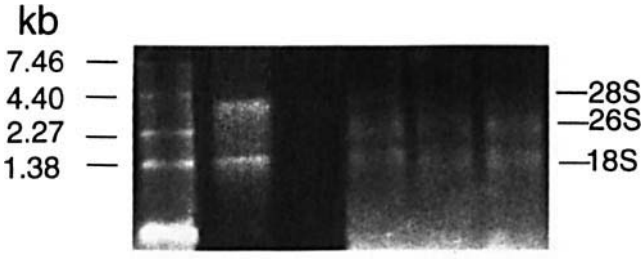

Figure 4. Ethidium bromide staining of Northern blot gels reveals integrity of total RNA in plant (English ivy) and animal (rat) extracts with visualization of ribosomal (r)RNA bands within the gels. (Lane 1) RNA ladder; (Lane 2) rat atria; (Lane 3) blank; (Lane 4) root; (Lane 5) stem; and (Lane 6) leaf.

amount of ANP mRNA cannot be determined until the actual sequence of the plant ANP genome is known.

Discussion

Southern blot analysis suggested that the ANP-like gene sequence is present in roots, stems, and leaves (i.e., in all plant tissues examined) of English ivy. The finding of the ANP-like gene sequence in each of the plant tissues is what one would expect if this gene were important for the movement of solutes and water within plants. The plant ANP-like genomic DNA of ≈2072 base pairs is similar to ANP genomic DNA found in vertebrate animals where ANP genomic DNA consists of 2207 bp in sheep (18). Use of restriction enzymes in this investigation revealed that *Taq* I restriction enzyme results in more ANP-like genomic DNA being digested suggesting that more of the sequence of CG A . . . 3′ 3′. . . AGC is present in plant ANP-like genomic DNA than the sequence G . . . 3′ 3′ . . . G′ that is recognized by the *Pst* I restriction enzyme.

Steady state ANP prohormone mRNA found within the stems of English ivy suggests that the ANP prohormone gene is being expressed within the stems of plants. The localization of ANP-like gene expression within the stems is where one would want a gene for peptide hormones to be if one were trying to design an ideal system to move water and salt(s) actively within plants. One function of atrial natriuretic–like peptides in plants is to enhance the flow of water up to their leaves and flowers (2) and if the stem of plants can synthesize these peptides, as they were demonstrated to be capable of doing in the present investigation, then these peptides would be available to help move water up to leaves and flowers. With respect to localization of ANP prohormone mRNA in the present investigation and the solute flow in plants, it is important to appreciate that gene products may be translocated to their sites of action *via* the conductive tissues in the plant. Thus, even if the stems are the main site of synthesis of the atrial natriuretic–like peptides, these peptides may have distant sites of action such as the stomata of leaves where they open the stomata to allow water to transpire out of the plant (2, 12).

The ability of the stem to produce atrial natriuretic–like peptides would appear to have been very important in the evolution of taller plants (i.e., trees) since it has been known for some time that trees could not have evolved to higher than 30 feet without some active process to enhance upward water movement as water could not have been pulled upward farther than 30 feet by the transpiration-osmosis mechanical mechanism of water flow (19). Thus, an active process mediated by a substance with water-flow properties would have to be present to allow plants to grow to heights greater than 30 feet (19). It has recently been demonstrated that trees as well as other plants contain the atrial natriuretic peptide–like hormonal system and that the ANP-like prohormone is present in trees as defined by high-performance gel permeation chromatography (11). The present investigation demonstrating that atrial natriuretic–like peptides are synthesized within the stem provides a mechanism for the flow of water in taller plants since these water flow–producing peptides would be immediately available from their storage form (i.e., the ANP-like prohormone) no matter how high the stem or trunk of a tree was to actively increase the flow of water and nutrients.

When one examines leaves of broad-leafed plants by HP-GPC followed by specific assays to the four portions of the ANP prohormone, what one discovers is that the four atrial natriuretic–like peptides are present within leaves of these plants such as the English ivy (2). This suggests that these peptides have been proteolytically cleaved from the ANP-like prohormone into their respective peptide hormones (2). It is important to note in this regard that peptides consisting of amino acids 1–30 (i.e., long-acting natriuretic peptide), 31–67 (vessel dilator), and 79–98 (kaliuretic peptide) of the ANP prohormone can be formed if a protease(s) would cleave the aspartic acid bonds in the ANP prohor-

mone. Plants contain such a proteolytic enzyme (i.e., papain 3E) which attacks and cleaves the aspartic acid bonds avidly (20) and, thus, could form proANPs 1–30, 31–67, and 79–98-like peptides found within plants (2).

To detect ANP prohormone messenger RNA in plants with a rat cDNA probe suggests that the ANP prohormone and the peptide hormones derived from this prohormone are similar in plants and animals. The 126 amino acid sequence in the ANP prohormone has been found to be highly conserved in all animal species studied (21–25). Thus, in rat and mouse, 93.8% of the amino acids are exactly the same and in the same sequence in their respective ANP prohormones (21–25). Comparison of the amino acid sequence of the human ANP prohormone with this prohormone in the dog reveals 89% of the 126 amino acids are the same and in the same sequence (22–26) whereas comparison of rat and human ANP prohormones reveals an 85% homology (21–25). Further, the ability to detect within invertebrates such as the oyster N-terminal and C-terminal ANF prohormone peptides and their close parallelism to standard curves for radioimmunoassays devised to the human amino acid sequence of the ANP prohormone (27) as well as their similar elution with high-performance gel permeation chromatography (13) suggests that the amino acid sequence ANP prohormone within invertebrates is similar to that found in vertebrates and similar to that of plants. These data plus the present Southern and Northern blot data of ANP-like gene expression would suggest that plants and animals evolved much more similarly than previously thought.

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