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Expansin-Like Molecules: Novel Functions Derived from Common Domains

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Abstract. An *Arabidopsis thaliana* transcript (*AtPNP-A*) encoding an immunoreactant plant natriuretic peptide (irPNP) analog was identified and isolated. The encoded protein shows similarity to CjBAp12, a functionally undefined protein from citrus that is induced in response to blight infection. CjBAp12 shows significant sequence identity to domains found in the cell wall loosening expansins but has tested negative for cell wall loosening activity. We have thus undertaken to establish the evolutionary and functional relationships of irPNP-like molecules within the superfamily of expansins, pollen allergens, and distantly related molecules such as endoglucanases. We show that irPNP-like molecules are related to expansins and fall in two groups; one includes CjBAp12 and the other AtPNP-A. Members of both groups share distinct sequence motifs (K[VI]VD and [LM]SxxAFxxI) but do not contain the tryptophan and tyrosine rich C-terminal putative polysaccharide-binding domain typical of expansins or bacterial cellulases and hemicellulases. We argue that both irPNP-like molecules and expansin have evolved from primitive/ancestral glucanase-like molecules that hydrolysed the cell wall. Importantly, we have previously demonstrated that irPNPs act on protoplasts, that is plant cells without cell walls as well as microsomes, indicating that these novel proteins specifically interact with the plasma membrane. It follows that the cell wall cannot be an obligatory substrate for irPNPs. Thus, both irPNP function and domain structure point to these molecules having a systemic role in H_2O and solute homeostasis.

Key words: Cell wall — Glucans — Pollen allergens — Expansins — Natriuretic peptides

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Introduction

Plant cells are contained by a rigid, exoskeleton-like structure, the cell wall. The major components of the wall are cellulose microfibrils consisting of an unbranched β-1,4-glucan polymer, hemicelluloses (branched glycans) that bind to cellulose to form a matrix, and acidic polysaccharides (pectins) that form ionic gels. Developmental processes (e.g. cell elongation) and any rapid physiological responses (e.g. stomatal movement) that require changes in cell shape thus necessitate temporary loosening of the cell walls. The expansins are proteins that promote cell wall loosening and extension and recently two families of expansins, α-expansins and β-expansins, have been recognized (for reviews see Cosgrove 1999; Cosgrove 2000a; Cosgrove 2000b). Expansins are functionally defined to induce long-term pH-dependent extension and enhance stress relaxation of plant cell walls. What makes expansin activity particularly remarkable is that the enzymes do not seem to have any apparent hydrolytic activity against the major cell wall constituents (McQueen-Mason et al. 1992; McQueen-Mason and Cosgrove 1995). However, expansins share structural motifs with the putative catalytic sites of

endoglucanases of the family-45 glycosidases, which in turn do not have expansin activity (Cosgrove 1999; Cosgrove 2000b). It would thus appear that the structural similarity between glucanases and expansins reflects an evolutionary relationship between two classes of enzymes that operate differently on a common substrate, the cell wall.

There has long been an interest in allergenic components from pollen and grass pollen, in particular, that induce hay fever and allergic asthma. A group of proteins termed the group-1 allergens were identified as the main causative agents of hay fever and allergic asthma elicited by grass pollen (Knox and Suphioglu 1996). The group-1 allergens are glycoproteins abundantly expressed on the surface of pollen grains and released upon hydration (Knox and Suphioglu 1996). The discovery that expansins bore some sequence similarity with group-1 pollen allergens hinted at a biological role for these allergenic proteins (Shcherban et al. 1995): cell wall loosening may indeed be an advantageous strategy for the pollen tube growing along the stigma and through the style as a prelude to fertilization. Such a role might also explain the ubiquity and abundance of the group I pollen allergens. Subsequent experiments have demonstrated that maize pollen group-I allergens have expansin activity and that this activity is essentially limited to cell walls from grasses (Cosgrove et al. 1997). This type of expansin is termed β-expansin and its preferred substrate is the monocotyledons, and specifically the grass cell wall: the α-expansins occur in monocotyledons and dicotyledons but seem less efficient on grass cell walls (Cosgrove 2000a). However, an intriguing question remains. If the full catalytic activity of β-expansins was limited to monocot cell walls, why would they occur in dicots? Different substrate specificities and biological roles for structurally related molecules from two classes (Monocotyledons and Dicotyledons) provide an opportunity to study the evolution of this family of molecules.

Furthermore, a molecule, CjBAp12 (previously referred to as a blight associated protein, p12), from citrus trees shares significant homology with expansins (Ceccardi et al. 1998). The molecule has a molecular mass of approximately 12 kDa and is thus considerably smaller than the classic expansins (molecular mass of ~25 kDa). The gene *CjBAp12* is expressed in root and stem tissue in response to a challenge from citrus blight. The protein itself accumulates in root, stem, and leaf tissues (Ceccardi et al. 1998), suggesting systemic mobility. However, despite marked sequence similarity with expansins, CjBAp12 has no apparent expansin-like activity (Ceccardi et al. 1998).

We have previously reported the immunoaffinity purification of novel, biologically active proteins from ivy (*Hedera helix*) and potato (*Solanum tuberosum*) with antibodies directed against vertebrate atrial natriuretic peptide (ANP) (Billington et al. 1997; Maryani et al. 2001) and termed them immunoreactant plant natriuretic peptides (irPNPs). Here we identify an *Arabidopsis thaliana* gene and transcript that encodes a plant natriuretic peptide. We investigate the evolutionary relationship between this protein and expansin-like molecules. Finally, these findings are discussed in the light of the increasing body of functional data available on irPNPs.

Materials and Methods

Total RNA from *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg erecta was extracted from tissue ground in liquid N_2 using the hot phenol extraction procedure outlined in Verwoerd et al. (1989). Ground tissue was placed into microfuge tubes containing 0.5 ml NETS buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris pH 7.4, 0.1% SDS) and 0.5 ml H_2O-saturated phenol and incubated at 80°C for 3–5 minutes. Samples were spun at 14 000 rpm for 10 min and supernatant transferred to tubes containing 0.5 ml salt saturated phenol. Tubes were vortexed, spun at 14 000 rpm for 10 min, and the supernatant collected. RNA was phenol extracted with salted phenol followed by a salted phenol/chloroform mix and lastly, chloroform. The RNA was ethanol precipitated and resuspended in 50–100 μl TE buffer.

The RT-PCR was performed using Superscript II (Gibco BRL) according to the manufacturer's instructions. The specific primers were: 5′-AAG AAA ATG ATA AAA ATG GCA G-3′ (forward), 5′-AAG AAT GAA ACT TAC GGT GTG T-3′ (reverse). All reactions were denatured at 94°C for one min and then 35 cycles of amplification were performed (30 s denaturation at 94°C, 30 s annealing at 52°C, and 60 s extension at 72°C), with a final extension at 72°C for ten min. The PCR-isolated gene and RT-PCR isolated cDNA fragments were blunt-ended using T4 DNA Polymerase (Gibco BRL) and cloned into the Sma I restriction site of the plasmid vector pBluescript SK (−) (Stratagene). Sequencing was undertaken using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and analyzed using the facilities at the Australian Genome Research Facility (AGRF).

Sequence alignments and phylogenetic trees were constructed using ClustalX (Thompson et al. 1997) and motif searches were performed against the Prints and Prosite databases (Attwood et al. 1994; Hofman et al. 1999). Protein secondary structures were predicted according to the method of Jones (1999).

Results

We have previously reported the isolation of irPNPs with antibodies directed against the C-terminal of atrial natriuretic peptides (α-hANP) (Billington et al. 1997; Gehring 1999). We have since partially sequenced C- and N-termini of several of these immunoaffinity purified plant natriuretic peptide immunoanalogues (irPNPs) from potato, *Solanum tuberosum* (Maryani et al. 2001), and identified a homologous sequence in *Arabidopsis thaliana* (accession No. AAD08935) that we term AtPNP-A. Figure 1a shows the genomic organization of *AtPNP-A* as predicted from the genomic sequence data. The genomic sequence is 478 bp long and contains one predicted 100 bp long intron. The predicted protein is thus expected to consist of 126 amino acids (approximately 14 kDa). Figure 1b confirms the length of the genomic PCR product and importantly shows a RT-PCR

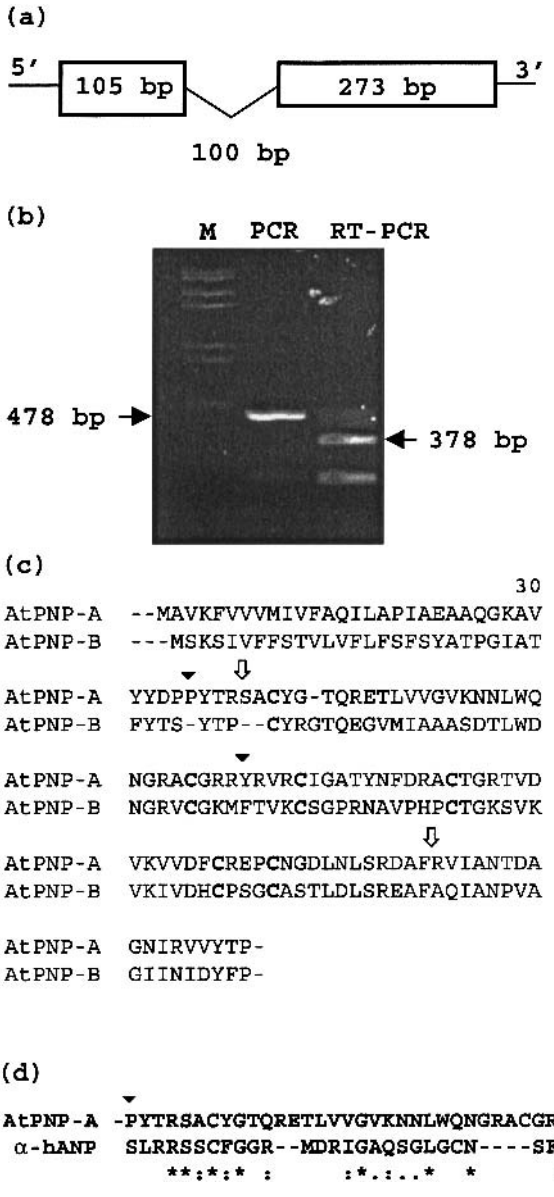

Fig. 1. **(a)** Intron and exon organization of the *AtPNP-A* encoding gene from *Arabidopsis thaliana.* **(b)** PCR and RT-PCR products from *Arabidopsis thaliana* DNA and RNA templates. **(c)** Sequence alignment of AtPNP-A and AtPNP-B from *Arabidopsis thaliana.* The conserved cysteines are in bold **(C),** the solid triangles (▼) delineate the sequence that is aligned with α-hANP in **(d);** The sequence between the open arrows (⇓) has been used to construct the dendrogram in Fig. 3. **(d)** Alignment of AtPNP-A and α-hANP; asterisks (*) identify identical amino acids, colons (:) are conservative amino acid substitutions, and dots (.) are semi-conservative amino acid substitutions.

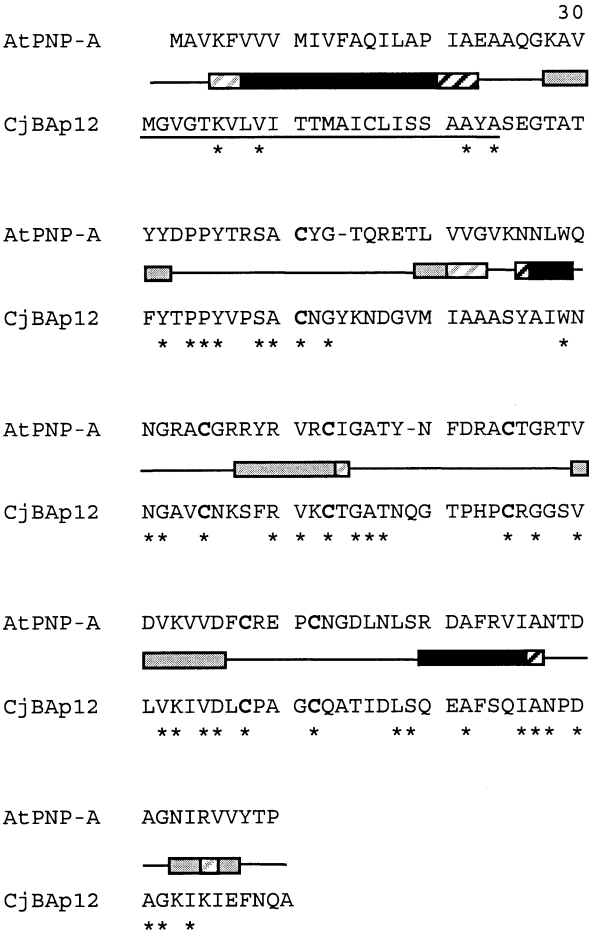

Fig. 2. Sequence comparison and predicted secondary structures of AtPNP-A from *Arabidopsis thaliana* and CjBAp12 from *Citrus jambhiri;* asterisks (*) indicate conserved amino acid residues and the underlined sequence delineates the hydrophobic signal peptide. Black boxes indicate α-helices (■), gray boxes are strands (β-sheets) (▩), and lines (——) represent random coils. The boxes are hashed when the predictions for the two molecules, AtPNP-A and CjBAp12, differ.

product of the predicted length (upper band). The sequence is not annotated in the database and the presence of a RT-PCR product implies that the gene is expressed at the transcription level in unstressed leaves. Figure 1c shows a second sequence from *Arabidopsis thaliana* that shows similarity (37% identity) with AtPNP-A. Hence, we refer to this sequence as AtPNP-B. This sequence is annotated as a blight-associated protein homolog (BAPH, accession no. CAB79756), since it shows a high degree (54% identity) of sequence similarity with CjBAp12 (accession no. AAD03398), a blight-induced protein of unknown function isolated from citrus, *Citrus jambhiri* (Ceccardi et al. 1998).

The cysteine residues in both proteins, AtPNP-A and AtPNP-B (Fig. 1C), are aligned which might suggest a conserved protein folding pattern. While CjBAp12 contains an additional cysteine in its putative signal peptide, all other cysteine residues align with the AtPNP-A and AtPNP-B (Figs. 1c, 2). IrPNPs were first isolated through immunoaffinity purification with an antibody directed against the biologically active C-terminus of human α-ANP (α-hANP). The alignment of AtPNP-A and human ANP (α-hANP, the antigen of the antibody used to isolate the potato homolog) (Fig. 1d) shows considerable similarity between the two molecules, with nine of the 28 amino acids of ANP being conserved and four conservative replacements.

A secondary structure prediction program (Jones 1999) was applied to test for similarities in coil, β-sheet

and helix domains (Fig. 2). The comparison between AtPNP-A and CjBAp12 shows near congruence in predicted secondary structure and might imply common, albeit unknown, functionality.

In order to obtain further information on the extent of this family of small proteins we queried the TIGR Gene Indices (http://www.tigr.org/tdb/tgi.shtml). The result of the search revealed a number of indices from different plant species notably rice (*Oryza sativa*) and soybean (*Glycine max*). An index is defined as a number of (partially overlapping) transcripts that represent either a single gene or possibly two or more genes with identical coding subregions. It is noteworthy that no related transcripts were found in either yeast (*Saccharomyces cerevisiae*) or slime mold (*Dictyostelium discoideum*). How then are these molecules, the irPNP-like proteins and CjBAp12, interrelated and related to other proteins, and can similarities with proteins of known function provide a key to understanding their hitherto unknown function? Homology searches using BLAST (Altshul et al. 1997) identified the expansin family as the only proteins with significant sequence similarity to irPNP-like molecules. The phylogenetic tree in Fig. 3, constructed with subsequences (see Fig. 1c) representing a best guess at the region common to both irPNP-like molecules and expansins, suggests firstly that irPNP-like proteins and CjBAp12 form a distinct group of proteins to the α-expansins and β-expansins. Secondly, AtPNP-B clusters with CjBAp12 in a group that contains both members of the mono- and dicotyledons. AtPNP-A is part of a different subgroup.

IrPNP-like and CjBAp12-like sequences are considerably shorter than the expansin sequences: ~12–14 kDa rather than ~25 kDa (Fig. 4a). Importantly, the sequence alignments reveal that the C-terminus of the expansins is consistently absent in irPNP-like molecules. The C-terminus of the expansins contains several tryptophans (four residues in ~70), which are also in conserved positions and exposed on the surface of the folded protein. This domain was assigned as a putative cellulose binding, and thus cell wall binding region of expansins (Cosgrove 1999). The latter is inferred by similarity to cellulose-binding domains of many microbial cellulases and hemicellulases; these domains have a role in enzyme immobilisation on insoluble carbohydrate substrates, and can confer carbohydrate-binding specificity but are not necessary for catalysis (Linder and Teeri 1997; Linder et al. 1998). Incidentally, fungal family-45 glycosidases (e.g. the β-1,4-endoglucanse from *Trichoderma reesei*) also contain a conserved C-terminal cellulose-binding domain (Saloheimo et al. 1994). The cellulose-binding domain from the fungus shows 38% sequence identity with α-expansins. More importantly the cysteine residues are aligned and two of the tryptophan residues align with tyrosine, another aromatic amino acid. This C-terminal domain of the ancestral family-45 glycosidases (glucanases) and the expansin family is absent in all irPNP-like molecules identified so far. It is noteworthy that expansins have two introns (Cosgrove 1999), the second close to the border of the two functional domains (endoglucanase-like and cellulose/polysaccharide-binding) (Fig. 4a). The second intron and the C-terminal cellulose-binding domain are both absent in the irPNP-like molecules.

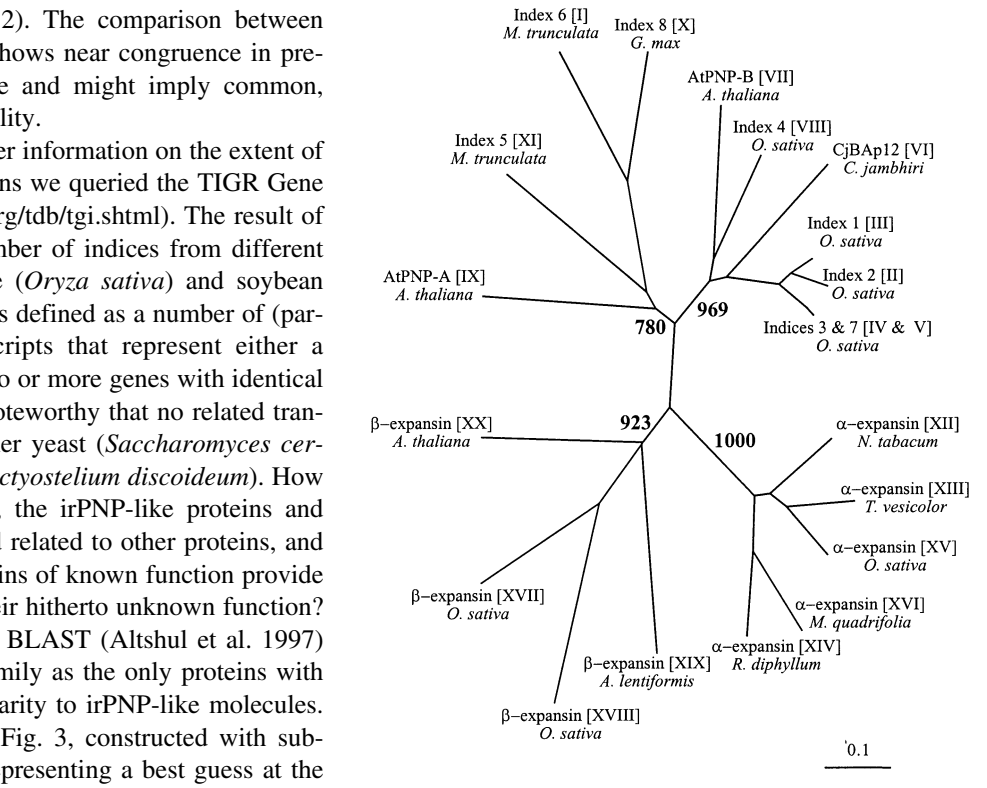

Fig. 3. Phylogenetic tree representing irPNP-like and CjBAp12-like molecules and α-expansins and β-expansins. The bold numbers represent bootstrap values from 1000 replicates. Indices are clustered EST sequences representing transcripts from one gene (or identical copies of one gene). The indices were identified by querying the TIGR database (http://www.tigr.org/tdb/tgi.shtml) with the complete AtPNP-A sequence (AAD08935). Sequence identification: I, TC29780; II, OSM110636; III, OSM11811; IV, OSM11298; V, AU101292; VI, AAD03398; VII, CAB79756; VIII, OSM110634; IX, AAD08935; X, TC64839; XI, BE943215; XII, AAC96081; XIII, T50660; XIV, AAF17571; XV, AAF62180; XVI, AAF17570; XVII, AAF72983; XVIII, AAB61710; XIX, BAB20817.

The sequence alignment (Fig. 4b) reveals a number of interesting features. First, the conservation of the cysteines is remarkable. Of the six cysteine residues in the irPNP-like molecules, the first four (solid arrows) are conserved in the representatives of all sequences. In addition, two glycine residues (solid squares) are highly conserved across all molecules and a third is conserved in most irPNP-like molecules (open square in square brackets). Furthermore, two amino acids, tryptophan and valine (open squares) are present in all irPNP-like molecules. Two highly conserved diagnostic motifs: A and B

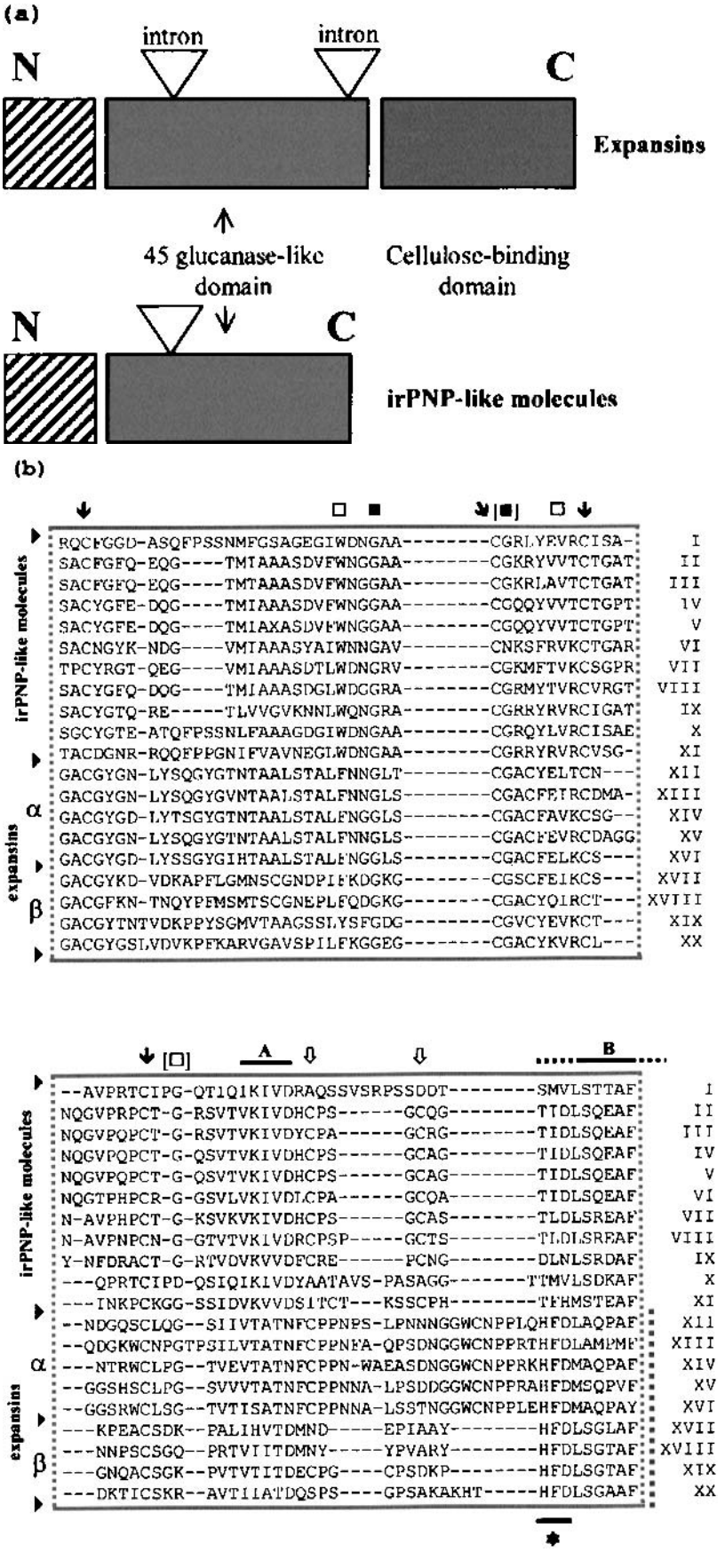

Fig. 4. **(a)** Domain comparisons of expansins and irPNP-like molecules. The open triangles (▽) signify introns and the color code is maintained in **(b). (b)** Alignment of sequences represented in the dendrogram (Fig. 3). Solid arrows (⬇) are cysteines conserved in all molecules but the out-group. The open arrow (⇓) indicates cysteines present in most irPNP-like molecules, but not expansins. Solid squares (■) indicate amino acids other than cysteines conserved across all molecules, solid squares in square brackets ([■]) indicate a high degree of conservation across all molecules. Open squares (□) are conserved amino acids other than cysteines in irPNP-like molecules, open squares in square brackets ([□]) indicate a high degree of conservation within the irPNP-like molecules and α-expansins. The asterisk (*) shows the HFD motif conserved between many expansins and the endoglucanases of the family-45 glycosidases and (A) and (B) delineate two conserved motifs in irPNP-like molecules.

(A: K[VI]VD and B: [LM]SxxAFxxI), have been identified in irPNP-like molecules. The second motif (B) includes the serine and phenylalanine that are conserved across the families. While expansins have only barely detectable (endo-) glucanase activity (Cosgrove 1999), most nevertheless contain a His-Phe-Asp (HFD) motif that is part of the catalytic site of the family-45 endoglucanases. This HFD motif is absent in irPNP-like molecules pointing to a further functional divergence of the group.

IrPNP-like molecules also share similarities with barley wound-induced (Barwin) proteins and it is remarkable that the region of closest homology between the irPNP-like molecules and Barwin proteins comprises both modified versions of the motifs A and B. In Barwin proteins, the K in motif A is replaced by an R (A: K/R[VI]VD) and motif B is modified to LxxxxFxxI. In addition, the glycine (open square) and the cysteine closest to motif A (open arrow) are also conserved between the Barwin proteins and irPNP-like molecules.

Discussion

What are natriuretic peptides and how do irPNPs and in particular AtPNP-A relate to them? Maintenance of water and solute homeostasis is a key requirement for all living systems. In vertebrates water and solute homeostasis is, in part, achieved by natriuretic peptides (NPs), a family of well characterized peptide hormones (for review see Thibault 1999). Several lines of evidence suggest the presence of a structurally related and biologically active natriuretic peptide hormone-like system in plants (for review see Gehring 1999). Most importantly, we have isolated and purified by immunoaffinity chromatography biologically active plant natriuretic peptide immuno-analogs from a number of different species including ivy (Billington et al. 1997) and potato (Maryani et al. 2001). IrPNPs have been shown to promote stomatal opening (Billington et al. 1997), to rapidly and specifically induce transient elevation of the second messenger cGMP (Pharmawati et al. 1998) and to modulate cation fluxes (Pharmawati et al. 1999) in maize (*Zea mays*) root stele tissue. Several irPNP-dependent processes have been observed in experimental systems that did not contain cell walls such as protoplasts or microsomal and plasma membrane vesicles. The processes include in vitro irPNP binding to isolated *Tradescantia multiflora* leaf microsomes (Suwastika et al. 2000), irPNP-dependent modulation of plasma-membrane H^+ gradients in potato leaf tissue vesicles (Maryani et al. 2000), increases of cGMP levels in response to irPNP in potato guard cell protoplasts (Pharmawati et al. 2001) and irPNP-dependent volume changes in protoplasts (Maryani et al. 2001). It does thus follow that irPNPs can act directly on the plasma membrane.

In order to study the molecular nature of these irPNP-like molecules we have isolated and partially sequenced irPNPs from potato (Maryani et al. 2001) and identified and isolated an *Arabidopsis thaliana* transcript (*AtPNP-A*) encoding an irPNP (Fig. 1). As expected, a section of sequence with homology to vertebrate ANP was found within *AtPNP-A* (Fig. 1d), thus linking the protein to the antibody and epitope used for its initial isolation. Sequence similarity of *AtPNP-A* was also observed to a functionally uncharacterized transcript from citrus, CjBAp12. Since CjBAp12 in turn shows significant homology with the expansins, it was decided to undertake a detailed survey of irPNP-like sequences deposited in databases and thus establish the phylogenetic position and domain organization of a potentially novel family of proteins that contain at least one member (*AtPNP-A*) that shares an epitope with ANP. Such an analysis we argued, may help to explain the absence of expansin activity in CjBAp12 and further interpret our results from the functional assays with ANP and irPNPs in cell wall-free systems (Suwastika et al. 2000; Maryani et al. 2000; Pharmawati et al. 2001; Maryani et al. 2001).

The most closely related molecules to irPNPs are expansins. Expansins have extended C-termini when compared to the irPNPs. Expansins are in turn related to glucanases and cellulases and in the case of glucanases and cellulases, the C-termini have been proven to be cell wall binding (Linder and Teeri 1997; Linder et al. 1998) and the same function has been suggested for the expansin C-terminus (Cosgrove 2000). Since expansins, the closest relatives of irPNPs, and the more distantly related glucanases and cellulases contain the C-terminus, it is reasonable to argue that irPNP-like molecules have in fact lost this domain. This conclusion is also in keeping with the fact that the second intron and third exon are absent in irPNP-like molecules. Such a domain loss, we speculate would result in an absence of wall binding and thus increased mobility of the molecule. The concept of increased mobility is supported by the fact that CjBAp12 appears to be a systemically mobile protein (Ceccardi et al. 1998), present, but not synthesized, in leaves. In addition, our in situ localization data in potato also identifies irPNP in conductive tissue (M.M. Maryani, result not shown); the conductive tissue is a highly unlikely place for irPNP synthesis but not for its transport.

These above observations and conclusions in itself do not explain the absence of expansin activity of CjBAp12 since the catalytic activity of expansin resides in the N-terminus. However, irPNP-like molecules also appear to be functionally further diverged from the ancestral endoglucanases since the HFD motif shared by the expansins and the endoglucanases is absent from the irPNP-like proteins (see Figure 4b).

Based on the sequence comparisons we propose that ancestral glucanases have given rise to the expansins and also have been recruited to serve in capacities entirely

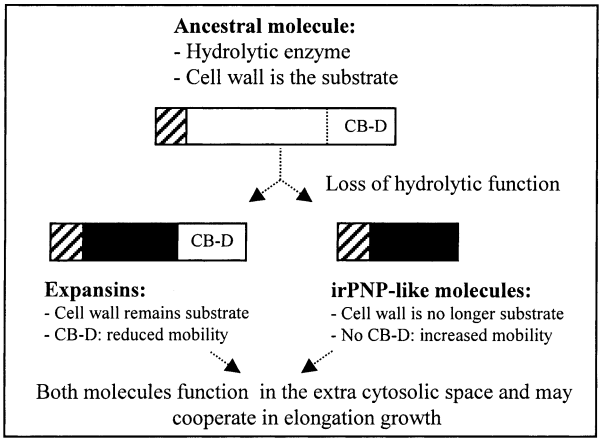

Fig. 5. Model of molecular evolution of irPNP-like molecules.

different from cell wall loosening. These recruited molecules may be part of stress responses and possibly function as extracellular signaling molecules that directly or indirectly affect water and ion transport. Such a conclusion is not only supported by the domain structure but by the fact, that irPNPs were initially isolated through the use of immunoaffinity to an antibody directed against ANP, a vertebrate signal peptide with a role in water and solute homeostasis (Billington et al. 1997; Gehring 1999). Natriuretic peptides, ANPs, and irPNPs have been shown to affect ion transport (Pharmawati et al. 1999; Maryani et al. 2000; Pharmawati et al. 2001) across plant cell membranes. Furthermore, water transport has been shown to be affected by ANP in vertebrate systems (Wolfensberger et al. 1994; Han et al. 1998) and both ANP and irPNP modulate osmoticum dependent water transport (Maryani et al. 2001) in protoplasts, an experimental system without cell walls. In summary, all evidence points towards a unique set of physiological functions for irPNP-like molecules that is entirely different from cell wall loosening (Fig. 5).

The data and hypothesis presented here further strengthen the case for the presence of a functional peptide based extracellular signaling system. This system may be derived from ancestral molecules that also gave rise to expansins. Furthermore, irPNP-like molecules from plants have been identified and isolated due to an epitope shared between the signaling vertebrate ANP and irPNPs. Moreover, ANP and irPNP induce a remarkable number of common physiological responses in plants. An interesting feature is that the irPNP-like molecules can cause enhanced osmoticum-dependent water uptake (Maryani et al. 2001; Pharmawati et al. 2001) and hence increases in cell turgor. Increasing cell turgor will pose stress on cell walls and may in turn signal the need for expansin-dependent wall loosening. It is conceivable, that irPNP-dependent swelling exerts the force required for cellular expansion. This observation provides fuel for the provocative suggestion that despite the divergent functions of expansins and irPNP-like molecules, their action is in fact cooperative. We are currently testing recombinant AtPNP-A with a view to further characterizing the biological role of this novel family of molecules.

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