AtPNP-A is a systemically mobile natriuretic peptide immunoanalogue with a role in *Arabidopsis thaliana* cell volume regulation

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Abstract **Cellular and physiological evidence suggests the presence of a novel class of systemically mobile plant molecules that are recognized by antibodies against vertebrate atrial natriuretic peptides (ANPs). In order to characterize the function of these immunoanalogues we have expressed the full-length recombinant (AtPNP-A[1–126]) and demonstrate that this molecule induces osmoticum-dependent H_2O uptake into protoplasts at nanomolar concentrations and thus affects cell volume. A similar response is also seen with a recombinant that does not contain the signal peptide (AtPNP-A[26–126]) as well as a short domain (AtPNP-A[33–66]) that shows homology to the vertebrate peptide. Taken together, these findings suggest that AtPNP-A has an important and systemic role in plant growth and homeostasis.**

Key words: Plant homeostasis; Cell volume regulation; Natriuretic peptide; Plant protoplast; *Arabidopsis thaliana*

1. Introduction

Plant natriuretic peptides (PNPs) are a novel class of molecules with biological activity at nanomolar concentrations. PNPs are recognized by antibodies against vertebrate atrial natriuretic peptides (ANPs) [1–3]. Vertebrate ANPs are a highly conserved and well studied group of peptide hormones with an important role in vertebrate salt and water homeostasis [4,5]. PNPs and vertebrate ANPs can elicit a number of common biological responses in plants [3,6] and one such response is the promotion of stomatal pore opening that causes increased transpiration and gas exchange. While PNPs and ANPs can thus be viewed as functional analogues, they are not likely to be related molecules in the evolutionary sense [7]. The weak sequence similarity between AtPNP-A, an *Arabidopsis thaliana* PNP, and human ANP (Fig. 1A) is the result of convergent evolution [7].

PNP-like molecules also share some sequence similarity with domain N-terminals part of the cell wall-loosening expansins and fall into two distinct groups [7] within the superfamily of expansins [7,8]. However, PNPs are significantly shorter molecules since they do not contain the extended C-terminus of expansins [7], a domain that, based on structural features and homology modeling, has been implicated in polysaccharide binding [9,10] and hence anchoring of expansins to their substrate. We have postulated that the absence of this putative wall anchor will result in increased extracellular mobility that in turn is a likely precondition for a systemic mode of action [7]. The association of PNPs with conductive tissues demonstrated by in situ hybridization and tissue printing [11] as well as the presence of biologically active PNP in xylem exudates [11] both lend strong support to this concept.

Furthermore, there are four major observations that suggest that contrary to expansins PNPs do not act on the wall. Firstly, a blight-induced PNP-like molecule from *Citrus jambhiri* (CjBAp12, accession number AAD03398) has no apparent expansin activity [12]. Secondly, ANP binding to plant microsomes in vitro can be displaced by PNPs demonstrating competition for binding sites on the plant membrane [13]. Thirdly, PNP can rapidly and significantly modulate ATP-dependent proton gradients in plasmalemma vesicles as well as promote Cl^- net uptake into membrane vesicles [14]. Finally, PNP can cause effects in protoplasts, i.e. plant cells with their walls removed. The effects include significant and rapid PNP-dependent elevations of the second messenger cGMP in potato guard cell protoplasts [15] and both ANP and PNP have been shown to affect H_2O net uptake into potato mesophyll protoplasts [16].

Here we report the synthesis of a recombinant *A. thaliana* PNP (AtPNP-A) and test its biological activity in a stomatal guard cell assay and in a protoplast system. In addition, we delineate the active domain and argue that to the best of our knowledge this molecule is the first peptidic plant signalling molecule that affects homeostasis.

2. Materials and methods

2.1. Preparation of recombinant AtPNP-A

The *AtPNP-A* gene [7] was amplified from pPNP-SETC3 by polymerase chain reaction and then cloned into the pCR T7/NT-TOPO expression vector (Invitrogen) which was maintained in TOP 10F′ *Escherichia coli* cells. For expression of the protein, BL 21 Star pLys S cells (Invitrogen) were transformed with the pCR T7/NT-AtPNPA construct and cultured on agar plates containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37°C. A single colony resuspended in 200 μl sterile distilled water then spread onto LB agar plates containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) and incubated at 37°C overnight was used to inoculate LB broth containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) and grown at 37°C on an orbital shaker (220 rpm). Expression was induced with 0.5 mM isopropyl-β-$_D$-thiogalactopyranoside (Sigma) when the optical density of the broth culture had reached mid-log phase ($OD_{600} = 0.6$).

The cleared *E. coli* lysate was prepared under denaturing conditions

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Abbreviations: ANP, atrial natriuretic peptide; PNP, plant natriuretic peptide

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as described in the manual (Protocol 10, QIAexpressionist, Qiagen). The purification of the recombinant was essentially performed as described in the manual. Firstly, 1 ml of the 50% Ni-NTA slurry was added to 4 ml of the cleared cell lysate and mixed gently by shaking on a rotary shaker for 60 min at 20°C. The lysate–resin mixture was loaded onto a column (100 mm × 10 mm), allowed to settle and the flow-through collected and stored at −20°C. The resin was washed twice with 4 ml of buffer C (100 mM Na_2HPO_4, 10 mM Tris–HCl, 8 M urea; pH 7.0) and then twice with urea wash buffer (8 M urea containing 500 mM NaCl, 20% glycerol, 20 mM Tris–HCl; pH 7.4). Subsequently, the column was equilibrated with 4 ml 6 M urea wash buffer, pH 7.4 before performing the gradient to refold the recombinant on the Ni-NTA matrix using a linear 6–1 M urea wash buffer gradient. After renaturation, proteins were eluted four times with 1 ml elution buffer (1 M urea wash buffer containing 250 mM imidazole; pH 7.4) and dialyzed (molecular cut-off: 10 000 Da) against water at 4°C for 48 h. The dialyzed sample was concentrated by freeze-drying, and the resultant powder taken up in 500 µl sterile ddH_2O.

2.2. Protoplast isolation and volume measurement

Protoplasts were prepared from 50 ml of *A. thaliana* cell suspension culture in mid-log phase grown in Murashige and Skoog Basal Salt with Minimal Organics (MSMO) medium (4.43 g MSMO, 50 µl kinetin, 3 g sucrose, 500 µl naphthaline acetic acid; pH 5.7 and made up to 1 l). Cells were spun at 4500 rpm for 10 min at room temperature and 10 ml enzymatic solution (0.8% w/v cellulase (Fluka), 0.2% w/v pectinase (Worthington), 0.08% w/v pectolyase (Sigma) prepared in 400 mM sorbitol; pH 4.8) was added to the cell pellet. The cells were resuspended and incubated in the dark at 37°C, at 40 rpm for 3 h prior to adding an additional 1 ml of enzymatic solution and further incubation for 2 h. The digest was then filtered through gauze and the filtrate containing the protoplasts centrifuged at 3500 rpm for 15 min. The supernatant was removed and the pellet resuspended in 500 µl 400 mM sorbitol, pH 4.8. The protoplasts were stored on ice until further treatment with different amounts of recombinant AtPNP-A. The protoplasts were incubated with the recombinant protein for 15 min at room temperature, visualized under the microscope with a calibrated ocular micrometer and pictures of the protoplasts were taken. The volumes of >50 randomly selected protoplasts of the controls and the samples were calculated and the results analyzed by one-way analysis of variance (ANOVA) and paired Student's *t*-test.

2.3. Stomatal guard cell assay

In each experiment three *A. thaliana* leaves were rinsed and submerged at 20–25°C in stomatal assay solution (10 mM PIPES (pH 6.3), 50 mM KCl, 1 mM $MgCl_2$ and 100 µM $CaCl_2$) in microtiter plate wells and treated at 20–25°C under incandescent light (430 nm at 35 W/m^2) for 30 min. Pore widths of >20 stomata from three separate leaves for each treatment were measured microscopically using a calibrated ocular micrometer and the results are the mean ± S.E.M. of >60 stomata subjected to ANOVA to establish differences in treatments.

3. Results and discussion

We have synthesized AtPNP-A (accession number AAD08935), a recombinant PNP from *A. thaliana* (AtPNP-A). Here we show the domain organization of AtPNP-A (Fig. 1A) and align AtPNP-A with human ANP to delineate the domain that shows similarity with ANP. When the full-length recombinant AtPNP-A[1–126] with a molecular weight of 13.9 kDa is added to the incubation medium in which whole *A. thaliana* leaves are submersed it promotes significant stomatal opening at 0.01 mg protein/ml which is equivalent to a concentration of 72 nM (Fig. 1B). Stomatal aperture changes play a key role in plant homeostasis and are caused by guard cell volume changes whereby volume increases lead to the opening of the pore and volume reduction leads to closure. This process is turgor-based and modulated by environmental conditions, plant hormones, ion channels and second messengers

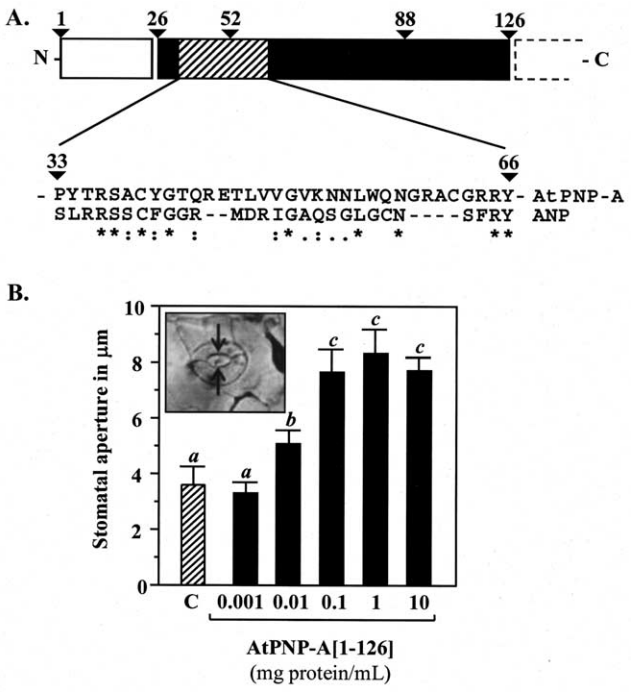

Fig. 1. Domain organization of AtPNP-A and effect of AtPNP-A[1–126] on stomatal aperture. A: In the representation of AtPNP-A domains, the open square is the signal peptide, the hatched square is the domain with similarity to human ANP and the C-terminus in broken lines is an extension not present in AtPNP-A but typical for expansins. Asterisks identify identical amino acids, colons are conservative amino acid substitutions and dots are semi-conservative amino acid substitutions. The numbers above the solid inverted triangles delineate the fragments tested for biological activity. B: Stomatal guard cell assay. *A. thaliana* leaves submerged in stomatal assay solution (see Section 2) were treated with 0.001, 0.01, 0.1, 1 and 10 mg AtPNP-A[1–126]/ml. Bars are the mean ± S.E.M. of apertures of >60 stomata, different letters signify significant ($P<0.01$) differences between treatments and the results are representative of four independent experiments. Inset: Stomatal pore with the opening delineated by two arrows. The bar is 10 µm.

[15,17]. Stomatal opening has previously been reported in response to both rat ANP [18] and immunoaffinity-purified PNPs [2] and these findings have suggested that an endogenous natriuretic peptide-like system does have a role in plant homeostasis. The results obtained with the recombinant AtPNP-A reported here (Fig. 1B) show that exogenous application to whole submerged leaves results in a biological response. However, it does not indicate if the molecule acts on the cell wall like the distantly related expansins [7] or the cell membrane.

To test the hypothesis if AtPNP-A acts on the cell membrane we have used the recombinant in a protoplast system (Fig. 2). *A. thaliana* protoplasts were prepared and suspended in 400 mM sorbitol (Fig. 2A) and treated with 50 ng AtPNP-A[1–126]/ml. This treatment leads to rapid (>20 min) and pronounced swelling and vacuolization as compared to the control. This effect is significant and concentration-dependent (Fig. 2B) and can occur at concentrations as low as 700 pM. It also indicates either that the protoplasts are more sensitive than guard cells in intact leaf tissue or that access of the recombinant is facilitated in the suspended protoplasts.

Volume increases are also caused by AtPNP-A[26–126] (Fig. 1A), a shorter recombinant protein that does not contain

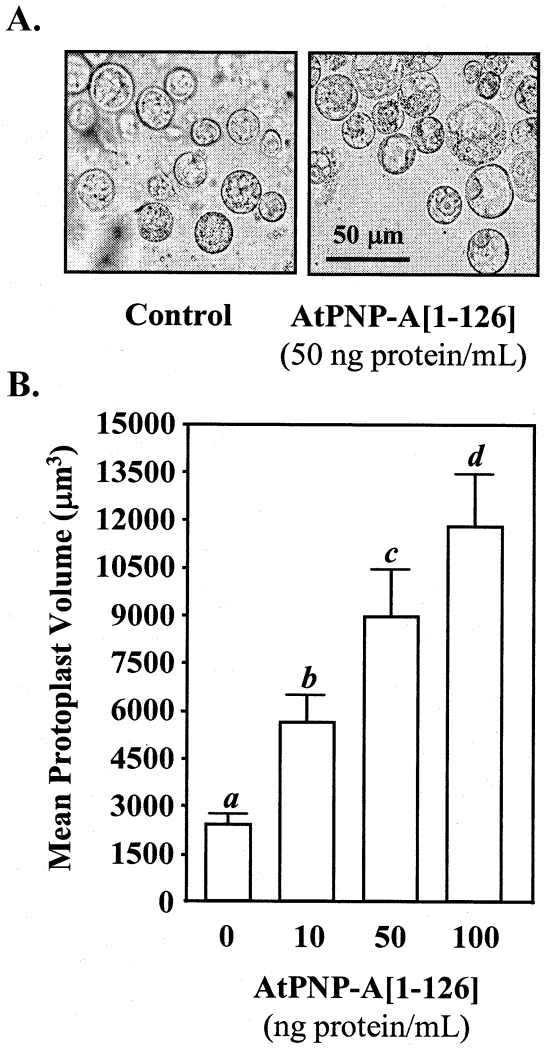

Fig. 2. Effect of AtPNP-A[1–126] on *A. thaliana* protoplasts. A: Protoplasts were extracted from *A. thaliana* cells grown in suspension culture, suspended in 400 mM sorbitol (left image) and treated 50 ng/ml AtPNP-A[1–126] for 15 min (right image). B: Protoplasts treated for 15 min with 0, 10, 50 or 100 ng AtPNP-A[1–126]/ml and photographed under a microscope. Diameters of randomly selected protoplasts were measured and volumes calculated assuming spherical shapes of protoplasts. The data shown are means ± S.E.M. of ≥ 50 protoplasts, different letters signify significant ($P < 0.01$) differences between treatments and the results are representative of three independent experiments.

the predicted N-terminal signal peptide [7] required for secretion of the protein into the extracellular space (Fig. 3A) and these increases are also concentration-dependent. In addition, a 34 amino acid long recombinant, AtPNP-A[33–66], that spans the domain of sequence similarity with the vertebrate ANP (Fig. 1A) is sufficient to induce significant protoplast swelling (Fig. 3B). No such response is seen with three control fragments, AtPNP[26–52], AtPNP[53–88] and AtPNP[89–126] (data not shown). These observations thus delineate the biologically active site required for protoplast swelling as being contained by AtPNP-A[33–66] and are consistent with previously reported ANP-dependent stimulation of osmoticum-dependent volume increases in potato mesophyll cell protoplasts [16].

When the osmotic pressure is increased by an increase in sorbitol concentration from 400 mM to 600 mM the protoplast volume decreases (Fig. 4A) and this decrease is a consequence of net H_2O efflux. This osmoticum-dependent shrinkage is completely prevented in the presence of 50 ng/ml AtPNP-A[1–126] (Fig. 4A). An identical response is seen with both shorter molecules AtPNP-A[26–126] and AtPNP-A[33–66] (Fig. 4A). It is not yet clear what the underlying mechanisms of this intriguing response to AtPNP-A are.

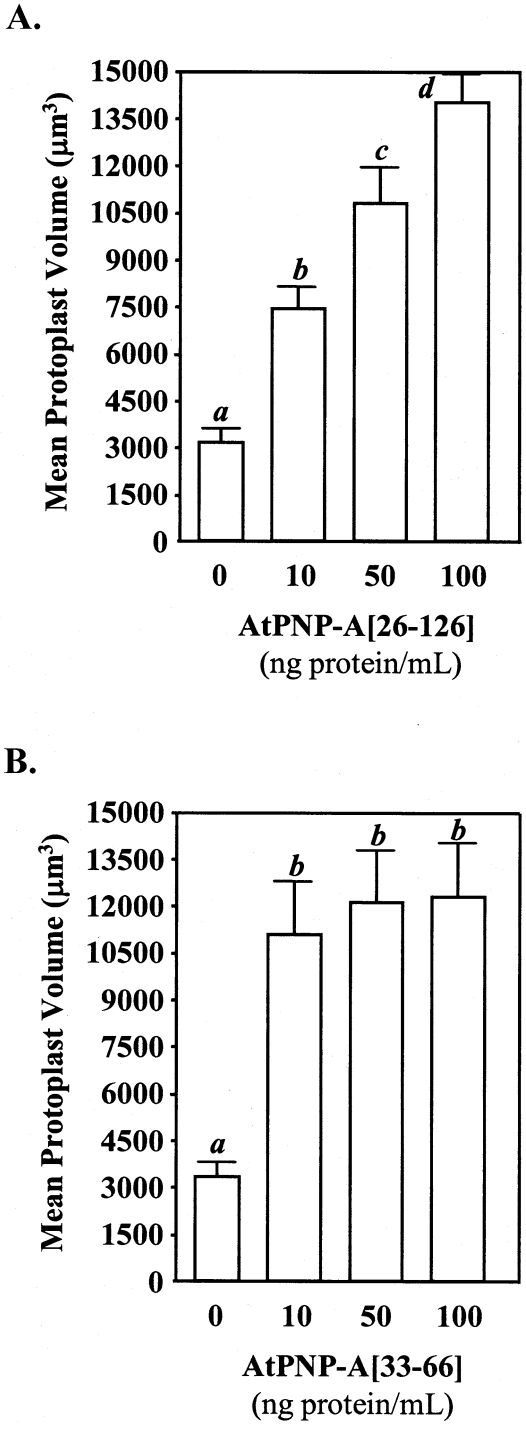

Fig. 3. Effects of recombinant peptide fragments on protoplast volume. A: Volume of protoplasts treated for 15 min with 0, 10, 50 or 100 ng AtPNP-A[26–126]/ml. B: Volume of protoplasts treated for 15 min with 0, 10, 50 or 100 ng AtPNP-A[33–66]/ml.

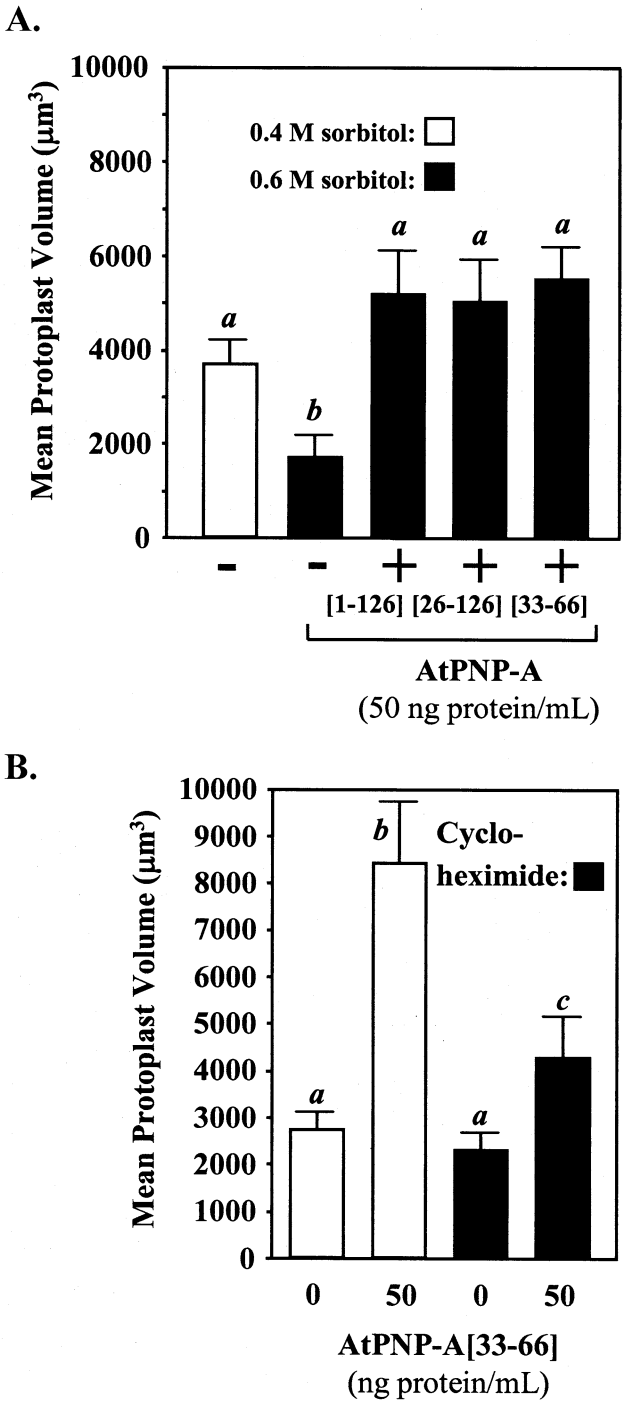

Fig. 4. Effects of increasing osmoticum concentration and the presence of AtPNP-A on protoplast volume and effect of cycloheximide on AtPNP-A-induced protoplast swelling. A: Protoplasts suspended in 400 mM sorbitol were exposed to 600 mM sorbitol in the presence or absence of 50 ng/ml AtPNP-A[1–126], AtPNP-A[26–126] or AtPNP-A[33–66] for 15 min. B: Protoplasts were suspended in 400 mM sorbitol and pretreated with cycloheximide (40 µg/ml) for 30 min prior to a 15 min treatment with 50 ng/ml AtPNP-A[33–66] followed by volume determination.

While there is evidence that immunoaffinity-purified PNPs can affect ion transport [14,19] it seems unlikely that a biophysical explanation such as a direct AtPNP-A-mediated modulation of ion channels could account for the observed phenomena. Firstly, immunoreactant PNP-dependent net K^+ influx in response to which H_2O uptake can occur is observed only after a delay of >20 min [19] and thus well after the swelling response has occurred. It is in fact likely that the delayed PNP-dependent net K^+ influx is a response to rather than the cause of H_2O net uptake. Furthermore, AtPNP-A may also cause membrane permeability changes or lead to the rapid synthesis and/or re-compartmentalization of cellular compatible solutes (e.g. proline or mannitol) to augment intracellular osmotic pressure which in turn would drive H_2O uptake. Secondly, we observe a significant reduction in swelling in the presence of cycloheximide (Fig. 4B). Since cycloheximide is a known inhibitor of cytoplasmic protein synthesis and has been used successfully to inhibit protein synthesis in plant protoplasts [20] the current finding would suggest that continuing de novo protein synthesis is at least in part required for the full response to AtPNP-A.

What is the biological role of PNP-like molecules? Immunological evidence for the presence of PNP-like molecules has been found in all angiosperm species tested to date [1–3,6,7] and ESTs encoding PNP-like molecules are reported in mono- and dicotyledonous plants [7]. While PNP-like molecules are expressed in unstressed plants (e.g. [7]), we have reported that in the brassicaceous weed *Erucastrum strigosum* these molecules are significantly up-regulated under salinity stress conditions [21]. We have also observed significant up-regulation in *A. thaliana* suspension culture cells in response to 150 mM NaCl and even more so in response to iso-osmolar amounts of sorbitol [21]. In addition, time-dependent increases in PNP in the xylem of the African sage *Plectranthus ciliatus* were registered after shoot removal [11] which causes severe homeostatic disturbances. In addition, ANP and PNP increase lateral water movement out of the conductive tissue (xylem) [22] and such a 'drawing' of water is compatible with the function of the recombinant AtPNP-As described here. Taken together, these observations establish PNP as osmotic stress-responsive and systemic in mode of action since the xylem is a highly unlikely place for protein synthesis but not for transport.

Finally, it is conceivable that PNP-dependent increases in cell turgor may also play an important role in plant growth and in particular cell elongation growth since PNP-dependent swelling may exert the force required for cellular expansion. The findings reported here provide fuel for the provocative suggestion that despite the divergent functions of wall-acting expansins [23,24] and the distantly related PNP-like molecules [7], their action may in fact be cooperative. A detailed analysis of AtPNP-A-deficient mutants or AtPNP-A-over-expressing transgenics will allow us to test this hypothesis and further explore the roles of this novel plant molecule.

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References

[1] Vesely, D.L., Gower, W.R. and Giordano, A.T. (1993) Am. J. Physiol. 265, E465–E477.
[2] Billington, T., Pharmawati, M. and Gehring, C.A. (1997) Biochem. Biophys. Res. Commun. 235, 722–725.
[3] Gehring, C.A. (1999) Ann. Bot. 83, 329–334.
[4] Kourie, J.I. and Rive, M.J. (1999) Med. Res. Rev. 19, 75–94.
[5] Kone, B.C. (2001) Cardiovasc. Res. 51, 429–441.
[6] Gehring, C.A. (2003) Int. J. Biochem. Cell Biol. 35, 1318–1322.

[7] Ludidi, N.N., Heazlewood, J.L., Seoighe, C.J., Irving, H.R. and Gehring, C.A. (2002) J. Mol. Evol. 54, 587–594.
[8] Li, Y., Darley, C.P., Ongaro, V., Fleming, A., Schipper, O., Baldauf, S.L. and McQueen-Mason, S.J. (2002) Plant Physiol. 128, 854–864.
[9] Linder, M. and Teeri, T.T. (1997) J. Biotechnol. 57, 15–28.
[10] Barre, A. and Rougé, P. (2002) Biochem. Biophys. Res. Commun. 296, 1346–1351.
[11] Maryani, M.M., Morse, M.V., Bradley, G., Irving, H.R., Cahill, D.M. and Gehring, C.A. (2003) J. Exp. Bot. 54, 1553–1564.
[12] Ceccardi, T.L., Barthe, G.A. and Derrick, K.S. (1998) Plant Mol. Biol. 38, 775–783.
[13] Suwastika, I.N., Toop, T., Irving, H.R. and Gehring, C.A. (2000) Plant Biol. 2, 1–3.
[14] Maryani, M.M., Shabala, S.N. and Gehring, C.A. (2000) Arch. Biochem. Biophys. 376, 456–458.
[15] Pharmawati, M., Maryani, M.M., Nikolakopoulos, T., Gehring, C.A. and Irving, H.R. (2001) Plant Physiol. Biochem. 39, 385–394.
[16] Maryani, M.M., Bradley, G., Cahill, D.M. and Gehring, C.A. (2001) Plant Sci. 161, 443–452.
[17] Blatt, M.R. (2000) Curr. Opin. Plant Biol. 3, 196–204.
[18] Gehring, C.A., Md Khalid, K., Toop, T. and Donald, J.A. (1996) Biochem. Biophys. Res. Commun. 228, 739–744.
[19] Pharmawati, M., Shabala, S.N. and Newman, I.A. (1999) Mol. Cell. Biol. Res. Commun. 2, 53–57.
[20] Houwing, C.J. and Jaspars, E.M.J. (2000) Arch. Virol. 145, 13–35.
[21] Rafudeen, M.S., Gxaba, N., Makgoke, G., Bradley, G., Pironcheva, G., Raitt, L., Irving, H. and Gehring, C. (2003) Physiol. Plant. (in press).
[22] Suwastika, I.N. and Gehring, C.A. (1998) Cell. Mol. Life Sci. 54, 1161–1167.
[23] Cosgrove, D.J. (2000) Nature 407, 321–326.
[24] Cosgrove, D.J., Li, L.C., Cho, H.T., Hoffmann-Benning, S., Moore, R.C. and Blecker, D. (2002) Plant Cell Physiol. 43, 1436–1444.