Application of Recombinant Technology in Protein Investigations

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1 General Introduction

Protein science is a rapidly expanding area of research in the biological sciences. It joins genomics, transcriptomics and metabolomics as an intact research subject termed proteomics employed to understand biological organisms in a systemic way. Proteomics deals with not only the end product of gene expression but also the functional mechanism of the product in an integrated pattern (Anderson & Anderson, 1998; Blackstock & Weir, 1999; Wilkins *et al.*, 1996). In protein science, one single analytical approach usually cannot generate sufficient information to clearly understand a research target, therefore multiple methodologies are needed for drawing a comprehensive and broader picture. Nowadays, many techniques such as X-ray crystallography, nuclear magnetic resonance spectroscopy, mass spectroscopy, various chromatographic techniques in conjunction with recombinant DNA technology and electrophoresis are being widely adopted for protein investigations (Alberts *et al.*, 2008; Lian & Roberts, 2012; Maurya *et al.*, 2009). Combined with the simultaneous development of bioinformatics and other novel techniques, these technologies have become powerful tools used in the characterization of protein structures and their functions, post-translational modifications, protein interactions, and purification of target proteins (Alberts *et al.*, 2008; Alterovitz *et al.*, 2008; Maron *et al.*, 2009; Ole, 2004).

Different research methodologies have distinct advantages that can be exploited for characterizing proteins. It is well known that there are two main barriers for studying proteins in more detail. One major challenge is that, obtaining a better understanding of how a target protein functions in a specified biological environment is complicated by the diverse and dynamic range of its living context. The other difficulty is that there are limitations associated with isolating large amounts of target protein from its natural environment. However, recombinant technology, which allows specific genes to be cloned and expressed in recombinant or chimeric forms, has proven helpful in mitigating such hindrances. Apart from aiding the successful isolation and purification of target proteins, recombinant technology also provides distinct and detectable means of tracking and monitoring protein activities *in vivo* (Alberts *et al.*, 2008; Brown, 2006).

For the above-mentioned reasons, this chapter describes various recombinant methodologies that are usually applied in protein science investigations. We selected five representative experiments from our two research themes: plant natriuretic peptides (PNPs) and receptor kinases. Plant natriuretic peptides are relatively newly identified signaling peptides in plants which are recognized by the antibody of human natriuretic peptide (a human hormone). Research findings indicate that PNPs play significant roles in regulating fluid homeostasis in plants, particularly under stressful conditions. There is general consensus amongst researchers that PNP possibly operates via a signaling pathway involving the second messenger guanosine 3', 5'-cyclic monophosphate (cGMP) (Gehring & Irving, 2003; Meier *et al.*, 2008; Wang *et al.*, 2010; Wang, Donaldson, *et al.*, 2011; Wang *et al.*, 2007; Wang, Gehring, *et al.*, 2011). Cyclic GMP production is catalyzed by a class of enzymes known as guanylate cyclases (GCs) that convert the nucleotide triphosphate, guanosine 5'-triphosphate (GTP) to cGMP. We have identified a group of leucine-rich repeat receptor like kinases that have a GC catalytic center that is embedded within the kinase domain (Kwezi *et al.*, 2007; Kwezi *et al.*, 2011). A lot of valuable information in these two research fields is obtained by employing recombinant protein technology.

The chapter contains a number of methodologies including recombinant protein purification and analysis, enzymatic reporter assays and fluorescent tag detection. Contents are formalized with various research aspects covering physiological, biochemical, molecular and cell biology fields.

2 Recombinant Protein Applied for Physiological & Biochemical Analyses

2.1 Experiment: Plant Natriuretic Peptide Induces Stomatal Opening

2.1.1 Introduction

Stomatal movements are used by plants in initiating adaptive responses to stressful environmental stimuli. PNP, a plant hormone which has been shown to significantly enhance guard cell opening in plants (Gehring & Irving, 2003) counteracts the action of abscisic acid (ABA), another plant hormone, which stimulates stomatal closure. Such a noteworthy antagonist reaction suggests that PNP and ABA are most likely to have a close interaction. Stomatal closure is necessary for the maintenance of plant water status during stressful environmental conditions that favor water loss. On the other hand, stomatal opening is influenced by a number of factors which include light, higher temperatures and low carbon dioxide concentrations. Therefore, sensitive regulation of stomatal movements is a pre-requisite for normal plant growth and development. In plants, there is a need to maintain this delicate balance between various survival demands particularly during stressful conditions. The interplay among plant hormones can reduce the dominance of any one molecule (Wang & Irving, 2011). Hence the final output of a physiological activity may be a combined result which is possibly favorable for the maintenance of plant homeostasis. This experiment shows the outcomes of AtPNP-A (a PNP identified from model plant *Arabidopsis thaliana*) and ABA acting on stomata simultaneously (Wang *et al.*, 2007).

In this experiment, recombinant protein AtPNP-A was expressed and purified from bacteria. A denaturing purification method was used because AtPNP-A was expressed as insoluble protein aggregates (inclusion bodies) in the bacteria. Then recombinant AtPNP-A was added to stomatal assay solution containing leaf sections of *Tradescantia multiflora* Sw. and different concentrations of ABA. After 1 or 2 hours of incubation, the results revealed that guard cell aperture was increased in response to AtPNP-A but closed by ABA (Figure 1A). In the presence of ABA and AtPNP-A together, the ABA effect on guard cell apertures was greatly reduced with higher stomatal opening rate (Figure 1B) and wider pore size (Figure 1C).

2.1.2 Materials

Buffers for denaturing protein purification

1. Buffer A: 100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, 0.2 mg/ml Lysozyme, 30 µg/ml DNase I, pH = 8.0
2. Buffer B: 100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, pH = 7.0
3. Buffer C: 20 mM Tris, 20 % (v/v) Glycerol, 500 mM NaCl, 8 M Urea, pH = 7.4
4. Buffer D: 20 mM Tris, 20 % (v/v) Glycerol, 500 mM NaCl, 6 M Urea, pH = 7.4
5. Buffer E: 20 mM Tris, 20 % (v/v) Glycerol, 500 mM NaCl, 1 M Urea, pH = 7.4
6. Buffer F: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH = 7.4
7. Buffer G: 20 mM Tris, 1 mM PMSF (phenylmethylsulfonyl fluoride), pH = 7.4

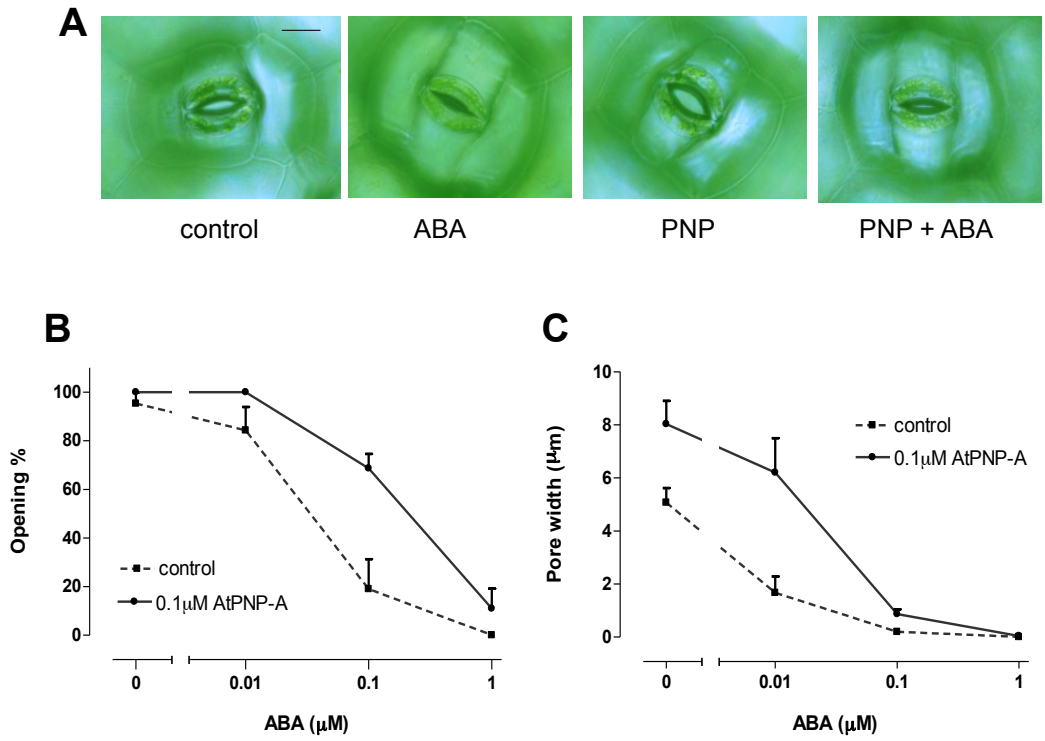

Figure 1: A *Tradescantia* guard cells after 1 h without treatment or with treatments of ABA (100 nM), AtPNP-A (100 nM) or both ABA and AtPNP-A (bar = 20 μm). **B** Addition of AtPNP-A (0.1 μM) in ABA treated leaves increasing stomatal opening rate after 2 h (guard cells with apertures greater than 0.5 μm counted as open). **C** Addition of AtPNP-A (0.1 μM) in ABA treated leaves enhancing guard cell aperture width. Figure reproduced with permission from Wang *et al.* (2007) http://www.publish.csiro.au/nid/102/paper/FP06316.htm)

Solutions for protein SDS-PAGE analysis

1. 5 × Loading buffer: 0.313 M Tris (pH 6.8), 10 % SDS, 0.05 % bromophenol blue, 50 % glycerol, stored at -20 ºC.
2. Stacking gel components: 6.4 ml distilled H_2O, 2.5 ml 0.5 M Tris-HCl (pH 6.8), 100 μl 10 % (w/v) SDS, 1 ml 40 % Acrylamide/Bis, 50 μl 10 % ammonium persulfate, and 10 μl TEMED (N, N, N', N',-tetramethyl-ethylenediamine).
3. Resolving gel components: 4.5 ml distilled H_2O, 2.5 ml 1.5 M Tris-HCl (pH 8.8), 100 μl 10 % (w/v) SDS, 3 ml 40 % Acrylamide/Bis, 50 μl 10 % ammonium persulfate, and 5 μl TEMED.
4. Destained solution I: 50 % (v/v) methanol, 10 % (v/v) acetic acid, 40 % (v/v) distilled H_2O.
5. Destained solution II: 5 % (v/v) methanol, 7 % (v/v) acetic acid, 88 % (v/v) distilled H_2O.
6. 10 × SDS running buffer: 15 g Tris, 72 g Glycine, 5 g SDS in 500 ml, pH ≈ 8.3.

7. Coomassie blue solution: 0.05 % (w/v) Coomassie blue, 50 % (v/v) methanol, 10 % (v/v) acetic acid, 40 % (v/v) distilled H_2O.

Solutions for protein western blot analysis

1. Transfer buffer: 3.03 g Tris, 14.4 g Glycine, and 200 ml Methanol made to 1000 ml, stored at 4 °C.
2. PBS solution: 8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4 dissolved in 1 liter of distilled water, pH 7.3.
3. PBST solution: 0.1 % Tween-20 in PBS solution.

Plant material

Tradescantia multiflora Sw. plants were grown in pots exposed to normal daylight. The youngest fully expanded leaves were used for stomatal assay.

Stomatal assay solution

10 mM PIPES [Piperazine-N,N'-bis(2-ethanesulphonic acid)], 50 mM KCl, 1 mM $MgCl_2$, 100 µM $CaCl_2$, pH = 6.3.

Instruments

Econo System (Bio-Rad), Vivaspin 20 (3000 MWCO PES) ultrafiltration spins column (Sartorius), centrifuge, Qubit fluorometer (Invitrogen), Quant-iT^{TM} protein assay kit (Invitrogen), electrophoresis chamber, membrane transfer cassette and chamber, calibrated ocular micrometer, and microscope.

2.1.3 Methods

Rapid screening of AtPNP-A expression

1. The expression vector pCR T7/NT-TOPO containing *AtPNP-A* gene (Morse et al., 2004) was transformed into BL21 Star (DE3) pLysS One Shot competent cells (Invitrogen) according to the manufacturer's guideline. Transformed cells were cultured on LB agar plate added with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C.
2. After 16 h incubation, several colonies from the plates were selected to separately inoculate 3 ml LB liquid culture containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) at 37 °C with shaking (220 rpm) overnight.
3. These overnight cultures were used to make bacterial stocks stored at -80 °C. Also 10 ml fresh LB medium (containing 100 µg/ml ampicillin and 34 µg/ml) with the addition of 0.5 ml overnight culture was grown at 37 °C with shaking (220 rpm) for rapid screening of protein expression. When the cell density reached about 0.4, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added into the medium. Samples (0.5 ml) were taken at 0 h, 1 h and 3 h time points for the assessment of protein expression. All bacterial cells were harvested after a 3 h incubation.
4. Bacterial cells were resuspended in 0.4 ml buffer A with gentle vortexing and centrifuged at 10000 - 15000 × g for 20 - 30 min. The supernatant was mixed with 0.1 ml 50 % Ni-NTA agarose on a stir wheel for 1 h. Thereafter the mixture was loaded to a filter column and allowed to flow through. The

column was washed three times with 0.6 ml buffer B and then recombinant AtPNP-A was eluted twice with 0.2 ml buffer F for expression assessment.

5. Eluted samples were initially mixed with 5 × loading buffer in 4:1 ratio and denatured by heating at 95 °C for 5 min. Denatured samples were separated in a stacking gel first and then in a resolving gel. Electrophoresis was run in 1 × SDS running buffer at 200 V for 35 min. Then the polyacrylamide gel was stained by Coomassie blue solution for 2 h. Thereafter the gel was destained in solution I for 1 h and continuously in solution II for 16 h. The destained gel was kept in distilled H_2O for checking (Figure 2A). The bacteria with the best levels of protein expression were kept as a stock.

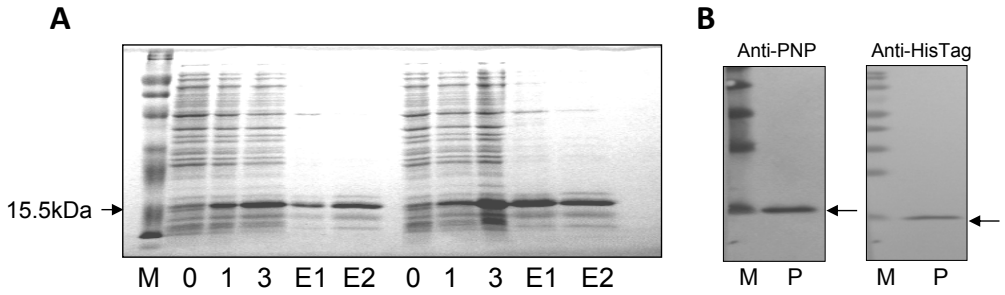

Figure 2: A SDS-PAGE analysis of two bacterial colonies in rapid screening of AtPNP-A expression. Lanes 0, 1, 3 represent lysates in 0 h, 1 h and 3 h time points; lanes E1 and E2 are the first and second eluted proteins. **B** Western blotting analysis of recombinant AtPNP-A using PNP and HisTag antibodies.

Batch purification of recombinant AtPNP-A

1. The stock with the best protein expression was streaked on a LB plate containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) and incubated at 37 °C overnight. Then one single colony was resuspended in 10 ml LB medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) at 37 °C with shaking (220 rpm) overnight. This overnight culture was used to inoculate 200 ml broth culture at 37 °C with shaking (220 rpm). At a cell density reaching about 0.4 (OD ≈ 0.4), 1 mM IPTG was added to induce protein expression. After 3 h shaking incubation, total bacterial cells were collected by centrifugation (5000 × g for 30 min at 4 °C) and pelleted cells were stored at 20 °C.

2. Pellets were thawed for 15 min on ice and resuspended in buffer A at 5 ml per gram wet weight. The continuing steps were completed at room temperature. Cells were stirred for 60 min on a rotary shaker and then centrifuged at 10000 × g for 30 min. Lysate was mixed with 50 % Ni-NTA agarose (4:1 ratio) on a stir wheel for 60 min.

3. The lysate-resin mixture was carefully loaded into an empty column with a cap still attached. The cap was removed, and the flow-through was allowed through. The column was washed with buffer B twice (2 × 4 ml) and then buffer C twice (2 × 4 ml). Finally the column was equilibrated with buffer D (4 ml).

4. Protein refolding was completed by gradual dilution of buffer D with buffer E. This procedure was performed using the Econo System (Bio-Rad). The gradient was set as follows: Buffer E was added to the running buffer (mixture of buffer D and E) for 0 % in 0 min, 20 % in 18 min, 50 % in 45 min, 80 % in 72 min, 100 % in 90 min, 100 % in 105 min. The flow rate was adjusted to approximately 2 ml/min.

5. After refolding, recombinant AtPNP-A was eluted using buffer F at least seven times (7 × 1 ml). Eluted protein was concentrated using Vivaspin 20 (3000 MWCO PES) ultrafiltration spin columns by centrifugation at 3000 × g for 2 h at 4 °C. A desalting step was followed by centrifugation with buffer G at 3000 × g for 60 ~ 90 min at 4 °C. Protein concentration was determined by Quant-iT^{TM} protein assay kit. Concentrated protein was stored at -20 °C in aliquots and also used for western blotting to confirm identity.

6. The concentrated proteins were run in polyacrylamide gels and then transferred to nitrocellulose membranes (code: RPN303D, Amersham Biosciences). The transfer was completed in an ice-cooled chamber (Bio-Rad) with pre-cooled transfer buffer. The running conditions were set at 100 V for 1 h.

7. Transferred membranes were first saturated in a saturation solution containing PBST solution and 1 % bovine serum albumin for 1 h and then probed with primary antibodies for 2 h at room temperature. AtPNP-A antibody (Wang et al., 2010) was prepared in the saturation solution with 1:50 ratio, while HisTag antibody (Novagen) was prepared in the saturation solution at 0.5 µg/ml. After being washed with PBST solution for three times at 5 min intervals, the AtPNP-A antibody probed membrane was incubated with secondary anti-rabbit IgG (from Sigma, 5 µl in 15 ml saturation solution), while the HisTag probed membrane was incubated with secondary anti-mouse IgG (from Sigma, 5 µl in 15 ml saturation solution). Both membranes were incubated for 1 h, and then washed first with PBST solution (3 × 5 min) and followed by PBS solution (1 × 5 min). Washed membranes were detected by incubating with TM/B peroxidase substrate solution (Chemicon / Millipore) for about 5 min (~ 10 ml per membrane). Both detected bands were of the expected size (Figure 2B).

Stomatal assay

1. Leaves from *T. multiflora* were cut into 2 × 10 mm^2 pieces. Three leaf sections (the sections for all treatments coming from the same leaves) were immersed into stomatal assay solution for each treatment and placed under white light at 23 °C.

2. The opening rate and pore width of about 20 stomata from each leaf section were checked under the microscope with a calibrated ocular micrometer (lower epidermis facing the microscope lens) after 1 or 2 h incubation.

3. Finally, all measured data was analyzed and compared.

2.1.4 Notes

1. Certain eukaryotic proteins are toxic to bacteria, causing premature termination of translation. This can be prevented by reducing rare coding sequence from recombinant protein while not affecting the protein function or replacing the original signal peptide (Sahdev *et al.*, 2008). In the above experiment, the signaling peptide of AtPNP-A was removed as it inhibited bacterial cell growth.

2. To determine the growth rate of bacterial culture, cell density (OD_{600}) can be measured every hour after 90 min shaking incubation. Induction of expression by adding IPTG mostly occurs at OD_{600} of 0.6. However, if bacterial growth is too slow, induction would be started at OD_{600} of 0.4. It is recommended to take a bacterial sample as 0 time control before the addition of IPTG. The optimal expression times have to be examined individually for different proteins. Induction times vary for different types of proteins.

3. In some cases, expression of recombinant proteins may lead to the formation of inclusion bodies which are insoluble cytoplasmic granules in bacteria. Extraction of insoluble proteins usually requires the use of denaturing agents such as urea. To test the solubility of a recombinant protein, native protein purification (see section 2.2.3 below) can be employed first where all fractions are analyzed by SDS-PAGE. If the protein of interest is in soluble fractions, then native purification should be adopted, otherwise denaturing purification is reccommended.

4. Protein folding pattern and inclusion body formation are strongly linked to the hydrophobic interactions that are temperature dependent. Generally protein expression at low temperature yields more soluble and properly folded products (Sahdev *et al.*, 2008). Thus bacterial induction growth may be completed at a range of 15 ~ 25 °C to enhance protein solubility.

5. The generation of recombinant protein fragments is often due to proteolytic degradation. It may be helpful to add the protease inhibitor PMSF or a protease inhibitor cocktail to decrease proteolysis especially during cell lysis and protein purification. To reduce the background proteins, 1 mM imidazole (or more if necessary) can be included in washing buffers. The viscosity may also be eliminated by either adding Dnase I or alternatively passing the solution three times through a needle attached to a syringe.

2.2. Experiment: Role of Phosphorylation on Protein Function

2.2.1 Introduction

Protein phosphorylation often plays a significant role in signal transduction processes of the cell through its regulation of protein function (Ciesla *et al.*, 2011; Olsen *et al.*, 2006). This phenomenon is known as phosphoregulation and it acts as an ‘on’ and ‘off’ switch in the regulation of many proteins thereby altering their biological function and activity. Most proteins under the influence of phosphoregulation exhibit different activities at different phosphorylation states. For instance, a protein may need to be in a phosphorylated state for it to perform its biological function at optimal activity with dephosphorylation of the protein representing a turned off state of the protein where biological activity is minimal.

Through the use of phosphomimetics, recombinant protein technology can be used to investigate the role of phosphorylation in regulating protein function or activity. Phosphomimetics is the mutagenic substitution of a protein’s phosphorylation sites with neutral or charged amino acid residues in order to mimic a desired phosphorylation state of a protein. Mutagenic substitutions of phosphorylation sites with a negatively charged amino acid residue, for example aspartate, would yield a mutant that mimics a phosphorylated form of the protein. By the same token, mutagenic substitutions with a neutral amino acid residue, for example alanine, would yield a dephosphorylated form of the protein. In order to carry out a phosphomimetic experiment, the phosphorylation sites of a target protein have to be defined and this is usually done by mass spectrometry. In this experiment, phosphorylation sites of an archetypical receptor kinase from *Arabidopsis thaliana*, were mutated to generate ‘on’ and ‘off’ phosphorylated states of the

protein (phosphomutants). The effect of phosphorylation on the regulation of this receptor kinase was investigated by measuring the kinase activity of the phosphomutants. The phosphomutants were recombinantly expressed in bacterial cells as His-tag fusions before affinity-purification on a Ni-NTA column (Figure 3A). A comparative analysis of the kinase activities of the two phosphomutants showed that the 'on' state phosphomutant had higher kinase activity relative to that of the 'off' state phosphomutant (Figure 3B).

2.2.2 Materials

Components for recombinant protein expression and purification

1. Media: SOC media, 50 mg/ml carbenicillin, Luria Broth (LB) media fortified with 10 mM $MgCl_2$ and 0.1 % glucose before use, 20 % filter-sterilized L-arabinose.
2. Protein expression vector (pDEST17) containing cDNA of the phosphomutants
3. BL21-AI *E. coli* competent cells for protein expression (obtained from Invitrogen)
4. Cell lysis/wash buffer: 100 mM NaH_2PO_4, 300 mM NaCl, 45 mM imidazole, pH 8.0
5. EDTA-free cocktail of protease inhibitor tablets (Roche), 100 mM PMSF stock solution
6. 10 mg/ml lysozyme stock solution.
7. Ni-NTA agarose beads for purification of His-tagged proteins.
8. Protein elution buffer: 100 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0.
9. Equipment: Sonicator, Rotary shaker, Spectrophotometer, Centrifugal concentrators

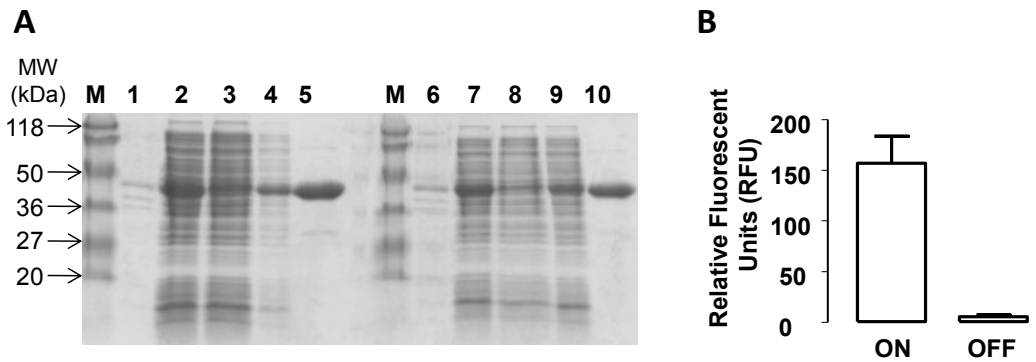

Figure 3: A SDS-PAGE analysis of protein fractions of the affinity purification of 'on' (lanes 1 - 5) and 'off' (lanes 6 - 10) phosphomutants of the receptor kinase**.** Lanes 1 and 6 shows the pellet fractions lanes 2 and 7 correspond to the crude lysate fractions, lanes 3 and 8 show the flow through fractions, lanes 4 and 9 show the column wash fractions and lanes 5 and 10 correspond to the fractions eluted from the Ni-NTA agarose column. The M lanes show the protein molecular weight marker. **B** Comparative analysis of the kinase activity of 'on' and 'off' state phosphomutants. Kinase activity was measured as relative fluorescence units (RFU) of the phosphorylated SOX peptide**.** Experiments were done in triplicate and the error bars represent standard error.

Kinase Assay components

1. Quantified recombinantly expressed protein sample.
2. Fluorescent Omnia® Ser/Thr Peptide 1 kit (Life Technologies).
3. FluoroNunc™ Maxisorp™ white 96 well microtiter plate
4. A fluorescence microplate reader capable of measuring fluorescence at excitation and emission wavelengths of 360 and 485 nm, respectively.

2.2.3 Methods

Recombinant protein expression

1. A 10 ml LB culture containing 50 µg/ml carbenicillin and 0.1 % glucose in 50 ml flask was inoculated with 500 µl of frozen glycerol stock containing a pDEST17 expression plasmid inserted with cDNA of the kinase. (see Note 1)
2. The culture was then incubated at 37 °C overnight in a shaking incubator. The 10 ml of the overnight starter culture was added to 500 ml of fresh LB in a 2 L flask with 50 µg/ml carbenicillin and the OD was monitored at 600 nm (OD_{600}).
3. When the OD_{600} reached a value between 0.4 and 0.5, protein expression was induced with 0.2 % L-arabinose and then the culture was allowed to grow at 25 °C for 4 hours.
4. Cells were harvested by centrifugation at 8000 g for 10 minutes and the supernatant was discarded.
5. The pellet was kept at -20 °C until needed for protein extraction.
6. 1 tablet of EDTA-free protease inhibitor cocktail and 1 ml of lysozyme solution from a 10 mg/ml stock solution were added per pellet from a 500 ml culture.
7. The mixture was incubated on ice with gentle shaking for 30 minutes.
8. After 30 minutes, the mixture was sonicated on ice for 10 sets of 10 second bursts at 300 W with a 10 second cooling period between each burst.
9. The sonicated lysate was then centrifuged at 10 000 g for 30 minutes at 4 °C in order to pellet cell debris. The supernatant contains the crude lysate of your bacterial extract and this fraction was retained for protein purification. (see Note 2)

Purification of recombinant protein

1. 4 ml of the crude lysate was added to 2 ml of 50 % equilibrated Ni-NTA slurry. (see Note 3).
2. The resultant mixture was then incubated on a rotary shaker at 4 °C for at least 1 hour.
3. The crude lysate and Ni-NTA mixture was loaded into a plastic column with a bottom cap and the bottom outlet was removed before collecting the flow through fraction. A 5 µl aliquot of the flow through fraction was saved for SDS-PAGE analysis.
4. The column was then washed twice with 5 ml of wash buffer and the wash fractions were collected. 20 µl of these fractions were saved for SDS-PAGE analysis.

5. The protein was eluted with 4 ml of elution buffer and 20 µl of the eluted fraction was kept for SDS-PAGE analysis.

6. The purification factions were analyzed by SDS-PAGE including the crude lysate fraction from the extraction procedure, so as to assess the expression profile of the recombinant protein (Figure 3A).

Determination of kinase activity of phosphomutants

1. Stock solutions for the kinase assay were set up using reagents provided in the Omnia® Ser/Thr Peptide 1 kit (Invitrogen).

2. 2 sets of triplicate experimental reactions were set up, with one set for each phosphomutant. A separate set of 3 control reactions was prepared using all the components of the kinase assay except the protein. For each set, a 4X master mix was prepared so as to minimize inconsistencies due to pipetting error. The total volume of each reaction was made up to 75 µl. (see Note 4)

3. The amount of protein that should be used for each reaction in a single well was determined. Each reaction should contain 1 µg of protein (i.e. 1 µg of protein per 75 µl reaction).

4. The master mix was then added to each well and made up to a total volume of 75 µl with the desired amount of protein.

5. Before measuring fluorescence, the excitation and emission wavelengths on the microplate reader were set at 360 and 485 nm, respectively.

6. The reaction was undertaken for 10 minutes with fluorescence readings being recorded for the entire duration of the reaction. For end point kinetics, the initial and the final values of the measured fluorescence units were used to calculate relative fluorescence units (RFU) for each reaction. The data was then analyzed using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA, USA). (see Note 5)

2.2.4 Notes

1. If starting up with colonies from a freshly streaked plate prepare 3 - 5 separate inoculations from isolated colonies. However a verified high protein yielding seed stock is preferable. Expression bacterial stocks kept at -80 °C in BL21-AI do lose their expression capability and it is sometimes worthwhile re-transfecting cells. It is imperative to test for expression if the seed stocks have been kept for more than 3 months. To propagate plasmid, keep in DH5α (bacterial stock at -80 °C) and also keep plasmid stocks for transfection (at -20 °C)

2. You can top up the sonicated mixture with water in order to meet centrifuge volume requirements. Keep 5 µl of the supernatant for SDS-PAGE analysis.

3. The Ni-NTA slurry should be equilibrated with buffer as it is often stored in a 20 % ethanol solution. To equilibrate the Ni-NTA beads wash with deionized sterile water using 5 column volumes (i.e. wash 2 ml resin with 10 ml water). Centrifuge this mixture at 2500 g for 5 minutes so as to pellet the beads. Remove water by discarding the supernatant and repeat this wash step to a total of 2 times using deionized water each time. Equilibrate the beads by the addition of 5 column volumes of lysis buffer before pelleting the beads by centrifugation. Discard the supernatant and add fresh lysis buffer to the beads.

4. When preparing the master mix for the experimental reactions leave room for the amount of protein to be added for each reaction. The protein is to be added immediately before beginning to record fluorescence units.

5. RFU was calculated as the difference between the initial and final fluorescence units for each reaction.

3 Enzymatic Reporter Used in the Assay of Gene Expression

3.1 Experiment: Plant Natriuretic Peptide Controls its Expression via Feedback Regulation

3.1.1 Introduction

Plants are a highly complicated and organized living organism. The role of each plant regulator often resides in the network and feedback systems (Wang & Irving, 2011). Specific activity of each plant regulator stimulates plant actions. Simultaneously, the activity of the regulator itself may be also modulated by diverse cellular compounds or molecular changes during the signaling process. As a plant regulator, PNP release may respond to numerous stimuli and then over time generate different responses. Besides other components, PNP is an element of its own signal transduction chain as well where it may have effects on PNP production. It is essential to elucidate the regulation at the molecular level to give an insight into the entire story of PNP expression and signaling.

Usually it is difficult to detect the transcriptional activity of a particular gene in the tissue where it functions. This problem can be circumvented by merging the upstream promoter sequences of any gene to the coding region of a reporter gene whose product is easily detectable for assessing gene expression. Currently one of the most widely applied reporter gene is luciferase. Luciferase is an oxidative enzyme originally isolated from fireflies. When luciferase acts on the appropriate luciferin substrate in the presence of ATP, light is emitted. The light can be detected by light sensitive apparatus such as a luminometer (Baldwin, 1996; Promega, 2009).

An example testing induction of *AtPNP-A* is given here. In the test, *luciferase (LUC)* gene is constructed under the control of *AtPNP-A* promoter. After the "*AtPNP-A* promoter::*LUC*" DNA is delivered into protoplasts, luciferase gene serves as a tool to document the transcriptional change of *AtPNP-A* gene in response to different amounts of recombinant AtPNP-A. From the outcome of LUC assay, *AtPNP-A* promoter showed concentration-dependent responses to recombinant AtPNP-A (Figure 4). Recombinant AtPNP-A at higher concentration (10 µg/ml) upregulated the *AtPNP-A* expression; but the highest concentration (20 µg/ml) of recombinant AtPNP-A was not different to the control. Apparently AtPNP-A controlled its own expression via a feedback loop. In the initial stage AtPNP-A induces itself via positive feedback; thus AtPNP-A can be amplified in a very short time. However, when AtPNP-A production reaches the threshold level, the self-regulation turns to negative feedback to suppress the continued expression of AtPNP-A avoiding any harmful effects of overexpression (Wang, Donaldson, *et al.*, 2011; Wang, Gehring, *et al.*, 2011).

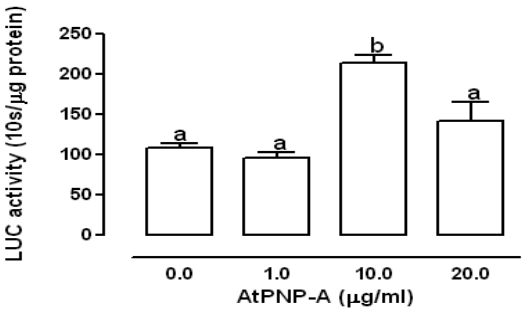

Figure 4: LUC activity was measured in protoplasts transfected with "*AtPNP-A* promoter::*LUC*" construct after 18 h incubation with different concentrations of recombinant AtPNP-A. Columns with different letters were significantly different ($P < 0.05$ one way ANOVA using Tukey-Kramer post-test). Figure reproduced with permission from Wang, Donaldson, *et al.* (2011).

3.1.2 Materials

Plant material

1. Seeds of *Arabidopsis thaliana* (Col-0) were washed on a sterile filter paper (inserted in a funnel) with 70 ~ 80 % ethanol and then 2 ml of sterilization solution [0.6 % (w/v) sodium hypochlorite, 0.1 % (v/v) Triton X-100]. The seeds were subsequently rinsed with sterile distilled water 3 ~ 5 times.
2. Sterilized seeds were placed on Murashige and Skoog (MS) basal medium (Sigma) supplemented with 3 % (w/v) sucrose and 0.4 % (w/v) agar. The medium was adjusted to pH 5.7. After stratification at 4 °C in the dark for 2 ~ 3 days, the seeds were cultured in 16 h light-period for 14 days at 23 °C. Thereafter the plantlets were transferred into soil with a 12 h light-period at 23 °C.
3. Leaves of 5 ~ 6 week-old plants were used for protoplast experiments.

Cloning kits

1. Taq enzyme (Qiagen)
2. dNTP (Astral Scientific Pty Ltd)
3. ultra-clean PCR clean-up DNA purification kit (MO BIO Laboratories Inc)
4. Gateway® BP Clonase™ II Enzyme Mix (Invitrogen)
5. Gateway® LR Clonase™ II Enzyme Mix (Invitrogen)

Solutions for protoplast transfection

1. Osmotic solution: 0.4 M mannitol, 3 mM MES, 7 mM $CaCl_2$, pH 5.7
2. Enzyme solution: 1 % (w/v) cellulase R-10 (Yakult, Japan), 0.3 % (w/v) Macerozyme R-10 (Yakult, Japan), 0.4 M mannitol, 3 mM MES, 7 mM $CaCl_2$, pH 5.7
3. Washing solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7
4. Transfection solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7

5. PEG solution: 4 g PEG 4000 (Fluka, #81240) mixed with 3 ml distilled H_2O, 2.5 ml 0.8 M mannitol and 1 ml 1 M $CaCl_2$, prepared freshly in use
6. Incubation solution: 0.4 M mannitol, 4 mM MES, 20 mM KCl, pH 5.7

Preparation for LUC assay

1. Luciferase Plant Cell Lysis Buffer (Promega)
2. Luciferase Assay Reagent (Promega)
3. NOVO Star microplate reader (BMG Labtechnologies)
4. Qubit® fluorometer (Invitrogen) and Quant-iT™ protein assay kit (Invitrogen).

3.1.3 Methods

Amplification of attB-PCR product

1. The Gateway cloning technology was utilized to construct the "*AtPNP-A* promoter::LUC" plasmid. According to the guidelines [Gateway® Technology (Version E), Invitrogen], two attB-PCR primers were designed to amplify 1547 bp full promoter region upstream to *AtPNP-A* gene (gene code: At2g18660).
2. Forward primer was:

   5′ - GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTTTATTTTACTTTTTGGGCT - 3′
3. Reverse primer was:

   5′ - GGGGACCACTTTGTACAAGAAAGCTGGGTCCATTTTCTTTAACTTGTTTGT - 3′
4. PCR was completed using the standard protocol described in the Qiagen Taq PCR handbook (Qiagen). A plasmid containing 2.5 kb *AtPNP-A* upstream region was used as DNA template (Wang, Gehring, et al., 2011). Approximately 2 ng to 20 ng plasmid DNA was added to 50 µl PCR reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP (Astral Scientific Pty Ltd), 0.4 µM of each primer, and 1.25 units Taq enzyme. The PCR was run in a MyCycler thermal cycler (Bio-Rad) with initial denaturation at 94 °C for 5 min; and then 30 cycles of DNA amplification (denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min); and final extension at 72 °C for 10 min; and maintenance at 4 °C for 5 min. PCR samples were checked by DNA electrophoresis in a 2 % agarose gel. Accurately sized PCR product was cleaned by the ultra-clean PCR clean-up DNA purification kit (MO BIO Laboratories Inc.).

Creating the entry vector by BP reaction

1. BP reaction was performed according to the guidelines (Gateway® BP Clonase™ II Enzyme Mix, Invitrogen). Plasmid pDONR 207 (Invitrogen) was used as a donor vector. BP mixtures were incubated overnight at 25 °C.
2. The BP reaction was stopped by incubation with proteinase K. Then 1 µl of each BP mixture was added to 50 µl of Std13 competent cells (Invitrogen) and gently mixed by tube-tapping. The mixed cells were incubated on ice for 15 min, then heated in a 42 °C water bath for 2 min, and followed by

15 min on ice again. Thereafter 1 ml of SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, made to 1 liter, pH 7.0; 2.5 ml of 1 M KCl and 10 ml of 1 M $MgSO_4$ added before use) was added to the transformed cells.

3. The transformed cells were incubated at 37 °C for 1 h with shaking (220 rpm) and then spun at 3000 × g for 2 min. About 800 µl of supernatant was discarded. Resuspended cells were plated respectively as 20 µl and 100 µl aliquots on B medium plates containing 10 µg / ml gentamycin, and were incubated at 37 °C for 16 h.

4. Three bacterial colonies were selected to separately inoculate 5 ml LB liquid culture containing 10 µg / ml gentamycin. After 16 h shaking (220 rpm) at 37 °C, plasmids were extracted from these cultures using the Miniprep kit (Qiagen).

5. Extracted plasmids were amplified by PCR using pDONR 201/207 primers (Invitrogen) to determine the sizes of inserts. PCR running conditions were set as the above attB-PCR running conditions except that the annealing temperature was tested at 50 °C, 52 °C, 54 °C and 56 °C respectively.

6. Plasmids with correct PCR sizes were sent for sequencing using:

   - pDONR 201/207 forward primer: 5′ – TCGCGTTAACGCTAGCATGGATCTC – 3′
   - pDONR 201/207 reverse primer: 5′ – GTAACATCAGAGATTTTGAGACAC – 3′.

7. Sequencing results were analysed using the following tools:

   - The *Arabidopsis* Information Resource (TAIR) (http://www.arabidopsis.org),
   - ApE Plasmid Editor (http://www.biology.utah.edu/jorgensen/wayned/ape)

Creating the expression vector by LR reaction

1. LR reaction was completed according to the guidelines (Gateway® LR Clonase™ II Enzyme Mix, Invitrogen). The *LUC* Trap-3 (GW) plasmid (GenBank accession No AY968054) (Calderon-Villalobos et al., 2006) was used as a destination vector. LR mixture was incubated at 25 °C overnight and then inactivated by proteinase K. The mixture was then transformed into DH5α competent cells (Invitrogen). The transformation procedure was the same as creating entry plasmids. Transformed cells were plated on LB medium containing 50 µg/ml kanamycin.

2. After 16 h incubation at 37 °C, two bacterial colonies were chosen to separately inoculate 5 ml LB liquid culture containing 50 µg / ml kanamycin. These cultures were incubated with 16 h shaking (220 rpm) at 37 °C.

3. Plasmids were extracted from the cultures using Miniprep kit (Qiagen). Extracted plasmids (~ 1 µg each) were digested with EcoR I enzyme (one cutting site in the *AtPNP-A* promoter region while another in the plasmid backbone) and then checked in a 2 % agarose gel. Plasmids with the correctly sized DNA bands were chosen for sequencing, and then analyzed. The sequencing primers were as followed:

   - Primer 1: (attB-PCR forward primer, located in the *AtPNP-A* promoter region)
     5′ – GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTTTATTTTACTTTTTGGGCT – 3′
   - Primer 2: (located in the *LUC* region), 5′ – AGTACTCAGCGTAAGTGATG – 3′

Protoplast transfection

1. Protoplasts were isolated from 5 ~ 6 week-old leaves using a protocol adapted from (Yoo et al., 2007). Leaves were cut into 1 mm-wide strips and plasmolyzed in osmotic solution for 30 min. Then about 600 mg leaf material was incubated in 10 ml enzyme solution for 2 ~ 3 h in the dark and slowly rotated (~50 rpm). After incubation, the enzyme solution was passed through a nylon filter (60 μm diameter) and spun at 100 × g for 5 min. Protoplast pellets were washed with washing solution and then spun at 100 × g for 5 min. Washed protoplasts were suspended in washing solution at 4 °C for 30 min and spun down at 100 × g for 5 min. Pelleted protoplasts were resuspended in transfection solution before protoplast transfection.

2. For protoplast transfection, all samples were pooled together to complete the transfection process first. Accordingly in each sample, 5×10^4 protoplasts were combined with 20 μg plasmid DNA. Then an equal volume of polyethyleneglycol (PEG) solution (PEG volume = DNA volume + Protoplast volume) was added and gently mixed with the protoplasts. After the protoplast mixture was incubated for 20 min, washing solution (washing solution volume = 4 × PEG solution volume) was separated into three aliquots and each was added to the mixture at 5 min intervals. Each addition of washing solution was followed with gentle mixing. Thereafter the mixture was spun at 100 × g for 5 min. Protoplast pellets were suspended in incubation solution. Finally transfected protoplasts were separated into aliquots in 1 ml solution each for different treatments. The samples were incubated in a 24-well plate (1 ml solution / well) at 23 °C in the dark for 18 h.

3. After 18 h of incubation, different samples were collected separately and spun at 100 × g for 5 min. Protoplast pellets of each sample were homogenized in 100 μl of Luciferase Plant Cell Lysis Buffer (Promega) by vigorous vortex for 10 s. Cell lysate was centrifuged at 13000 × g for 30 s and the supernatant was stored at -80 °C for LUC reporter gene assay.

Luciferase (LUC) assay

1. Cell lysate (from -80 °C) was defrosted at 4 °C and then added to a 96-well Geriner plate (Geriner Bio-One) with 20 μl per well.

2. A NOVO Star microplate reader (BMG Labtechnologies) was used to detect LUC activity. The auto-injection system was programmed for measurement. The injector of the NOVO Star microplate reader automatically injected 100 μl of Luciferase Assay Reagent (Promega) into every sample well, and then the measurement head detected luminescence intensity from the injected well immediately.

3. LUC activity was normalized by 10 second of time period and 1 μg of protein amount. Protein concentrations of samples were determined by a Qubit® fluorometer (Invitrogen) using a Quant-iT™ protein assay kit (Invitrogen).

3.1.4 Notes

1. To reduce variance, it is better to prepare buffers once which are enough for all protoplast transfection experiments.

2. Depending on the response of promoter to stimulus, plasmid DNA can be increased up to 40 μg for 5×10^4 protoplasts transfection.

3. In the beginning of new signal stimulation, it is recommended to test a broad range of incubation times and dose gradients to find the optimal expression conditions.
4. The LUC activity is sensitive to temperature and other factors, so it is helpful to store all samples at -80 °C first and then test their LUC activities at the same time.

4 Fluorescent Tag Fused to Detect Protein Cellular Localization

4.1 Experiment: Plant Natriuretic Peptides Are Mobile Molecules

4.1.1 Introduction

Fluorescent proteins are popular molecular probes used for protein visualization. Through recombinant DNA technology, a fluorescent protein tag can be fused to a target protein. Fluorescent fusion protein possesses unique advantages in objective imaging such as the use of green fluorescent protein (GFP) in live organisms. Generally GFP is composed of 238 amino acids (~ 27 kDa) and was first isolated from the jelly fish *Aequorea victoria* (Chalfie *et al.*, 1994). It fluoresces green when exposed to blue light. A hexapeptide containing a cyclic-tripeptide (serine, dehydro-tyrosine and glycine) portion, which is covalently linked through the protein's peptide backbone, is necessary for fluorescence. This unique structure is supposed to be formed via post-translational modification (Cody *et al.*, 1993; Heim *et al.*, 1994). Besides the original jellyfish GFP (wtGFP), currently a wide variety of engineered GFPs are available for imaging experiments. Modified forms of GFP show various excitation and emission ranges, and are more sensitive in application. The most commonly used variants are red-shifted GFPs such as enhanced green fluorescent protein (EGFP) and enhanced yellow fluorescent protein (EYFP). EGFP has a maximum excitation at 484 nm (wavelength) and a maximum emission at 507 nm; while EYFP has a maximum excitation at 514 nm and a maximum emission at 527 nm (Cormack *et al.*, 1996; Delagrave *et al.*, 1995; Heim *et al.*, 1994). Fluorescent protein usually can be fused to either the N-terminus or the C-terminus of a broad range of target proteins without affecting their native functions. Also its fluorescence is not species-specific and is relatively resistant to photo-bleaching (Stearns, 1995).

Although the function and structure of AtPNP-A had been studied actively, little was known about the cellular processing of AtPNP-A. To broaden and deepen the understanding of AtPNP-A processing, it is vital to clarify the cellular localization of AtPNP-A. In the following experiment, GFP was employed as a detectable tag for AtPNP-A visualization (Wang, Gehring, *et al.*, 2011). Using the Gateway cloning technology, AtPNP-A containing signaling peptide was engineered to be C-terminus fused with GFP (signalPNP::GFP). After direct onion epidermal transformation, it was found that AtPNP-A was secreted outside the cells under the direction of its signaling peptide (Figure 5).

4.1.2 Materials and Methods

1. Similarly, Gateway cloning technology was employed to construct the "signalPNP::GFP" expression vector. In accord with the Gateway guidelines (basic procedures detailed in section 3.1.3), full length *AtPNP-A* DNA was cloned into the vector p2GWF7.0 (Karimi *et al.*, 2005; Karimi *et al.*, 2002) as the "signalPNP::GFP" expression vector for subsequent experiment.

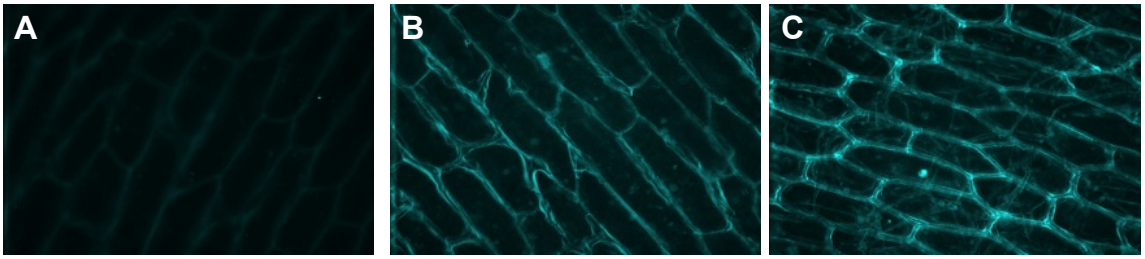

Figure 5: **A** Control onion lower epidermal explants (mock transfection without the construct) show little fluorescence. **B** & **C** Fluorescent protein (signalPNP::GFP) was detected in extracellular area after 24 h and 48 h transfection respectively. Figure reproduced with permission from Wang, Gehring, et al. (2011).

2. Lower onion epidermis was peeled into thin sections (about 5 × 20 mm^2). These sections were sterilized by 70 % ethanol and 0.6 % sodium hypochlorite for 10 s each, followed by washing three times with sterile water and once with MS medium containing 0.3 % sucrose.
3. The sterile sections were cultured on the MS medium with 0.3 % sucrose and 0.4 % agar in the dark at 25 °C for 24 h.
4. Thereafter the sections were dipped into osmotic solution (MS medium containing 0.4 M mannitol and 0.3 % sucrose) for 10 min, and then transferred into transformation solution (MS medium with 0.3 % sucrose and 2 µg/mL "signalPNP::GFP" plasmid while control without the plasmid). The transformation was carried out in the dark for 8 h with gentle shaking (50 rpm).
5. After incubation, onion sections were washed by MS medium containing 0.3 % sucrose briefly and cultured onto fresh solid MS medium as above in the dark at 25 °C.
6. Transformation results were checked using fluorescent microscopy (excitation wavelength = 488 nm) at 24 h and 48 h respectively.

4.1.3 Notes

1. To adopt the above transformation method, plasmid DNA size should be smaller than 10 kb or PCR product may be directly tried.
2. Onion epidermal layers need to be peeled as thin as possible (single layer is the best).

4.2 Experiment: Mammalian Cells Enabled to Express Plant Natriuretic Peptides

4.2.1 Introduction

In the human body, natriuretic peptides are involved in regulating blood volume, blood pressure, ventricular hypertrophy, pulmonary hypertension, fat metabolism and long bone growth (Potter *et al.*, 2006). Obviously, natriuretic peptides serve as important regulators for human health. The plant regulator AtPNP-A shares a homologous part of its molecular structure with hANP (Wang *et al.*, 2010) and is predicted to probably play similar roles as ANP (Meier *et al.*, 2008).Therefore it was of interest to see

whether AtPNP-A could be expressed not only in plant cells but also in mammalian cells. This is also a crucial first step to test the possibility of utilizing AtPNP-A to mimic ANP in future health applications. Herein mammalian cells are adopted as a research platform to track the AtPNP-A cellular processing (Wang *et al.*, 2010). Using Gateway cloning technology, AtPNP-A without its signaling peptide (26-126 amino acids) was constructed to be C-terminus fused with GFP (PNP::GFP). The construct was transfected into mammalian cells. The results showed that AtPNP-A could be expressed in mammalian cells and did not affect the continuing growth of the transfected mammalian cells (Figure 6).

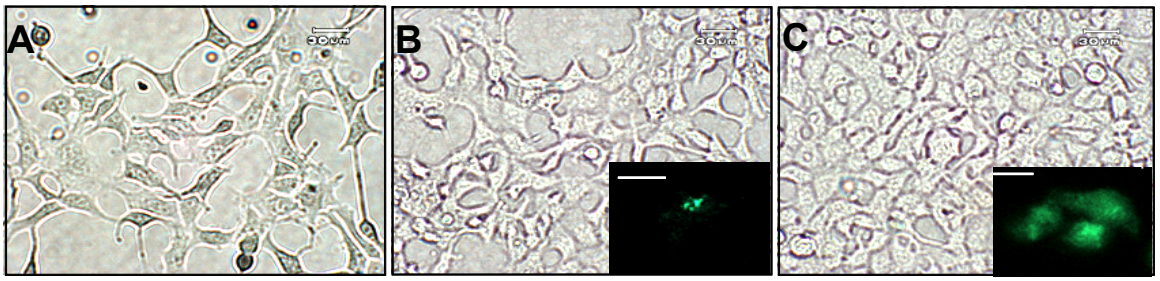

Figure 6: Fusion protein (PNP::GFP) expressed in HEK-293T cells. **A** 24 h cultured cells before transfection. **B** Growing cells after 24 h transfection (inset: confocal image showing PNP::GFP protein mainly located with vesicular regions such as endoplasmic reticulum and Golgi apparatus, bar = 20 µm). **C** Confluent cells after 48 h transfection (inset: confocal image showing PNP::GFP protein distributed throughout the cells, bar = 20 µm). Figure reproduced with permission from Wang *et al.* (2010).

4.2.2 Materials and Methods

Culture of mammalian cells

1. The mammalian cell line 293T/17 [HEK 293T/17], which originates from human kidney embryonic cells, was obtained from the American Type Culture Collection (ATCC), the Global Bioresource Center (ATCC® No: CRL-11268™). HEK 293T/17 cells were cultured in Dulbecco's Modified Eagle's Medium (GIBCO®, Invitrogen) supplemented with 10 % fetal bovine serum (GIBCO®, Invitrogen) at 37 °C in a 5 % CO_2 incubator.
2. For cell subculture, the old culture medium was discarded and cells were washed briefly with sterile 1 × PBS solution (composition as section 2.1.2). Following the removal of PBS solution, a small amount (2 ~ 3 ml) of Trypsin-EDTA (0.25 % Trypsin, 0.5 mM EDTA in 1 × PBS) was added to the flask / plate to disperse the cell layer at 37 °C in a 5 % incubator for 5 min, and then 6 ~ 8 ml culture medium was added to inhibit trypsin activity. The cells were gently aspirated into a 15 ml tube and spun down at 200 × g for 5 min. Cell pellets were suspended in new culture medium in a dilution ratio of 1:4 to 1:8. The medium was renewed every 2 ~ 3 days.
3. The cell viability and density were checked by staining with 0.4 % Trypan Blue.

Mammalian cell transfection

1. Applying Gateway cloning technology (basic procedures detailed in section 3.1.3), *AtPNP-A* gene without its signaling fragment was constructed into plasmid pcDNA™6.2/C-EmGFP (Invitrogen) as the "PNP::GFP" expression vector for mammalian cell transfection.
2. HEK 293T/17 cells were set to a density of 2×10^5 / ml and cultured in a Costar 6-well plate (Corning Inc.) with 2 ml medium per well.
3. Transfection was undertaken the following day. The "PNP::GFP" plasmid DNA (2.5 μg) was added to 500 μl Opti-MEM® I reduced serum medium (Invitrogen) with thorough mixing; and then 6.25 μl Lipofectamine™ LTX reagent (Invitrogen) was added to the diluted DNA with thorough mixing again. This mixture (for one well) was incubated at room temperature for 30 min, and then added to the well containing cells with back and forth gentle rocking for a while. The cells were incubated at 37 °C in a 5 % CO_2 incubator.
4. Medium was changed after 4 h; and expression status was checked by confocal microscopy after 24 h.

4.2.3 Notes

1. Cells should be 50 ~ 80 % confluent at the time of transfection. So the cell growth needs to be monitored by preceding tests. Accordingly the cell density is adjusted when cells are subcultured the day prior to transfection.
2. If microscope is of the upright style, a sterile cover slip can be put inside each well for cell culture. When required, the cover slip is simply taken out, placed on a slide and covered with some culture medium and another cover slip for checking expression status.

5 Concluding Remarks

Currently there is no single technology likely to solve complete problems in protein science. Recombinant technology contributes some important advances in protein science research such as enabling the design of simplified experiments with easier and more thoroughly controlled procedures. Utilization of recombinant technology with the support of other state of the art research platforms will accelerate the pace of protein science. The experimental protocols described here provide a basic guide on the application of recombinant technology and/or some hints for researchers to design and scale their experiments according to their situations.

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