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Short communication

The *Arabidopsis thaliana* natriuretic peptide AtPNP-A is a systemic regulator of leaf dark respiration and signals via the phloem

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ABSTRACT

Plant natriuretic peptides (PNPs) belong to a novel class of peptidic signaling molecules that share some structural similarity to the N-terminal domain of expansins and affect physiological processes such as water and ion homeostasis at nano-molar concentrations. Here we show that a recombinant *Arabidopsis thaliana* PNP (AtPNP-A) rapidly increased the rate of dark respiration in treated leaves after 5 min. In addition, we observed increases in lower leaves, and with a lag time of 10 min, the effect spread to the upper leaves and subsequently (after 15 min) to the opposite leaves. This response signature is indicative of phloem mobility of the signal, a hypothesis that was further strengthened by the fact that cold girdling, which affects phloem but not xylem or apoplastic processes, delayed the long distance AtPNP-A effect. We conclude that locally applied AtPNP-A can induce a phloem-mobile signal that rapidly modifies plant homeostasis in distal parts.

Introduction

Natriuretic peptides (NPs) are a family of peptide hormones in vertebrates that have a role in osmoregulation (Toop and Donald, 2004). The first evidence for the presence of NP in plants was that radioimmunoassays recognized molecules from extracts of Florida Beauty (Vesely and Giordano, 1991) and that exogenous application of human atrial NP (ANP) can increase plant transpiration and solute flow (Vesely et al., 1993). Subsequently, rat ANP was demonstrated to bind isolated leaf membranes (Gehring et al., 1996; Suwastika et al., 2000) and induce stomatal opening in a concentration, conformation and guanosine 3′,5′-cyclic monophosphate (cGMP) dependent manner (Pharmawati et al., 1998).

Plant NPs (PNPs) have since been immunoaffinity purified from a number of different species (Billington et al., 1997; Maryani et al., 2001; Rafudeen et al., 2003). Partial sequencing of these immunoreactant PNPs (irPNPs) led to the identification of two *Arabidopsis* sequences that show some similarity to expansins (Ludidi et al., 2002). PNPs have an N-terminal signal peptide directing them to the extracellular space – a location experimentally confirmed by the identification of *Arabidopsis thaliana* PNP (AtPNP-A) in the apoplast (Boudart et al., 2004). However, PNPs have lost the domain thought to be involved in cell wall binding, thus affording them increased mobility (Ludidi et al., 2002).

Exogenous application of recombinant and native PNPs promotes a number of physiological responses, including radial water movements out of the xylem, osmoticum-dependent protoplast swelling, transient increases in cGMP and ion H^+, K^+ and Na^+, fluxes (Gehring and Irving, 2003). PNPs also cause changes in the proteome consistent with their role as modulators of photosynthesis (Gottig et al., 2008; Garavaglia et al., 2010). In summary, these observations led to the hypothesis that PNPs are systemically mobile regulators of homeostasis. Since PNP responses have only been measured in isolated plant tissues, we set out to determine whether PNP treatment can systemically modify whole plant homeostasis.

Materials and methods

Preparation of recombinant AtPNP-A

AtPNP-A (AT2G18660) without its signal peptide was cloned into the pCRT7/NT-TOPO expression vector and maintained in TOP 10F′ *Escherichia coli* cells (Invitrogen, USA) (Morse et al., 2004). For expression purposes, pCRT7/NT-AtPNP-A was transformed into BL 21 Star pLys S *E. coli* cells (Invitrogen, USA) and cultured on LB agar supplemented with 100 mg/mL ampicillin and 34 mg/mL chloramphenical at 37 °C overnight. A single colony was grown at 37 °C in 100 mL LB until OD_{600} = 0.6 was reached. Protein expression was

Abbreviation: PNP, plant natriuretic peptide; LB, Lysogeny broth.

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induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside over 3 h. Cells were harvested by centrifugation and the 6xHis-tagged recombinant AtPNP-A purified as detailed previously (Morse et al., 2004). Protein refolding was performed using AKTA fast protein liquid chromatography equipment (Amersham Biosciences, UK) programmed to run a linear refolding gradient in which 8 M urea gradient buffer was linearly diluted to 0 M urea with refolding buffer (200 mM NaCl, 50 mM Tris–Cl, pH 8.0, 500 mM glucose, 0.05% (w/v) poly-ethyl glycol, 4 mM reduced glutathione, 0.04 mM oxidized glutathione, 100 mM non-detergent sulfobateine, 0.5 mM phenylmethanesulfonylfluoride (PMSF)) over 90 min. The renatured recombinant AtPNP-A was eluted in 2 mL elution buffer (200 mM NaCl, 50 mM Tris–Cl; pH 8.0, 250 mM imidazole, 20% (v/v) glycerol, 0.5 mM PMSF). Finally, recombinant AtPNP-A was concentrated and desalted using a centriplus filtration device (Millipore Corporation, USA) with a molecular weight cut-off of 3.0 kDa. The concentration of the purified protein was determined according to the Bradford method (Bradford, 1976) and its integrity evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

Plant growth conditions, leaf treatments and assaying procedures

Leaf dark respiration experiments were conducted on 8-month-old soft South African perennial forest sage, *Plectranthus ecklonii* Benth. Plants were grown in peat-based potting soil (*Stanler Farms*, South Africa) under green house conditions (average day/night temperature and humidity: 23/16 °C and 30/70%; range of day time irradiances and CO_2 concentrations: 150–650 μmol s^{-1} m^{-2} and 380–390 ppm, respectively). Leaf dark respiration was measured during the day, using a portable infra-red gas analyzer (LCpro+, ADC Bioscientific Ltd, UK). Prior to treatment, each leaf was enclosed in the leaf chamber (enhancing the darkness in the chamber by covering it with a black cloth) and the basal leaf dark respiration rate measured at 0 μmol photons m^{-2} s^{-1}. Leaves were then treated with 50 μL recombinant AtPNP-A solution or 50 μL sterile dH_2O (control), by evenly spreading 25 μL over the abaxial and 25 μL over adaxial leaf surfaces using a micropipette tip. Immediately (within 20 s) following treatment, the leaf was enclosed in the leaf chamber and its induced leaf dark respiration rate at 0 μmol photons m^{-2} s^{-1} recorded. For each leaf the leaf dark respiration rate was calculated as the difference between the induced and basal dark respiration rate.

Dose and time response assays

For the dose response assay, leaves were treated with 50 μL of either a 20 μg/mL recombinant AtPNP-A solution (1.0 μg protein); 40 μg/mL AtPNP-A (2.0 μg); 60 μg/mL AtPNP-A (3.0 μg); 80 μg/mL AtPNP-A (4.0 μg); 100 μg/mL AtPNP-A (5.0 μg) or 120 μg/mL AtPNP-A (6.0 μg) followed by measurement of leaf dark respiratory rates as described above. For the time-dependent assay, leaves were treated with 50 μL of a 100 μg/mL recombinant AtPNP-A solution or 50 μL of sterile dH_2O (control) and leaf dark respiration rates measured at 0, 2, 4, 6, and 8 min post-treatment.

Systemic response assay

To measure systemic responses to AtPNP-A, three adjacent pairs of leaves were selected per plant and four leaves labeled as shown in Fig. 2A. The basal dark respiration rates of these four leaves were measured as described. One leaf of the middle pair (leaf 1) was then treated with 50 μL of a 100 μg/mL recombinant AtPNP-A or 50 μL of sterile dH_2O (control) followed immediately by measurement of the induced leaf dark respiration rate. After 5 min, dark respiratory responses were measured in one leaf from the lower pair (leaf 2), in the opposite leaf of the middle pair (leaf 3) and in one leaf from the upper pair (leaf 4). Additional measurements were recorded for the systemic leaves (2, 3 and 4) at 10 and 15 min post-treatment of leaf 1.

Cold girdling assay

The systemic response assay was repeated with the petiole segments of the systemic leaves (2, 3 and 4) chilled by non-invasive ice-cold rings during the entire assaying period. Chilling was achieved by circulating ice-cold dH_2O around each leaf petiole in 1.0 mm (internal diameter) × 0.8 mm (wall thickness) plastic tubing using a small three-way (P-3) peristaltic pump (Pharmacia Fine Chemicals, Sweden) at a flow rate of 1.0 mL/min.

Statistical analysis

Results are the mean of the six biological replicates ($n = 6$) subjected to analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, Statsgraphics Corporation, USA). Where the ANOVA revealed significant differences, the means were separated using a *post hoc* Student–Newman–Keuls (SNK) multiple range test.

Results

In order to explore whether PNP treatment influences plant physiology, AtPNP-A was cloned, expressed and purified (Fig. 1A). The effects of AtPNP-A were investigated in the South African soft perennial forest sage, *P. ecklonii* Benth., a species that has been shown to respond to recombinant PNPs from different species (Maryani et al., 2003; Gottig et al., 2008). Recombinant AtPNP-A treatment increased the rate of leaf dark respiration in a dose-dependent manner, attaining a maximum rate of 5.33 ± 0.47 mmol s^{-1} m^{-2} in response to 5.0 μg of protein (Fig. 1B) corresponding to approximately 0.4 nmol AtPNP-A. At this dose, the response to AtPNP-A occurred rapidly (within 20 s) inducing a similar maximum rate of 5.42 ± 0.19 mmol s^{-1} m^{-2} 2 min after treatment, an effect sustained for a further 6 min (Fig. 1C), showing that treatment of *P. ecklonii* with nmol amounts of AtPNP-A stimulates the rate of leaf dark respiration in a concentration and time dependent manner.

To determine whether AtPNP-A responses could be initiated systemically, the effect of AtPNP-A treatment on the rate of dark respiration was measured in distal leaves. Three adjacent pairs of leaves were selected per plant. One leaf from the middle pair was treated with recombinant AtPNP-A, after which dark respiratory responses were measured in the treated leaf, in a leaf from the upper pair, in a leaf from the lower pair and in the opposite leaf of the middle pair (Fig. 2A). The treated leaf responded immediately to AtPNP-A with a threefold increase in the rate of dark respiration from 1.59 ± 0.22 mmol s^{-1} m^{-2} to 5.83 ± 0.43 mmol s^{-1} m^{-2} (Fig. 2B–D). In the distal leaves, a small but significant stimulation of the leaf dark respiration rate was first observed in the lower leaf, measuring 2.59 ± 0.45 mmol s^{-1} m^{-2} five min after treatment (Fig. 2B). In this leaf, the rate of dark respiration reached a maximum of 4.95 ± 0.02 mmol s^{-1} m^{-2} 10 min after treatment (approximately 86% of the response of the treated leaf) and remained elevated at 4.48 ± 0.23 mmol s^{-1} m^{-2} 15 min after treatment (Fig. 2C and D). Next, the upper leaf responded with an increased dark respiratory rate of 3.64 ± 0.20 mmol s^{-1} m^{-2} 10 min after treatment (approximately 58% of the treated leaf response) that was sustained 15 min after treatment (Fig. 2C and D). Finally, a small but consistent improvement in the rate of dark respiration was recorded 15 min after treatment in the leaf opposite to the treated leaf at 2.72 ± 0.03 mmol s^{-1} m^{-2} (approximately 39% of the response of the treated leaf) (Fig. 2D). Water control treatments