Bacterial and plant natriuretic peptides improve plant defense responses against pathogens

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Running title: PNPs increase plant defenses

Keywords: plant natriuretic peptide, AtPNP-A, XacPNP, plant defense, *Pseudomonas syringae* pv. *tomato*, *Xanthomonas axonopodis* pv. *vesicatoria*, *Xanthomonas citri* subsp. *citri*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/mpp.12560

SUMMARY

Plant natriuretic peptides (PNPs) are implicated in the regulation of ions and water homeostasis, and their participation in the plant immune response has been also proposed. *Xanthomonas citri* subsp. *citri* contains a gene encoding a PNP-like protein (*XacPNP*) that has no homologues in other bacteria. XacPNP mimics its *Arabidopsis thaliana* homolog AtPNP-A by modifying host responses to create favorable conditions for pathogen survival. However, the ability of XacPNP to induce plant defense responses was never investigated. Here, and to further study the role of XacPNP *in vivo*, *A. thaliana* lines over-expressing *XacPNP* and also, lines over-expressing *AtPNP-A* or *AtPNP-A* deficient plants were generated. Plants over-expressing *XacPNP* as well as *AtPNP-A* showed larger stomatal aperture and were more resistant to saline or oxidative stress than *PNP* deficient lines. First, and to further study the role of PNP in biotic stress responses, *A. thaliana* leaves were infiltrated with pure recombinant XacPNP and showed enhanced expression of genes related with the defense response and a higher resistance to pathogen infections. Moreover, *AtPNP-A* expression increased in *A. thaliana* upon *Pseudomonas syringae* pv. *tomato* (Pst) infection. These evidences led us to analyze the transgenic plants responses to pathogens. Plants over-expressing *XacPNP* and *AtPNP-A* were more resistant to Pst infection compared to control plants, while *PNP* deficient plants were more susceptible and showed a stronger HR when challenged with a non-host bacteria. Therefore, *XacPNP*, acquired by horizontal gene transfer, is able to mimic PNP functions even in the increase of plant defense responses.

INTRODUCTION

Natriuretic peptides (NPs) are a family of peptides involved in the regulation of salt and water homeostasis in vertebrates. The effects of NPs are mediated by their cognate signaling receptors. These receptors are guanylyl cyclases and as a result of ligand binding, intracellular cGMP concentrations increase (Potter, 2011). Plants possess heterologous proteins to animal NPs, named plant natriuretic peptides (PNPs) which are involved in a number of responses essential for plant homeostasis and growth (Gehring & Irving, 2003). Several activities have been observed for PNPs. Purified PNP application causes stomatal opening and activation of membrane H^+-ATPase in leaves, transient elevation of cGMP levels in roots tissue, increased osmoticum-dependent volume in leaf protoplasts and modulation of ions influx (H^+, K^+ and Na^+) across plasma membranes in roots (Pharmawati *et al.*, 1998a, Pharmawati *et al.*, 1998b, Pharmawati *et al.*, 1999, Suwastika & Gehring, 1999, Maryani *et al.*, 2003, Morse *et al.*, 2004, Ludidi *et al.*, 2004). The presence of a signal sequence in PNPs suggests that they are secreted into the plant apoplast; further, the immunodetection of PNPs in the conductive tissue of plants and the increase in cGMP levels due to PNP application have suggested that PNP function should be mediated via a receptor involving cGMP as a second messenger (Billington *et al.*, 1997, Suwastika *et al.*, 2000). Also, transiently transfected onion epidermal cells with PNP signal peptide fused to GFP showed that PNP-GFP is located in the extracellular space (Wang *et al.*, 2011) (for a review see (Gehring & Irving, 2013)).

The *Arabidopsis thaliana* most characterized PNP is AtPNP-A, encoded by At2g18660 gene. Recently, a receptor for AtPNP-A has been identified as a leucine-rich repeat protein with guanylyl cyclase activity that allows cGMP-dependent signaling

(Turek & Gehring, 2016). Several efforts have been conducted to elucidate AtPNP-A function, particularly during the stress response. A proteomic analysis of AtPNP-A-treated plant cells revealed that proteins involved in oxidation-reduction processes and in response to salt stress were over-represented. Consistently, it has been proposed that AtPNP-A has a key role in oxidation-reduction processes as well as in response to salt stress (Turek *et al.*, 2014). Further, AtPNP-A is able to modulate its own expression which enables the tuning of transcript and protein levels (Wang et al., 2011). It has also been reported that PNPs signaling employs a systemic signal to alter photosynthesis and respiration integrating the response to the whole plant (Ruzvidzo *et al.*, 2011). Besides, a gene ontology analysis of *AtPNP-A* and the 25 most expression correlated genes revealed an over-representation of genes associated with plant defense response related to both abiotic or biotic stress, suggesting the involvement of this type of molecules in plant defense responses (Meier *et al.*, 2008).

The phytopathogen *Xanthomonas citri* subsp. *citri* (Xcc) is the causal agent of the worldwide distributed citrus canker disease and this bacterium has a gene coding for a PNP-like protein named *XacPNP* (XAC2654; Q8PJ87) (Gottig *et al.*, 2008). Remarkably, no significant similarity between XacPNP and other bacterial proteins has been found to date even if homologues in phytopathogenic fungus have been found as Ave1 (avirulence on Ve1) present in *Verticillium dahliae, Verticillium alboatrum, Colletotrichum higginsianum*, *Cercospora beticola*, and *Fusarium oxysporum* f. sp. *lycopersici* (de Jonge *et al.*, 2012). Formerly, it has been proposed that *XacPNP* has been acquired in an ancient lateral gene transfer event from plants (Nembaware *et al.*, 2004) and evolutionary analysis of Ave1 proteins showed that these fungal proteins are

more related to plant PNPs than to XacPNP suggesting that also Ave1 proteins have been horizontally acquired from plants. (de Jonge *et al.*, 2012).

Previous results support the hypothesis that XacPNP behaves as a PNP mimicking the host protein. The pure recombinant XacPNP protein elicits physiological responses in plants similar to AtPNP-A (Gottig *et al.*, 2008) such as the increase in photosynthesis efficiency in plant tissues infiltrated with this peptide (Garavaglia *et al.*, 2010b). In line with these results, a comparative proteomic study of citrus leaves infected with wild type Xcc and a *XacPNP* deletion mutant revealed that photosynthetic proteins are under-represented in the tissue infected with the mutant strain and this can be reverted by the exogenous application of pure recombinant XacPNP or AtPNP-A (Garavaglia *et al.*, 2010a). During citrus infections *XacPNP* is expressed in Xcc and serves to keep the plant tissue hydrated and in a healthier state allowing the biotrophic pathogen to survive longer periods in the infected tissue and this also occurs when the bacterial gene is replaced by the plant *AtPNP-A* (Gottig *et al.*, 2008). Therefore, the main known function for XacPNP, much like PNPs, has been the modulation of plant homeostasis.

Despite the similar activities observed for XacPNP and AtPNP-A, the observation that *AtPNP-A* co-expresses with *A. thaliana* defense response genes, led us to wonder whether these proteins contribute to increase the plant defense response, besides their homeostatic function. Here, and since little is known about the *in vivo* role of PNPs, the effect of these proteins in the defense response of *A. thaliana* was analyzed. Accordingly, we analyzed whether AtPNP-A functions *in vivo* modulating plant defense responses. Further, we hypothesized that the bacterial PNP might mimic PNPs in the response to biotic stress. Transgenic *A. thaliana* lines over-expressing

AtPNP-A or bacterial *XacPNP*, as well as *AtPNP-A* deficient plants were obtained to evaluate these hypothesis. These plants were challenged with *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) that has been widely used to infect *A. thaliana* seedlings (Yao *et al.*, 2013) and in addition, the non-host interaction with *X. axonopodis* pv. *vesicatoria* (Xav) was evaluated. These studies allowed us to gain deeper insight about these peptides' function in plant defense responses and unveil that a bacterial PNP might be useful as a new candidate gene for crop improvement.

RESULTS

A. thaliana plants with modified expression of *XacPNP* and *AtPNP-A*.

To study if XacPNP could trigger plant defense responses and to analyze the participation of AtPNP-A *in vivo* in the defense response, *A. thaliana* plants with modified expression of these peptides were obtained. Transgenic Arabidopsis lines expressing *XacPNP* (named 35S:*XacPNP*) or *AtPNP-A* (named 35S:*AtPNP-A*) were obtained by transforming Col-0 *rdr6-11* plants since the background is deficient in silencing, allowing efficient overexpression of these genes (Mecchia *et al.*, 2013). The genes were cloned under the control of the 35S CaMV constitutive promoter in the binary vector pCHF3 (Fig. 1a). In addition, a mutant T-DNA insertion line (SALK_000951C) with an insertion in the second intron of *AtPNP-A* (*atpnp-A*) (Alonso *et al.*, 2003), as well as RNAi lines with reduced levels of *AtPNP-A* (*AtPNP-A*-RNAi) were characterized (Fig. 1a). To estimate the levels of *XacPNP* and *AtPNP-A* expression, RNA was extracted from 10 days-old plants obtained in independent transformation events with 35S:*XacPNP*, 35S:*AtPNP-A* or empty vector (EV) pCHF3 and RT-PCR and quantitative RT-PCR (qRT-PCR) assays were performed (Figure S1).

Figure 1b shows 35S:*XacPNP* and 35S:*AtPNP-A* lines with the highest expression. *AtPNP-A* expression levels were similar between 35S:*AtPNP-A* independent lines and at least 7-fold higher than the expression of endogenous *AtPNP-A* (Fig. 1b), 35S:*XacPNP* lines showed levels of expression of *XacPNP* of more than a hundred times when compared to the endogenous *AtPNP-A* in control plants (Fig. 1b). The expression of *AtPNP-A* in several RNAi lines and in *atpnp-A* lines (derived from SALK) was also determined and no transcripts were detected by RT-PCR (Figure S1). By qRT-PCR, no peaks were detected in *atpnp-A* lines whereas in RNAi lines levels of expression of less than 3% compared to Col-0 plants (with the empty pCHF3) were observed (Fig. 1b). The lines 35S:*XacPNP*$_2$, 35S:*XacPNP*$_3$, 35S:*AtPNP-A*$_1$ and 35S:*AtPNP-A*$_3$, with the highest expression levels and *atpnp-A*$_2$, *atpnp-A*$_4$, *AtPNP-A*-RNAi$_7$ and *AtPNP-A*-RNAi$_{11}$ were chosen for further analyses of PNP roles.

PNPs increase stomatal opening

Phenotypic characterization of 35S:*AtPNP-A*, 35S:*XacPNP*, *AtPNP-A*-RNAi and *atpnp-A* lines showed no significant changes in morphology, root length, leaf area, rosette fresh weight and flowering time, compared to their respective control lines or EV ones (Figure S2). We previously reported that treatment with recombinant XacPNP or AtPNP-A resulted in rapid and significant increase in stomatal conductance and aperture (Gottig *et al.*, 2008). Thus, stomata opening were measured in control and transgenic lines as well as in control treated with naphthalene acetic acid (NAA) and abscisic acid (ABA) that cause stomatal opening and closure, respectively. Stomata aperture was significantly increased in PNP over-expressing plants, while plants deficient in PNP showed reduced stomata opening ($p < 0.05$) (Fig. 2).

PNPs protect plant against saline and oxidative stress

Then, and in view of the proposed role for PNPs in regulating water and salt homeostasis, the response to saline stress was evaluated in the different *A. thaliana* lines. Plants were exposed to 150 mM NaCl one week after germination. Experiments were performed *in vitro* in MS 0.5X-agar due to the lack of reproducibility when they were performed in soil. Phenotypic characterization of the different lines in response to NaCl stress was evaluated. Three days post-treatment, chlorosis and dehydration signals were evident mostly in *atpnp-A* and *AtPNP-A*-RNAi plants, whereas, 35S:*XacPNP* and 35S:*AtPNP-A* lines showed better fitness (Fig. 3a). To further evaluate injury, remaining chlorophylls were quantified, where 35S:*XacPNP* displayed about 70% of remaining chlorophyll and 35S:*AtPNP-A* showed about 50% compared to the untreated control ($p < 0.05$) (Fig. 3b). In the case of *atpnp-A* and *AtPNP-A*-RNAi, the contrary was observed, showing 20% and 30% of remaining chlorophylls on average relative to the control, respectively ($p < 0.05$) (Fig. 3b). Next, the behavior of the different lines subjected to oxidative stress was evaluated. For this purpose, plants were challenged with the herbicide methyl viologen (MV), a superoxide-generating agent (Youngman & Dodge, 1979). Two days after treatment, chlorotic lesions caused by the herbicide were observed. Chlorosis was even greater in *atpnp-A* and *AtPNP-A*-RNAi, while 35S:*XacPNP* and 35S:*AtPNP-A* showed similar damage compared to control lines (Fig. 3c). The amount of remaining chlorophylls was calculated and 35S:*XacPNP* and 35S:*AtPNP-A* plants showed slightly higher, though no significantly different ($p < 0.05$), levels of pigments compared to controls. In contrast, PNP deficient lines showed almost three times less remaining chlorophyll than controls ($p < 0.05$) (Fig. 3d). Ion

leakage was also quantified and plants expressing XacPNP showed the lowest ion leakage percentage, with a value of 18% against 38% observed in control plants ($p < 0.05$). The levels of ion leakage of 35S:*AtPNP-A* plants were similar to control plants. PNP deficient plants were the most damaged displaying values of 75-82% while controls were about 40% (Fig. 3e).

XacPNP triggers *A. thaliana* defense responses

To analyze the participation of XacPNP in plant biotic stress triggering plant defense responses, *A. thaliana* Col-0 leaves were infiltrated with the recombinant pure XacPNP protein and at 6 hour post-inoculation (hpi), transcript levels of several *A. thaliana* genes related to plant defense responses were analyzed. RNA was extracted and real-time quantitative reverse-transcriptase polymerase chain reactions (RT-qPCR) were performed. Several defense marker genes were analyzed such as *MITOGEN ACTIVATED PROTEIN KINASE 3* (*MAPK3*, At3g45640), *MAP KINASE KINASE 4* (*MKK4*, At1g51660), *WRKY30* transcription factor (*WRKY30*, At5g24110), *GLUTATHIONE-S-TRANSFERASE 1* (*GST1*, At1g02930), *PHENYLALANINE AMMONIA-LYASE 1* (*PAL1*, At2g37040), *PATHOGENESIS RELATED 1* (*PR1*, At2g14610) and *PR5* (At1g75040). RT-qPCR analysis showed that all transcripts were significantly ($p < 0.05$) more abundant in plants treated with XacPNP than in the 6XHis-Trx-infiltrated controls with a major up-regulation for *GST1*, related to the oxidative stress response (Fig. 4a).

Then, and to further evaluate whether XacPNP is involved in the defense response, the ability of this protein to enhance *A. thaliana* Col-0 resistance to Pst was evaluated. Leaves were pre-infiltrated with 5 µM XacPNP and 6XHis-Trx as a control.

The pre-infiltrated leaves were then infiltrated with Pst at 5 x 10^6 CFU/ml and bacterial growth at 3 and 6 days post-inoculation (dpi) was monitored. At both times analyzed, XacPNP was able to induce defense responses reducing significantly ($p < 0.05$) the population of bacteria by about two orders of magnitude (Fig. 4b).

AtPNP-A expression is induced in _A. thaliana_ upon Pst infection.

AtPNP-A expression in leaves infected with Pst was quantified. For this, plants were infected with Pst at 5 x 10^6 CFU/ml and at 0, 2 and 4 dpi tissue samples were collected, RNA was extracted and expression levels of *AtPNP-A* were quantified. As a marker of infection, expression of *PR1* was also evaluated. *AtPNP-A* and *PR1* expression showed a significant increase ($p < 0.05$) with a similar pattern in infected *A. thaliana* tissues relative to non-infected tissues (Fig. 4c). Altogether these results suggest that XacPNP can promote defense responses in *A. thaliana* and that AtPNP-A also contributes to the plant defense response.

PNPs increase defense responses both in host and non-host interactions

Then, the response of *A. thaliana* lines with modified levels of PNPs to pathogens was evaluated. Flood-inoculation with the virulent pathogen Pst at 1 x 10^6 CFU/ml was performed. This method allows reproducibility of the phenotypes observed and is more similar to natural infection than inoculation by infiltration (Ishiga *et al.*, 2011). The different lines showed similar infection phenotypes 3 dpi, except for 35S:*XacPNP* lines that displayed less damaged tissue (Fig. 5a). To better characterize the infection process, *in planta* bacterial growth measurements were performed. At 3 and 5 dpi, PNPs overexpressing plants showed a decrease of 1 or 2 orders of magnitude in bacterial

number compared to control lines, whereas PNP deficient lines, exhibited an increased bacterial growth at both times, reaching populations of 10^{12} CFU/mg of tissue at 5 dpi while control lines reached only 10^{10} CFU/mg (Fig. 5b). To understand the differences in disease phenotypes and taking into consideration the augment in defense gene expression in recombinant XacPNP-treated leaves (Fig. 4a), the expression of different genes related to defense responses was analyzed at 24 hpi. 35S:*XacPNP* and 35S:*AtPNP-A* plants showed a major induction of *MKK4, WRKY30, PAL1* and *PR1* genes than control plants whereas in PNP deficient lines, the opposite occurred (Fig. 5c).

Then, inoculation with Xav at 5 x 10^7 CFU/ml was assayed. In this case, all the lines showed the typical defense response known as Hypersensitive Response (HR), being the symptoms observed similar in 35S:*XacPNP* and 35S:*AtPNP-A* plants compared to control lines at 24 hpi, however, *atpnp-A* and *AtPNP-A*-RNAi plants showed more damaged tissue (Fig. 6a). This was also evidenced by the detection of hydrogen peroxide (H_2O_2) at 18 hpi, assessed by staining the plants with 3,3'-diaminobenzidine (DAB). Microscope observations (Fig. 6b) and quantification of DAB staining intensities (Fig. 6c) showed that in PNP deficient lines H_2O_2 production was significantly greater than in control lines and in PNP overexpressing lines this production was diminished ($p < 0.05$).

Finally, ion leakage was analyzed in these Xav-treated plants and also a significant major damage was observed in *atpnp-A*$_4$ and *AtPNP-A*-RNAi lines, while in 35S:*XacPNP* ion leakage was even lower than in control plants ($p < 0.05$). In the case of of 35S:*AtPNP-A* no significant differences compared to the control were observed (Fig. 6d).

DISCUSSION

Plant natriuretic peptides are known for modulating salt and water homeostasis (Gehring & Irving, 2013). Besides, the participation of AtPNP-A in the plant defense response against pathogens has been proposed taking into account that it is co-expressed along with genes involved in this response (Meier *et al.*, 2008, Turek *et al.*, 2014). Also, the recently found AtPNP-A receptor which is a leucine rich-repeat receptor-like kinase and belongs to a family of proteins associated with plant defense responses to pathogens (Turek & Gehring, 2016), strengthen the idea that PNPs may be involved in the response to plant pathogens.

Previously, we demonstrated that *XacPNP* as well as *AtPNP-A* expressed in *X. citri* subsp. *citri* are able to limit necrotic progress, improve photosynthetic efficiency and water homeostasis during the disease in citrus leaves (Gottig *et al.*, 2008, Garavaglia *et al.*, 2010a). In this work, we are going beyond, revealing a role for PNPs in plant defense responses. The observations that: (i) pure XacPNP induces the expression of defense genes in Arabidopsis plants, (ii) pretreatment with recombinant XacPNP makes these plants more resistant to Pst and (iii) *AtPNP-A* shows a similar pattern of expression than *PR1* upon infection, indicate that PNPs have a role in plant defense responses. Moreover, and for the first time, transgenic *A. thaliana* lines with modified levels of PNPs expression were obtained to further evaluate the role of PNP in defense responses. These plants displayed different phenotypes regarding stomatal aperture. Previously, we have observed that pure XacPNP or AtPNP-A proteins are able to induce stomata opening (Gottig *et al.*, 2008). Accordingly, plants overexpressing *XacPNP* or *AtPNP-A* have an increase in stomatal aperture compared to control plants

and deficient PNP lines showed a clear impairment in stomatal aperture, giving support to the function previously observed for PNPs (Gottig *et al.*, 2008). The active closure of stomata upon bacterial contact is a widespread defense response in plants exposed to potential pathogens. Stomata have a function in the early phases of innate immunity; in particular, stomata are closed 1 to 2 h after bacterial recognition, preventing their entry in the host tissue (Melotto *et al.*, 2006). Hereof, Arabidopsis mutants that keep their stomata open in response to Pst, and thus not responding as a barrier to bacterial invasion, do not show significantly higher susceptibility to the pathogen (Zeng *et al.*, 2011). Accordingly, 35S:*XacPNP* and 35S:*AtPNP-A* plants, though showing increased stomatal aperture than wild type plants, did not display increased susceptibility, or *PNP* deficient plants with stomata less opened did not show more resistance to Pst. These results suggest that the stomata aperture of the different plant lines is not determining bacterial entrance in this instance.

PNPs were also able to protect *A. thaliana* against saline stress that is related to the known function of PNP as regulator of K^+, Na^+, and H^+ fluxes. In this work another function has been attributed to PNPs and it is the protection against oxidative stress. A proteomic analysis of PNP-treated leaves showed an overrepresentation of the gene ontology (GO) terms “oxidation-reduction process”, “translation” and “response to salt stress” (Turek *et al.*, 2014). These results together with our findings put forward the participation of PNPs in modulating oxidative cell responses, besides its role in regulating ion fluxes.

In plant-pathogen interactions, the challenge with Pst that causes disease, revealed that transgenic plants overexpressing *XacPNP* or *AtPNP-A* were more resistant to this pathogen compared to control plants by impairing bacterial growth, maybe through the

increased expression of defense genes. By the contrary, *PNP* deficient plants showed the opposite effect being more susceptible to Pst. In the of case of the non-host response challenged with Xav, *PNP* deficient plants displayed stronger HR than both *PNP* over-expressing and control plants. Even if HR is related to the defense response, this stronger HR observed might be a consequence of larger tissue damage in the absence of PNP, since PNP has a role in tissue health maintenance The role of this peptide as a molecule that maintains plant tissues in a healthier state and that also induces the defense response may be an adaptive reaction of the plant to fight against pathogens without the detrimental of damaging the plant own tissue. In general, 35S:*XacPNP* plants displayed a more noticeable phenotype than 35S:*AtPNP-A* plants and this may be due to the higher levels of expression observed in the first ones. It has been shown that AtPNP-A is able to modulate its own expression (Wang *et al.*, 2011). Albeit a Col-0 line impaired in gene silencing was used, and that expression was under 35S CaMV promoter, *AtPNP-A* may have been subjected to additional regulation, considering that *XacPNP* is a bacterial gene not encoded in the plant genome. It is worth to mention that XacPNP is able to exert its function while infiltrated as pure protein or while expressed by the plant. The recent finding of AtPNP-A receptor protein opens the question whether XacPNP interacts with this receptor and if it has the same affinity as the Arabidopsis peptide. Both peptides share the conserved active site (Nembaware *et al.*, 2004) and this region in AtPNP-A is sufficient for the interaction with the receptor to occur (Turek & Gehring, 2016), suggesting that they may have similar affinity for the receptor protein and therefore the overexpression of *XacPNP* that is higher than the overexpression of *AtPNP-A* may explain the more pronounced phenotypes observed in 35S:*XacPNP* plants Our results reinforce the idea that XacPNP mimics its cognate in

plants and even if it has been adapted to be expressed in the unique bacterium Xcc, it conserves its biological activity as its plant counterpart.

In summary, we reveal a role for AtPNP-A and the bacterial XacPNP in biotic and abiotic stress responses and propose that PNPs are part of a concerted action to cope with environmental changing conditions that plants have to adapt ranging from oxidative stress and salt stress to the challenge of a pathogen attack. Moreover, this work enhances the understanding of a bacterial-acquired gene that mimics AtPNP-A function in defense responses to plant pathogens.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis plants ecotype Columbia Col-0 *rdr6-11* (At3g49500) (Mecchia *et al.*, 2013) were used for construction of plants overexpressing PNPs, in order to achieve higher gene expression by avoiding gene silencing (Butaye *et al.*, 2004). Arabidopsis plants ecotype Columbia (Col-0) was used for construction of *AtPNP-A*-RNAi experiments and Col-0 (SALK_000951C) insertional mutant was purchased from the Arabidopsis Biological Resource Center. Homozygous mutants from this line were isolated by PCR-based genotyping using the gene-specific PCR primers (AtPNPs, see Table S1). Seeds were surface sterilized with ethanol 70% (v/v) Tween-20 0.1% (v/v) for 10 min and spread on Murashige & Skoog (MS) basal medium plates. Seeds on plates were stratified for 2 days and then kept in growth chambers at 22 °C under fluorescent light, with 16:8 photoperiods. Seedlings were transferred to soil pots and kept in the same conditions until harvested.

Arabidopsis transformation

A. thaliana transgenic plants overexpressing *XacPNP* or *AtPNP-A* and RNA interfering *AtPNP-A* lines were obtained. *AtPNP-A* was amplified from Col-0 WT cDNA using 35S:AtPNP oligonucleotides (Table S1) and it was subcloned into *Kpn*I-*Bam*HI of pCHF3 binary vector under the control of cauliflower mosaic virus (CaMV) 35S promoter. For *XacPNP* overexpression, the plant PNP signal peptide was fused to *XacPNP* coding sequence. The signal peptide was amplified using PNP-SP primers and *XacPNP* with 35S:XacPNP ones. Both PCR products were used as templates in a second round of amplification and the final fusion product was subcloned into pCHF3 binary vector under the control of the 35S CaMV promoter. For RNA interfering lines, *AtPNP-A* was amplified using AtPNP-A-RNAi primers and the product was subcloned into *Asc*I-*Swa*I and *Xba*I-*Bam*HI sites from vector pFGC5941, which allows efficient silencing of a gene of interest (www.arabidopsis.org). The resulting constructs pCHF3-*XacPNP*, pCHF3-*AtPNP-A* and pFGC5941-*AtPNP-A* were introduced into *Agrobacterium tumefaciens* GV3101 pMP90 strain by electroporation. Agrobacterium mediated plant transformation using floral dip was used to obtain transgenic lines in several independent transformations for each construct (Zhang *et al.*, 2006). T1 seeds were selected on plates containing kanamycin when the vector pCHF3 was used or by spraying the herbicide BASTA to soil grown plants when the vector pFGC5941 was used. Homozygous T3 or T4 seeds were obtained and several independent lines were analyzed for each construct. For phenotype controls, Arabidopsis lines were obtained using both Col-0 and Col-0 *rdr6-11* plants transformed with empty pCHF3 and pFGC5941 vectors (EV).

RNA preparation, RT-PCR and RT-qPCR

Total RNA from leaves was isolated using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. RT-PCRs and RT-qPCRs were performed as described previously (Sgro *et al.*, 2012), with the specific oligonucleotides detailed in Table S1. Values are the means of three biological replicates with three technical replicates each.

Stomatal opening assay

Leaves from 3 to 4-weeks-old plants were used. Individual leaves were incubated in 1:1 ethanol: KOH 5 % (p/v) at 60˚C for 30 min, then washed and cleared using ethanol. Leaves were stained with 1 % (p/v) safranin solution to increase contrast. NAA 1 μM and ABA 50 μM were used as controls. In this case, leaves were incubated for 2 h in 10 mM MES, 10 mM KCl, pH 6.5, supplemented with each hormone. Pore widths of around 100-150 stomata were measured under the microscope with a calibrated ocular micrometer.

Saline and oxidative stress treatments

Seeds from the different *A. thaliana* lines were grown in MS 0.5X-agar 0.8% for 7 days. Then, they were transplanted to MS plates as controls and to MS plates with the addition of NaCl 150 mM to evaluate saline stress or MV 30 μM for oxidative stress.

Bacterial strains and growth conditions

Pseudomonas syringae pv. *tomato* DC3000 (Pst) and *Xanthomonas axonopodis* pv. *vesicatoria* (Xav) were grown at 28 °C in LB (Luria Bertani) medium and Silva Buddenhagen (SB) medium (5 g/l sucrose, 5 g/l yeast extract, 5 g/l peptone, and 1 g/l glutamic acid, pH 7.0), respectively, with constant agitation at 200 rpm on a rotating shaker.

Plant inoculation and *in planta* growth assays

Plant flood-inoculation was performed as previously described (Ishiga *et al.*, 2011) Col-0 or Col-0 *rdr6-11* plants were grown in Petri dishes containing MS 0.5X, vitamins, phytagel 0.3% (p/v) for two weeks. Bacterial cultures were diluted to the required concentrations in sterile water supplemented with Silwett L-77 0.025% (v/v) and incubated with the plants for 3 min, then plates were drained and incubated in growth chamber. Bacterial growth assays were performed by grinding previously weighed leaves in a 10 mM $MgCl_2$ solution, diluted, and plated in SB or LB plates to determine CFU/mg of fresh weight. Experiments were repeated three times and plants from two plates were evaluated per bacterial strain in each experiment. For the analysis of Pst growth in leaves pre-infiltrated with XacPNP, leaves were infiltrated with needleless syringes with 5 μM purified XacPNP and 5 μM 6XHis-Trx as a control. Proteins were purified as described previously (Gottig et al., 2008). After 6 h these leaves were infiltrated with Pst suspension at 10^6 CFU/ml. Growth assays were performed from 10 infiltrated leaves for each treatment at the indicated times as detailed above.

Chlorophyll and ion leakage measurements

These methods were performed as previously described (Dunger *et al.*, 2012). Chlorophyll extraction was done 3 days post treatment after saline stress and 2 days post treatment after oxidative stress. Values are expressed as the remaining percentage of chlorophylls compared to control infiltrated tissue. Ion leakage in Xav inoculated plants was performed 18 h post treatment by placing leaf discs in 2 ml of water. In methyl viologen-treated leaves, assay was done 8 h post treatment. Conductivity measurements were taken from each tube. Conductance of boiled tissue was measured and the values were used as representative of 100% of ion content (Dunger *et al.*, 2005). The experiments were performed with three leaves and repeated three times.

DAB staining

Plants from the different lines were flood-inoculated with Xav 10^7 CFU/ml and after 1 day, leaves were cut and the petioles were submerged in a 0.1% (w/v) DAB solution and kept in the dark overnight. The leaves were cleared in ethanol and observed and photographed in an optical microscope. DAB intensity was calculated from the digital photographs by the number of brown pixels relative to the total number of pixels covering the plant material, using Photoshop CS3 software. Average DAB measurements were calculated from at least 5 photographs from three independent experiments.

Statistical analysis

In all figures, bars are the mean of the data and error bars are the standard deviation. All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD test when stated.

ACKNOWLEDGEMENTS

We thank Diego Aguirre (IBR-CONICET) for plant technical assistance. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica [PICT2013-0625] and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP2014-2016. BSG, NG and JO are staff members and FAF and CG are fellows of CONICET.

SUPPORTING INFORMATION

Table S1. List of oligonucleotides used in this work.

Figure S1. Further expression analysis of PNPs modified plants.

Figure S2. Phenotypic analysis of plants with modified levels of PNPs.

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FIGURE LEGENDS

Figure 1. Construction of *A. thaliana* lines with modified levels of PNPs and analysis of PNPs expression. (a) Schematic representation of the constructions used to obtain the transgenic lines. *AtPNP-A* sequences are represented in light violet and *XacPNP* in blue. (b) Relative expression of PNPs analyzed by RT-qPCR of selected lines of transgenic plants and compared to the endogenous *AtPNP-A* levels in control plants. rdr6 EV means Col-0 *rdr 6-11* bearing pCHF3 and Col-0 EV, Col-0 with pFGC5941 (EV, empty vector). Nd, not detected. Values are the means of three biological replicates with three technical replicates each. Error bars indicate standard deviations. The data were analyzed with ANOVA followed by Tukey test. Bars with different letter are significantly different ($p < 0.05$).

Figure 2. PNPs promote stomatal aperture. Quantification of stomatal apertures in leaves of *A. thaliana* transgenic lines. As control (C), Col-0 and Col-0 *rdr6-11* were analyzed and they showed similar opening values and thus the average value was considered as control. Naphthalene acetic acid (NAA) 1 μM and abscisic acid (ABA) 50 μM were used as controls of aperture and closure, respectively. Bars are the means of apertures of 100 stomata, and the results are representative of three independent experiments. Error bars indicate standard deviations. The data were analyzed with ANOVA followed by Tukey test. Bars with different letter are significantly different ($p < 0.05$).

Figure 3. PNPs protect *A. thaliana* against saline and oxidative stress. (a) One week-old plants were transplanted to 150 mM NaCl-containing plates. Representative photographs after 3 days of treatment are shown. EV, empty vector. (b) Chlorophylls were quantified at 3 days post treatment and referred to chlorophylls in the control

treatment. (c) One week-old plants were transplanted to 30 μM MV-containing plates and representative photographs after 2 days of treatment are shown. (d) Chlorophylls were quantified at 2 days post treatment and referred to chlorophylls in the control. (e) Ion leakage quantification of leaves from the lines stated after 8 h incubation with 30 μM MV. In (b), (d) and (e), values represent an average of 5-10 leaves per line and the experiments repeated three times. Error bars are standard deviations. The data were analyzed for statistical differences by one-way ANOVA followed by Tukey test. Bars with different letter are significantly different ($p < 0.05$).

Figure 4. Induction of the defense response by XacPNP and *AtPNP-A* expression pattern upon Pst infection. (a) RT-qPCR of *A. thaliana* genes related with defense responses. RNA was extracted from leaves infiltrated with 5 μM XacPNP at 6 hpi. Bars indicate the expression levels of the indicated gene relative to the control treatment. As control, 5 μM 6XHis-Trx was used. (b) Enhancement of the defense response mediated by XacPNP. Quantification of Pst growth at 0, 3 and 6 dpi in leaves pre-infiltrated with 5 μM XacPNP and 6XHis-Trx as control. (c) RT-qPCR of *AtPNP-A* and *PR1* in *A. thaliana* leaves infected with Pst at 0, 2 and 4 dpi. Values are the means of three biological replicates with three technical replicates each. Error bars indicate standard deviations. The data were analyzed with ANOVA ($p < 0.05$).

Figure 5. PNPs protect *A. thaliana* against Pst. Two-three weeks-old plants were flood-inoculated with Pst. (a) Representative images of plants 3 dpi are shown. Numbers represent independent lines. EV, empty vector. (b) Quantification of bacterial populations' size at the times stated. Bars indicate the mean bacterial population at log CFU/mg tissue. An average of 4 plants was used for each line. The data were analyzed by ANOVA followed by Tukey test ($p < 0.05$). Bars with different letter are

significantly different ($p < 0.05$). For the sake of clarity letter for the population at 0 dpi was omitted. (c) RT-qPCR of *A. thaliana* genes related with defense responses at 24 hpi. Numbers on x-axis represent independent transgenic lines. Bars indicate the gene expression levels, relative to infected control plants that were not included in the figure. In all cases, experiments were repeated three times and error bars indicate standard deviations. The data were analyzed by ANOVA followed by Tukey test. Bars with different letter are significantly different ($p < 0.05$). Comparisons were performed among different lines for each gene.

Figure 6. PNPs alleviate HR symptoms in the interaction of *A. thaliana* with Xav. Four weeks-old plants were flood-inoculated with Xav. (a) Representative images of plants 2 dpi are shown. Col-0 and Col-0 EV showed similar symptoms as Col-0 rdr6 EV (rdr6 EV) and due to the large data were not included. EV, empty vector. (b) DAB detection of H_2O_2 accumulation in leaves inoculated with Xav at 1 dpi. Representative photographs of DAB staining leaves are shown. (c) Quantification of DAB staining from the microscopic images was done as described in Materials and Methods. The means were calculated from 5 photographs from three independent experiments for each line. Error bars indicate standard deviations. Mock indicates a treatment with sterile water and silwett L-77 0.025% (v/v). nd, not detected. The data was analyzed by ANOVA followed by Tukey test. Bars with different letter are significantly different ($p < 0.05$). (d) Ion leakage quantification at 18 hpi. Values represent an average of 4 plants assayed for each line and the experiment repeated three times. Error bars are standard deviations. The data was analyzed by ANOVA followed by Tukey test. Bars with different letter are significantly different ($p < 0.05$).