Bacterial and plant natriuretic peptides improve plant defence responses against pathogens

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SUMMARY

Plant natriuretic peptides (PNPs) have been implicated in the regulation of ions and water homeostasis, and their participation in the plant immune response has also been proposed. *Xanthomonas citri* ssp. *citri* contains a gene encoding a PNP-like protein (*XacPNP*) which has no homologues in other bacteria. XacPNP mimics its *Arabidopsis thaliana* homologue AtPNP-A by modifying host responses to create favourable conditions for pathogen survival. However, the ability of XacPNP to induce plant defence responses has not been investigated. In order to study further the role of XacPNP *in vivo*, *A. thaliana* lines over-expressing *XacPNP*, lines over-expressing *AtPNP-A* and *AtPNP-A*-deficient plants were generated. Plants over-expressing *XacPNP* or *AtPNP-A* showed larger stomatal aperture and were more resistant to saline or oxidative stress than were *PNP*-deficient lines. In order to study further the role of PNP in biotic stress responses, *A. thaliana* leaves were infiltrated with pure recombinant XacPNP, and showed enhanced expression of genes related to the defence response and a higher resistance to pathogen infections. Moreover, *AtPNP-A* expression increased in *A. thaliana* on *Pseudomonas syringae* pv. *tomato* (*Pst*) infection. This evidence led us to analyse the responses of the transgenic plants to pathogens. Plants over-expressing *XacPNP* or *AtPNP-A* were more resistant to *Pst* infection than control plants, whereas *PNP*-deficient plants were more susceptible and showed a stronger hypersensitive response when challenged with non-host bacteria. Therefore, *XacPNP*, acquired by horizontal gene transfer, is able to mimic PNP functions, even with an increase in plant defence responses.

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

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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 signalling receptors. These receptors are guanylyl cyclases and, as a result of ligand binding, intracellular cyclic guanosine monophosphate (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 and Irving, 2003). Several activities have been observed for PNPs. Purified PNP application causes stomatal opening and the activation of membrane H^+-ATPase in leaves, transient elevation of cGMP levels in root tissue, increased osmoticum-dependent volume in leaf protoplasts and modulation of ion influx (H^+, K^+ and Na^+) across plasma membranes in roots (Ludidi *et al.*, 2004; Maryani *et al.*, 2003; Morse *et al.*, 2004; Pharmawati *et al.*, 1998a,b, 1999; Suwastika and Gehring, 1999). 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 as a result of 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). In addition, in transiently transfected onion epidermal cells with a PNP signal peptide fused to green fluorescent protein (GFP), PNP-GFP is located in the extracellular space (Wang *et al.*, 2011) (for a review, see Gehring and Irving, 2013).

The most well-characterized PNP of *Arabidopsis thaliana* is AtPNP-A, encoded by the At2g18660 gene. Recently, a receptor for AtPNP-A has been identified as a leucine-rich repeat protein with guanylyl cyclase activity which allows cGMP-dependent signalling (Turek and 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 the response to salt stress were over-represented. Consistently, it has been proposed that AtPNP-A plays a key role in oxidation–reduction processes and in the 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 PNP signalling employs a systemic signal to alter photosynthesis and respiration, integrating the response to the whole plant (Ruzvidzo *et al.*, 2011). In addition, a gene ontology analysis of *AtPNP-A* and the 25 most expression-correlated genes has revealed an over-representation of genes associated with the plant defence response related to both abiotic and biotic stress, suggesting the involvement of this type of molecule in the plant defence response (Meier *et al.*, 2008).

The phytopathogen *Xanthomonas citri* ssp. *citri* (*Xcc*) is the causal agent of the worldwide-distributed citrus canker disease, and this bacterium possesses a gene coding for a PNP-like protein, named *XacPNP* (XAC2654; Q8PJ87) (Gottig *et al.*, 2008). Remarkably, no significant similarities between XacPNP and other bacterial proteins have been found to date, although homologues in phytopathogenic fungi have been found, i.e. 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* was acquired in an ancient lateral gene transfer event from plants (Nembaware *et al.*, 2004), and evolutionary analysis of Ave1 proteins has shown that these fungal proteins are more related to plant PNPs than to XacPNP, also suggesting that Ave1 proteins have been horizontally acquired from plants (de Jonge *et al.*, 2012).

Previous results have supported 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 an increase in the efficiency of photosynthesis 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 were under-represented in the tissue infected with the mutant strain, and this could be reversed 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, is the modulation of plant homeostasis.

Despite the similar activities observed for XacPNP and AtPNP-A, the observation that *AtPNP-A* is co-expressed with *A. thaliana* defence response genes led us to wonder whether these proteins contribute to increase the plant defence response, in addition to their homeostatic function. As little is known about the *in vivo* role of PNPs, in this article, we investigated the effect of these proteins in the defence response of *A. thaliana*. Accordingly, we analysed whether AtPNP-A functions *in vivo* to modulate plant defence responses. Further, we hypothesized that the bacterial PNP may 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 hypotheses. These plants were challenged with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) which has been widely used to infect *A. thaliana* seedlings (Yao *et al.*, 2013) and, in addition, the non-host interaction with *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) was evaluated. These studies allowed us to gain a deeper insight into the function of these peptides in the plant defence response and to reveal that a bacterial PNP may be useful as a new candidate gene for crop improvement.

RESULTS

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

To study whether XacPNP could trigger plant defence responses and to analyse the participation of AtPNP-A *in vivo* in the defence 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, as the background is deficient in silencing, allowing efficient over-expression of these genes (Mecchia *et al.*, 2013). The genes were cloned under the control of the 35S *Cauliflower mosaic virus* (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) and RNA interference (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-day-old plants obtained in independent transformation events with 35S:*XacPNP*, 35S:*AtPNP-A* or empty vector (EV) pCHF3, and reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) assays were performed (Fig. S1, see Supporting Information). Figure 1b illustrates that 35S:*XacPNP* and 35S:*AtPNP-A* lines show the highest expression. *AtPNP-A* expression levels were similar between 35S:*AtPNP-A* independent lines and at least seven-fold higher than the expression of endogenous *AtPNP-A* (Fig. 1b). 35S:*XacPNP* lines showed levels of expression of *XacPNP* of more than 100 times those of 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 (Fig. S1). By qRT-PCR, no peaks were detected in *atpnp-A* lines, whereas, in RNAi lines, levels of expression of less than 3% relative 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
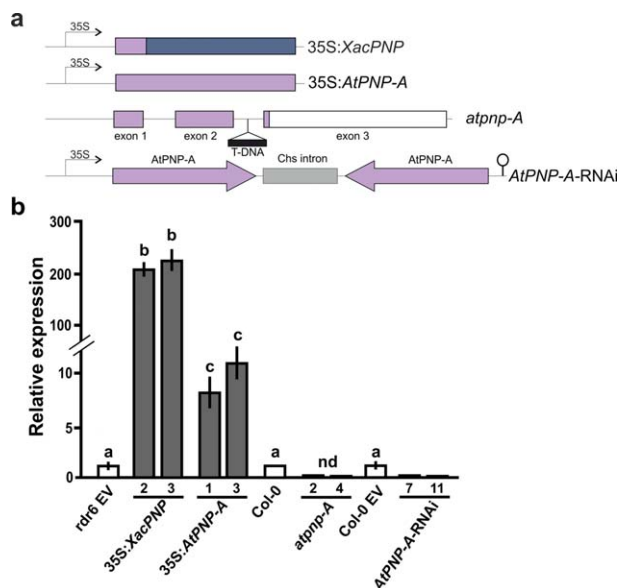
Fig. 1 Construction of *Arabidopsis thaliana* lines with modified levels of plant natriuretic peptides (PNPs) and the analysis of PNP expression. (a) Schematic representation of the constructions used to obtain the transgenic lines. *AtPNP-A* sequences are represented in light violet and *XacPNP* in blue. (b) Relative expression of PNPs analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of selected lines of transgenic plants and compared with the endogenous *AtPNP-A* levels in control plants. rdr6 EV denotes Col-0 *rdr6-11* bearing pCHF3, and Col-0 EV denotes 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 analysed by analysis of variance (ANOVA) followed by Tukey's test. Bars with different letters are significantly different ($P < 0.05$).

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 the roles of PNPs.

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 with their respective control lines or EV lines (Fig. S2, see Supporting Information). We have reported previously that treatment with recombinant XacPNP or AtPNP-A results in a rapid and significant increase in stomatal conductance and aperture (Gottig *et al.*, 2008). Thus, stomatal opening was measured in control and transgenic lines, as well as in controls treated with naphthalene acetic acid (NAA) and abscisic acid (ABA), which cause stomatal opening and closure, respectively. The stomatal aperture was increased significantly in PNP-over-expressing plants, whereas plants deficient in PNP showed reduced stomatal opening ($P < 0.05$) (Fig. 2).

PNPs protect plants against saline and oxidative stress

In view of the proposed role of PNPs in the regulation of water and salt homeostasis, the response to saline stress was evaluated in the different *A. thaliana* lines. Plants were exposed to 150 mM NaCl at 1 week after germination. Experiments were performed *in vitro* in 0.5 X MS (Murashige and Skoog) agar because of the lack of reproducibility when they were performed in soil. Phenotypic characterization of the different lines in response to NaCl stress was evaluated. At 3 days post-treatment, chlorosis and dehydration signals were evident mainly 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, the remaining chlorophyll was quantified, with 35S:*XacPNP* lines displaying about 70% remaining chlorophyll and 35S:*AtPNP-A* lines showing about 50% compared with the untreated control ($P < 0.05$) (Fig. 3b). In the case of *atpnp-A* and *AtPNP-A*-RNAi, on average, only 20% and 30% of remaining chlorophyll were observed relative to the control, respectively ($P < 0.05$) (Fig. 3b). Next, the behaviour 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 and Dodge, 1979). Two days after treatment, chlorotic lesions caused by the herbicide were observed. Chlorosis was greater in *atpnp-A* and *AtPNP-A*-RNAi, whereas 35S:*XacPNP* and 35S:*AtPNP-A* showed similar damage to the control lines (Fig. 3c). The amount of remaining chlorophyll was calculated, and 35S:*XacPNP* and
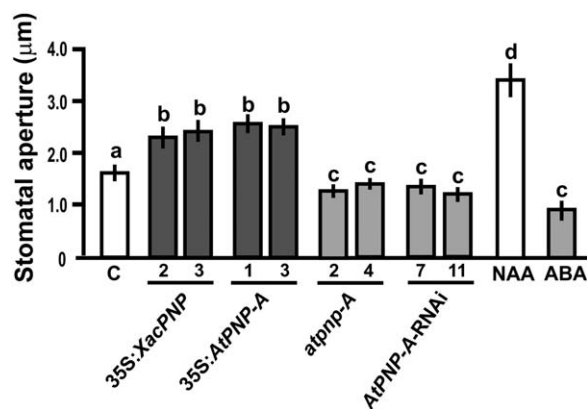

Fig. 2 Plant natriuretic peptides (PNPs) promote stomatal aperture. Quantification of stomatal apertures in leaves of *Arabidopsis thaliana* transgenic lines. As control (C), Col-0 and Col-0 *rdr6-11* were analysed and showed similar apertures; thus, the average value was considered as the control. Naphthalene acetic acid (NAA) at 1 μM and abscisic acid (ABA) at 50 μM were used as controls of aperture and closure, respectively. Bars are the means of the apertures of 100 stomata, and the results are representative of three independent experiments. Error bars indicate standard deviations. The data were analysed by analysis of variance (ANOVA) followed by Tukey's test. Bars with different letters are significantly different ($P < 0.05$).
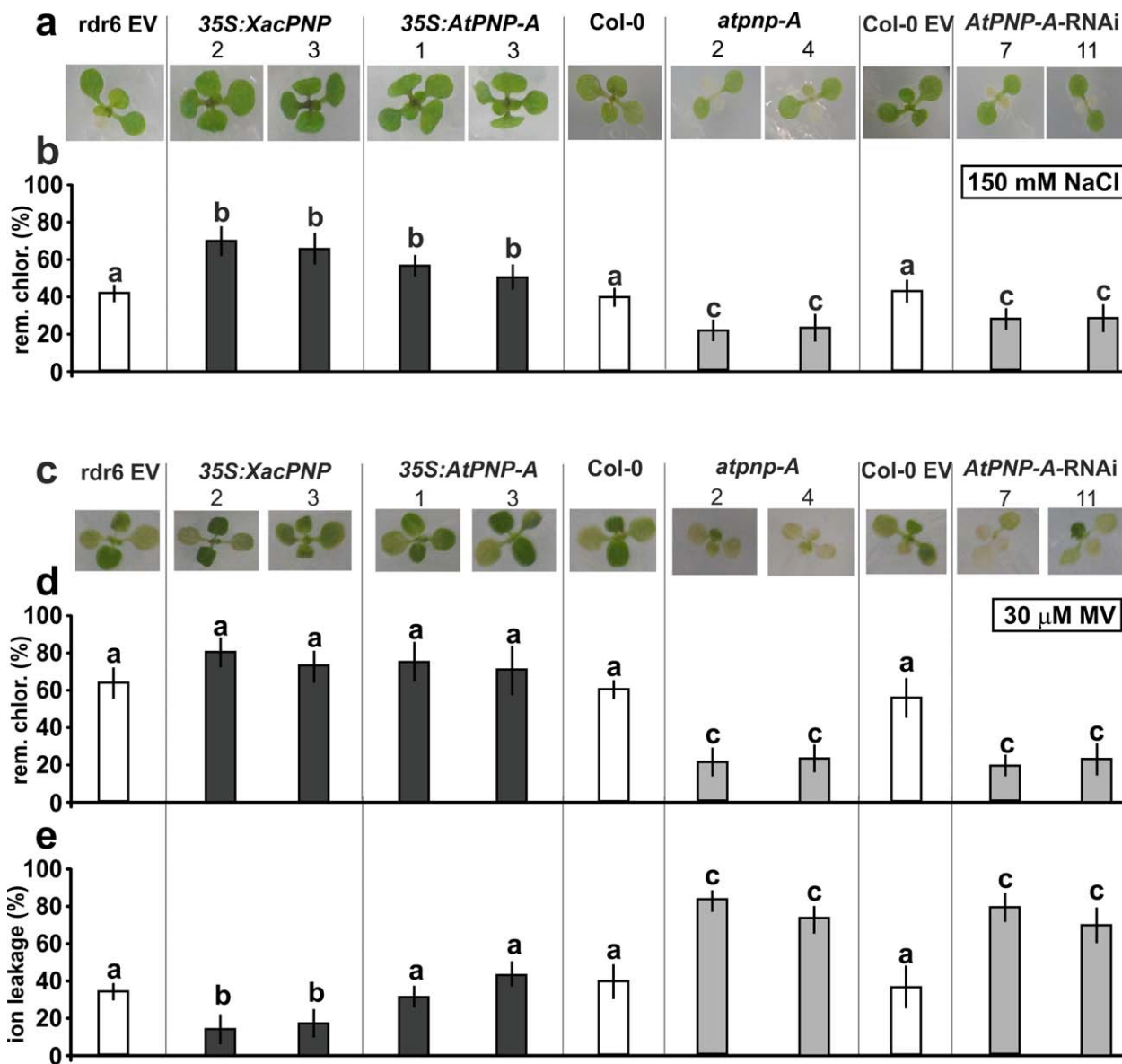
Fig. 3 Plant natriuretic peptides (PNPs) protect *Arabidopsis thaliana* against saline and oxidative stress. (a) One-week-old plants were transplanted to 150 mM NaCl-containing plates. Representative photographs are shown after 3 days of treatment. EV, empty vector. (b) Chlorophyll (rem. chlor.) was quantified at 3 days post-treatment and is shown with reference to chlorophyll in the control treatment. (c) One-week-old plants were transplanted to 30 μM methyl viologen (MV)-containing plates and representative photographs are shown after 2 days of treatment. (d) Chlorophyll was quantified at 2 days post-treatment and is shown with reference to chlorophyll in the control. (e) Ion leakage quantification of leaves from the lines stated after 8 h of incubation with 30 μM MV. In (b), (d) and (e), values represent an average of 5–10 leaves per line and the experiments were repeated three times. Error bars are standard deviations. The data were analysed for statistical differences by one-way analysis of variance (ANOVA) followed by Tukey's test. Bars with different letters are significantly different ($P < 0.05$).

35S:*AtPNP-A* plants showed slightly higher, but not significantly different ($P < 0.05$), levels of pigments compared with the 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 percentage of ion leakage, with a value of 18%, versus 38% observed in control plants ($P < 0.05$). The levels

of ion leakage of 35S:*AtPNP-A* plants were similar to those of control plants. PNP-deficient plants were the most damaged, displaying values of 75%–82%, with controls about 40% (Fig. 3e).

XacPNP triggers *A. thaliana* defence responses

To analyse the participation of XacPNP in plant biotic stress triggering plant defence responses, *A. thaliana* Col-0 leaves were

infiltrated with the recombinant pure XacPNP protein and, at 6 h post-inoculation (hpi), the transcript levels of several *A. thaliana* genes related to plant defence responses were analysed. RNA was extracted and real-time qRT-PCR was performed. Several defence marker genes were analysed: *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). qRT-PCR analysis showed that all transcripts were significantly ($P < 0.05$) more abundant in plants treated with XacPNP than in the $6 \times$ His-Trx-infiltrated controls (His, histidine; Trx, thioredoxin), with a major up-regulation for *GST1*, related to the oxidative stress response (Fig. 4a).

To further evaluate whether XacPNP is involved in the defence 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 $6 \times$ His-Trx as a control. The pre-infiltrated leaves were then infiltrated with *Pst* at 5×10^6 colony-forming units (CFU)/mL, and bacterial growth at 3 and 6 days post-inoculation (dpi) was monitored. At both times analysed, XacPNP was able to induce defence 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* on *Pst* infection

AtPNP-A expression in leaves infected with *Pst* was quantified. Plants were infected with *Pst* at 5×10^6 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, the 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 defence responses in *A. thaliana* and that AtPNP-A also contributes to the plant defence response.

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

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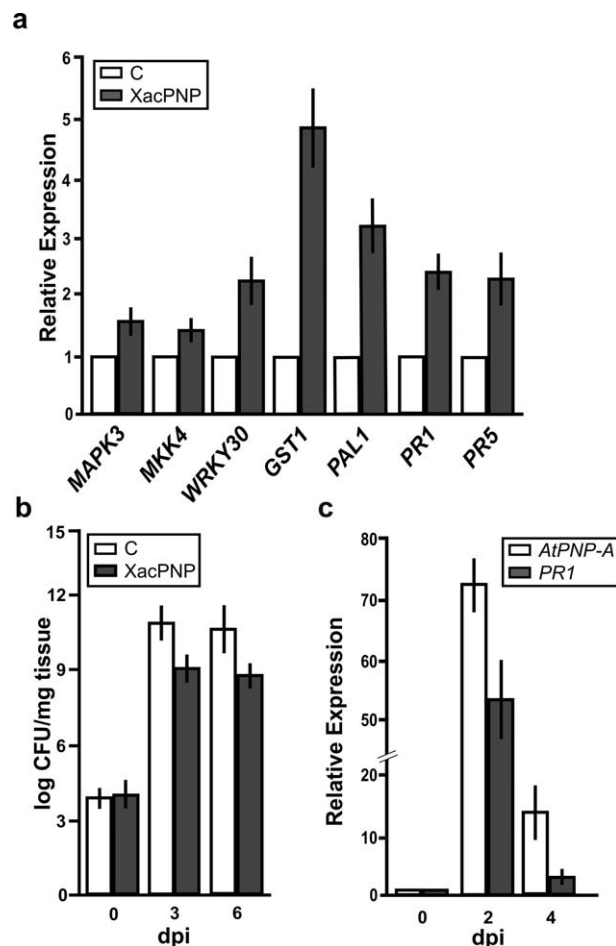

Fig. 4 Induction of the defence response by XacPNP, and *AtPNP-A* expression pattern, on *Pseudomonas syringae* pv. *tomato* (*Pst*) infection. (a) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of *Arabidopsis thaliana* genes related to the defence response (see text for gene definitions). RNA was extracted from leaves infiltrated with 5 μM XacPNP at 6 h post-inoculation (hpi). Bars indicate the expression levels of the indicated gene relative to the control treatment. As control, 5 μM $6 \times$ His-Trx was used (His, histidine; Trx, thioredoxin). (b) Enhancement of the defence response mediated by XacPNP. Quantification of *Pst* growth at 0, 3 and 6 days post-inoculation (dpi) in leaves pre-infiltrated with 5 μM XacPNP and $6 \times$ His-Trx as control. CFU, colony-forming unit. (c) qRT-PCR 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 analysed by analysis of variance (ANOVA) ($P < 0.05$).

At 3 and 5 dpi, PNP-over-expressing plants showed a decrease by one or two orders of magnitude in bacterial number compared with 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, with control lines reaching only 10^{10} CFU/mg (Fig. 5b). To understand the differences in disease phenotypes and taking into consideration the increase in defence
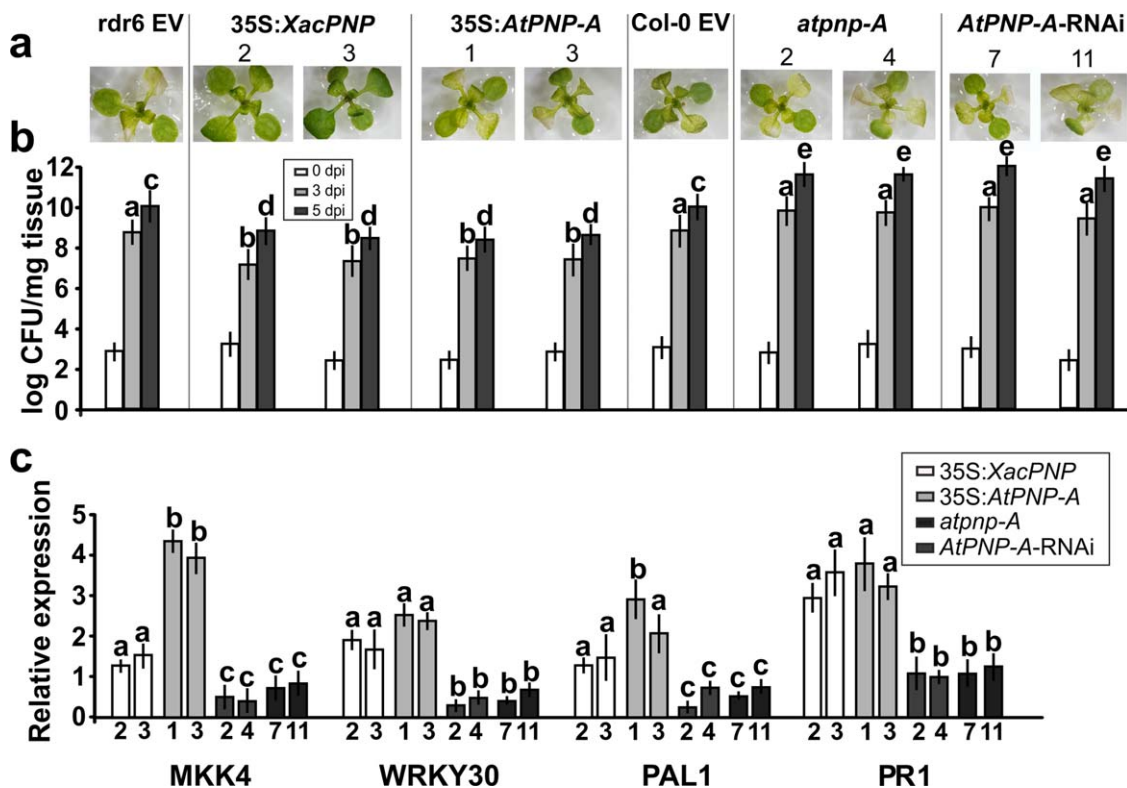
Fig. 5 Plant natriuretic peptides (PNPs) protect *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* (*Pst*). Two 3-week-old plants were flood inoculated with *Pst*. (a) Representative images of plants at 3 days post-inoculation (dpi). Numbers represent independent lines. EV, empty vector. (b) Quantification of bacterial population size at the times stated. Bars indicate the mean bacterial population as log colony-forming units (CFU)/mg tissue. An average of four plants was used for each line. The data were analysed by analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$). Bars with different letters are significantly different ($P < 0.05$). For the sake of clarity, letters for the population at 0 dpi were omitted. (c) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of *A. thaliana* genes related to the defence response at 24 h post-inoculation (hpi). Numbers on the *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 analysed by analysis of variance (ANOVA) followed by Tukey's test. Bars with different letters are significantly different ($P < 0.05$). Comparisons were performed among different lines for each gene.

gene expression in recombinant XacPNP-treated leaves (Fig. 4a), the expression of different genes related to the defence response was analysed at 24 hpi. 35S:*XacPNP* and 35S:*AtPNP-A* plants showed a major induction of *MKK4*, *WRKY30*, *PAL1* and *PR1* genes relative to control plants, whereas, in PNP-deficient lines, the opposite occurred (Fig. 5c).

Inoculation with *Xav* at 5×10^7 CFU/mL was assayed. In this case, all the lines showed the typical defence response, known as the hypersensitive response (HR), with the symptoms observed being similar in 35S:*XacPNP* and 35S:*AtPNP-A* plants compared with 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). Microscopic 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-over-expressing lines, this production was diminished ($P < 0.05$).

Finally, ion leakage was analysed in *Xav*-treated plants and significant major damage was observed in *atpnp-A4* and *AtPNP-A*-RNAi lines, whereas, in 35S:*XacPNP*, ion leakage was lower than in control plants ($P < 0.05$). In the case of 35S:*AtPNP-A*, no significant differences relative to the control were observed (Fig. 6d).

DISCUSSION

It is known that PNPs modulate salt and water homeostasis (Gehring and Irving, 2013). In addition, the participation of AtPNP-A in the plant defence response against pathogens has been proposed, taking into account that it is co-expressed together with genes involved in this response (Meier *et al.*, 2008; Turek *et al.*, 2014). Moreover, the recently found AtPNP-A receptor, which is a leucine-rich repeat receptor-like kinase
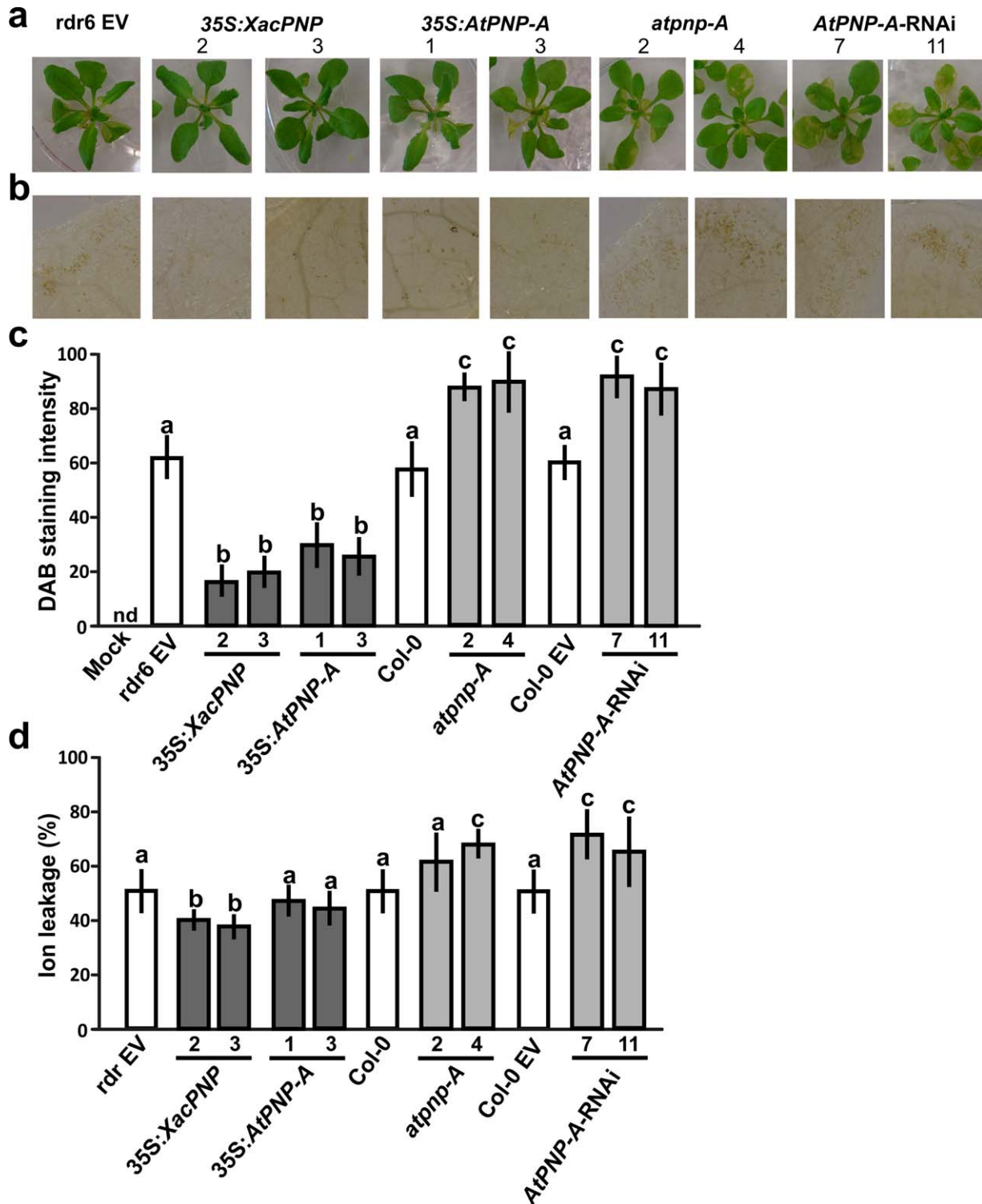
and belongs to a family of proteins associated with plant defence responses to pathogens (Turek and Gehring, 2016), strengthens the idea that PNPs may be involved in the response to plant pathogens.

Previously, we have demonstrated that *XacPNP* and *AtPNP-A*, expressed in *Xac*, are able to limit necrotic progress and to improve photosynthetic efficiency and water homeostasis during disease in citrus leaves (Garavaglia *et al.*, 2010a; Gottig *et al.*,

2008). In this work, we have revealed a role for PNPs in plant defence responses. The following observations indicate that PNPs play a role in plant defence responses: (i) pure XacPNP induces the expression of defence 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 to *PR1* on infection. Moreover, and for the first time, transgenic *A. thaliana* lines with modified levels of PNP expression were obtained to further evaluate the role of PNP in defence responses. These plants displayed different phenotypes with regard to stomatal aperture. Previously, we have observed that pure XacPNP or AtPNP-A proteins are able to induce stomatal opening (Gottig *et al.*, 2008). Accordingly, plants over-expressing *XacPNP* or *AtPNP-A* show an increase in stomatal aperture relative to control plants and PNP-deficient lines show a clear decrease in stomatal aperture, providing support for the function previously observed for PNPs (Gottig *et al.*, 2008). The active closure of stomata on bacterial contact is a widespread defence response in plants exposed to potential pathogens. Stomata play a role in the early phases of innate immunity; in particular, stomata are closed 1–2 h after bacterial recognition, preventing their entry into the host tissue (Melotto *et al.*, 2006). However, Arabidopsis mutants that keep their stomata open in response to *Pst*, and thus do not respond 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, although showing increased stomatal aperture to wild-type plants, do not display increased susceptibility, and *PNP*-deficient plants, although showing decreased stomatal aperture, do not show more resistance to *Pst*. These results suggest that the stomatal aperture of different plant lines does not determine bacterial entrance in this instance.

PNPs were also able to protect *A. thaliana* against saline stress, which is related to the known function of PNPs as regulators of K^+, Na^+ and H^+ fluxes. In this work, another function has been attributed to PNPs, i.e. 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, indicate the participation of PNPs in the modulation of oxidative cell responses, in addition to their role in the regulation of ion fluxes.

In plant–pathogen interactions, challenge with *Pst*, which causes disease, revealed that transgenic plants over-expressing *XacPNP* or *AtPNP-A* were more resistant to this pathogen relative to control plants; bacterial growth was impaired, possibly through the increased expression of defence genes. In contrast, *PNP*-deficient plants showed the opposite effect, being more susceptible to *Pst*. In the case of the non-host response challenged with *Xav*, *PNP*-deficient plants displayed stronger HR than both *PNP*-over-expressing and control plants. Although HR is related to the defence response, this stronger HR might be a consequence of larger tissue damage in the absence of PNP, as 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 defence response may be an adaptive reaction of the plant to fight against pathogens without the need to damage its own tissue. In general, 35S:*XacPNP* plants displayed a more noticeable phenotype than 35S:*AtPNP-A* plants, and this may be a result of the higher levels of expression observed in the former. It has been shown that AtPNP-A is able to modulate its own expression (Wang *et al.*, 2011). Although a Col-0 line impaired in gene silencing was used, and expression was under the control of the 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 mentioning that XacPNP is able to exert its function when infiltrated as a pure protein or when expressed by the plant. The recent finding of the AtPNP-A receptor protein leads to the question of whether XacPNP interacts with this receptor and whether it has the same affinity as the Arabidopsis peptide. Both peptides share a conserved active site (Nembaware *et al.*, 2004) and this region in AtPNP-A is sufficient for interaction with the receptor to occur (Turek and Gehring, 2016); this suggests that they may have similar affinity for the receptor protein, and therefore the over-expression of *XacPNP*, which is higher than the over-expression of *AtPNP-A*, may explain the more pronounced phenotypes observed in 35S:*XacPNP* plants. Our results reinforce the idea that XacPNP mimics its cognate protein in plants and, although it has been adapted to be expressed in the unique bacterium *Xcc*, it conserves its biological activity in its plant counterpart.

In summary, we have revealed a role for AtPNP-A and bacterial XacPNP in biotic and abiotic stress responses, and we propose that PNPs are part of a concerted action to cope with the environmentally changing conditions to which plants must adapt, ranging from oxidative stress and salt stress to the challenge of a pathogen attack. Moreover, this work enhances our understanding of a bacterial-acquired gene that mimics AtPNP-A function in defence 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 the construction of plants over-expressing PNPs, in order to achieve higher gene expression by avoiding gene silencing (Butaye *et al.*, 2004). Arabidopsis plants ecotype Columbia (Col-0) were used for the construction of *AtPNP-A*-RNAi experiments, and the Col-0 (SALK_000951C) insertional mutant was purchased from the Arabidopsis Biological Resource Center, Columbus, OH, USA. Homozygous mutants from this line were isolated by PCR-based genotyping using the gene-specific PCR primers (AtPNPs, Table S1, see Supporting Information). Seeds were surface sterilized with ethanol 70% (v/v) and Tween-20 0.1% (v/v) for 10 min, and spread on Murashige and 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 h : 8 h photoperiods. Seedlings were transferred to soil pots and kept in the same conditions until harvest.

Arabidopsis transformation

Arabidopsis thaliana transgenic plants over-expressing *XacPNP* or *AtPNP-A* and RNAi *AtPNP-A* lines were obtained. *AtPNP-A* was amplified from Col-0 WT cDNA using 35S:AtPNP oligonucleotides (Table S1) and subcloned into *Kpn*I-*Bam*HI of the pCHF3 binary vector under the control of the CaMV 35S promoter. For *XacPNP* over-expression, the plant PNP signal peptide was fused to the *XacPNP* coding sequence. The signal peptide was amplified using PNP-SP primers and *XacPNP* using 35S:XacPNP primers. Both PCR products were used as templates in a second round of amplification, and the final fusion product was subcloned into the pCHF3 binary vector under the control of the 35S CaMV promoter. For RNAi 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 the 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 analysed 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 qRT-PCR

Total RNA from leaves was isolated using TRIzol® reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions. RT-PCRs and qRT-PCRs 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 3–4-week-old plants were used. Individual leaves were incubated in 1 : 1 ethanol–KOH 5% (w/v) at 60 °C for 30 min, and then washed and cleared using ethanol. Leaves were stained with 1% (w/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 2-(N-morpholino) ethanesulfonic acid (MES) and 10 mM KCl (pH 6.5), supplemented with each hormone. The 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 0.5 X MS (Murashige and Skoog) agar 0.8% for 7 days. They were then 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) to investigate oxidative stress.

Bacterial strains and growth conditions

Pst and *Xav* were grown at 28 °C in Luria–Bertani (LB) 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 described previously (Ishiga *et al.*, 2011). Col-0 or Col-0 *rdr6-11* plants were grown in Petri dishes containing 0.5 X MS (Murashige and Skoog) agar, vitamins and phytagel 0.3% (w/v) for 2 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, the plates were drained and incubated in a 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 the fresh weight (CFU/mg). 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 6 × His-Trx as a control. Proteins were purified as described previously (Gottig *et al.*, 2008). After 6 h, these leaves were infiltrated with a *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 experiments were performed as described previously (Dunger *et al.*, 2012). Chlorophyll extraction was performed at 3 days post-treatment after saline stress and at 2 days post-treatment after oxidative stress. Values are expressed as the remaining percentage of chlorophyll compared with control infiltrated tissue. Ion leakage in *Xav*-inoculated plants was performed at 18 h post-treatment by placing leaf discs in 2 mL of water. In MV-treated leaves, assay was performed at 8 h post-treatment. Conductivity measurements were taken from each tube. The 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 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 (Adobe Systems Incorporated, San Jose, CA, USA). Average DAB measurements were calculated from at least five photographs from three independent experiments.

Statistical analysis

In all figures, bars show the mean of the data and error bars show the standard deviation. All data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test when stated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 List of oligonucleotides used in this work.

Fig. S1 Analysis of the expression levels of plant natriuretic peptides (PNPs) in genetically modified plants. Reverse transcription-polymerase chain reaction (RT-PCR) (a) and quantitative RT-PCR (qRT-PCR) (b) performed on selected lines of transgenic plants with modified levels of PNPs. EV represents Col-0 rdr6-11 plants transformed with pCHF3 when the expression of either AtPNP-A or XacPNP was analysed in 35S:*XacPNP* and 35S:*AtPNP-A* lines (top), whereas EV represents Col-0 plants transformed with pFGC5941 when the expression of AtPNP-A was analysed in AtPNP-A-RNAi plants. C is the PCR-positive control using *Xanthomonas citri* ssp. *citri* (*Xcc*) DNA as template. nd, not detected; tub, tubulin.

Fig. S2 Phenotypic analysis of plants with modified levels of plant natriuretic peptides (PNPs). Plants were grown in soil in similar conditions and the root length, fresh weight of rosettes and area of leaf 5 of 28-day-old plants were determined. The flowering time was established as the day on which the first flower appeared. No significant differences were observed. Ten plants were assayed for each line and the experiment was repeated twice. Error bars are standard deviations. The data were analysed for statistical differences by one-way analysis of variance (ANOVA) ($P < 0.05$).