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A plant natriuretic peptide-like gene in the bacterial pathogen *Xanthomonas axonopodis* may induce hyper-hydration in the plant host: a hypothesis of molecular mimicry

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Abstract

Background: Plant natriuretic peptides (PNPs) are systemically mobile molecules that regulate homeostasis at nanomolar concentrations. PNPs are up-regulated under conditions of osmotic stress and PNP-dependent processes include changes in ion transport and increases of H₂O uptake into protoplasts and whole tissue.

Presentation of the hypothesis: The bacterial citrus pathogen *Xanthomonas axonopodis* pv. Citri str. 306 contains a gene encoding a PNP-like protein. We hypothesise that this bacterial protein can alter plant cell homeostasis and thus is likely to represent an example of molecular mimicry that enables the pathogen to manipulate plant responses in order to bring about conditions favourable to the pathogen such as the induced plant tissue hyper-hydration seen in the wet edged lesions associated with *Xanthomonas axonopodis* infection.

Testing the hypothesis: We found a *Xanthomonas axonopodis* PNP-like protein that shares significant sequence similarity and identical domain organisation with PNPs. We also observed a significant excess of conserved residues between the two proteins within the domain previously identified as being sufficient to induce biological activity. Structural modelling predicts identical six stranded double-psi β barrel folds for both proteins thus supporting the hypothesis of similar modes of action. No significant similarity between the *Xanthomonas axonopodis* protein and other bacterial proteins from GenBank was found. Sequence similarity of the *Xanthomonas axonopodis* PNP-like protein with the *Arabidopsis thaliana* PNP (AtPNP-A), shared domain organisation and incongruent phylogeny suggest that the PNP-gene may have been acquired by the bacteria in an ancient lateral gene transfer event. Finally, activity of a recombinant *Xanthomonas axonopodis* protein in plant tissue and changes in symptoms induced by a *Xanthomonas axonopodis* mutant with a knocked-out PNP-like gene will be experimental proof of molecular mimicry.

Implication of the hypothesis: If the hypothesis is true, it could at least in part explain why the citrus pathogen *Xanthomonas campestris* that does not contain a PNP-like gene produces dry corky lesions while the closely related *Xanthomonas axonopodis* forms lesions with wet edges. It also suggests that genes typically found in the host, horizontally transferred or heterologous, can help to explain aspects of the physiology of the host-pathogen interactions.

Background

Plant natriuretic peptides (PNPs) are a novel class of plant molecules with biological activity at nanomolar concentrations. The PNP-dependent responses include concentration-dependent promotion of stomatal opening [1], rapid and transient increases in cellular cGMP levels [2] and modulation of K⁺, Na⁺ and H⁺ net fluxes [3] in *Zea mays* root tissue. PNPs also induce rapid increases in osmoticum-dependant H₂O uptake into *Solanum tuberosum* and *Arabidopsis thaliana* protoplasts [4,5]. We have also observed PNP-dependant increases in lateral H₂O movement out of the conductive tissue (xylem) into the neighbouring parenchyma [6] and such a 'drawing' of water into cells and tissues together with an up-regulation under conditions of drought and NaCl stress are compatible with a role for these molecules in plant homeostasis. Incidentally, a PNP-like protein from *Citrus jambhiri* (CjBAP12) is expressed in root and stem tissue in response to a challenge from citrus blight [7] which proliferates in the conductive tissue of the host and severely affects host homeostasis eventually resulting in xylem plugging and consequent shoot wilting and host death. It is conceivable that the expression CjBAP12 is an early host response to counteract the pathogen induced limitation of water and nutrient availability.

Several lines of evidence suggest that PNPs can act systemically. Firstly, PNPs are associated with conductive tissues as demonstrated by *in situ* hybridisation and tissue printing [8]. Secondly, biologically active PNP was isolated from xylem exudates [8], a tissue that is associated with transport and not protein synthesis. Amino acid sequence comparisons and structural modelling predict that PNPs do not contain the putative polysaccharide-binding C-terminal domain typical for the related expansins that act on the cell wall [9-11]. The absence of such a domain presumably results in increased extracellular mobility which in turn is a precondition for a systemic mode of action.

Here we report the discovery of a gene in the completely sequenced genome of the plant pathogenic bacterium *Xanthomonas axonopodis* [12] that encodes a protein with significant sequence similarity to an *Arabidopsis thaliana* PNP (AtPNP-A) A. We propose that the presence of a PNP-like protein in *Xanthomonas axonopodis* has enabled the pathogen to affect plant homeostasis. Furthermore, we have investigated the origin of this PNP-like protein encoding gene in *Xanthomonas axonopodis* and have found evidence consistent with the possibility that it has been acquired by the bacterium through horizontal gene transfer. This has led us to search for other genes that show evidence of horizontal transfer with a view to establishing how many genes may have been acquired by *Xanthomonas axonopodis* through horizontal gene transfer from plants.

Presentation of the hypothesis

We found a *PNP-like* gene in the bacterial citrus pathogen *Xanthomonas axonopodis* pv. Citri str. 306 that has significant sequence similarity to the PNP encoding genes and hypothesise that the encoded protein can alter homeostasis of the host plant. Since PNP-like molecules are exported into the extracellular space, act systemically and promote significant ion and H₂O uptake into cells it is very possible that the pathogen uses its PNP-like molecule to induce cell and tissue hyper-hydration in the host. Such hyper-hydration is typically seen in the wet rim of the lesions caused by *Xanthomonas axonopodis* and may suggest that PNP-like molecule benefits the pathogen by facilitating access to water and nutrients while severely disturbing the homeostasis of its host.

Testing the hypothesis

The closest homologue of the *Xanthomonas axonopodis* protein NP_642965.1 that motivated this study was the *Arabidopsis thaliana* protein AtPNP-A that we have previously shown to have an important role in plant homeostasis [13]. The alignment of the two protein sequences (Figure 1) shows that they are similar in length (AtPNP-A: 126 amino acids; *Xanthomonas axonopodis* PNP-like protein: 144 amino acids) and that both contain N-terminal transmembrane signal peptides to direct the molecules into the extracellular space, a precondition for a systemic role. Importantly, the molecules show a significantly greater amount ($p < 0.05$ using a Fishers' Exact Test) of conservation at sites between amino acids 33 and 66 of AtPNP-A (Figure 1) that we have previously identified as critical and sufficient for homeostatic function [5]. Within the entire length the of the domain (Figure 1) the identity is 36.4%, the similarity is 43.2% and the gaps are 22.7%.

The observed similarity between the two proteins could be due to an ancient horizontal gene transfer event [14] from the plants to bacteria or to convergent evolution. However, we believe that lateral transfer is more likely because the bacterial and the plant genes also show some similarity outside of the region that we have shown to be essential and sufficient for the function of the protein (Figure 1). This similarity in domains not essential for osmotic function suggests that the overall similarity between the two molecules is not just a result of shared function but reflects common ancestry.

A bootstrapped phylogenetic tree constructed using the *Xanthomonas axonopodis* protein NP_642965.1 and its closest homologues (Figure 2) reveals that, if the bacterial gene is indeed a product of horizontal gene transfer, the transfer event is likely to have occurred after the divergence of AtPNP-A from the rest of the expansin protein family. If indeed a plant is the source of this gene through horizontal transfer, it is likely to be the result of a

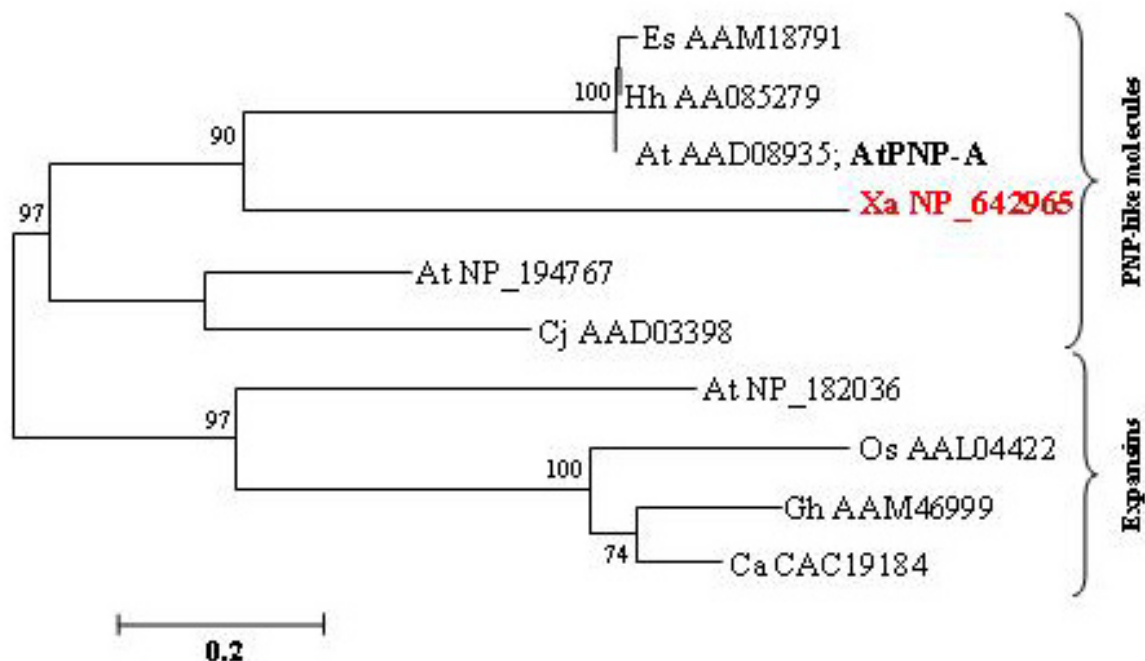


Figure 2

CLUSTALW [26] was used for the multiple sequence alignment of the full length *Xanthomonas axonopodis* PNP-like protein and its homologs obtained from BLASTp searches against the NCBI database. An alignment of 287 amino acids with a score of 17253 was obtained and after discarding all columns with gaps the alignment length was reduced to 89 amino acids with a score of 9705. The Neighbor-Joining tree of the 89 amino acid alignment was constructed using MEGA2 [33]. Bootstrap values are shown on the branches and the root was placed at the mid-point of the tree. Sequences are named by abbreviations of the species name followed by the NCBI accession number. Abbreviations: At – *Arabidopsis thaliana*, Ca – *Cicer arietium*, Cj – *Citrus jambhiri*, Es – *Erucastrum strigosum*, Gh – *Gossypium hirsutum*, Hh – *Hedera helix*, Os – *Oryza sativa*, Xa – *Xanthomonas axonopodis* pv. Citri str. 306 (in red).

The sequence conservation between AtPNP-A and the *Xanthomonas axonopodis* PNP-like molecule is greatest in the domain spanning the $\beta 2$ and $\beta 3$ strands which both flank the α helix (Figure 1 and 3). In AtPNP-A this structure ($\beta 2 - \alpha$ helix - $\beta 3$) has been demonstrated to be within the 33 amino acid long domain that is critical and sufficient for conferring biological activity [5]. This domain also contains the first psi loop which is likely to be a part of the functional framework of AtPNP-A and the *Xanthomonas axonopodis* PNP-like molecule.

We also carried out a screen of all proteins from *Xanthomonas axonopodis* in order to discover whether other genes from this bacterial pathogen showed evidence of unexpected tree topology thus indicating horizontal gene transfer [14] from plants. All known proteins from the completely sequenced genomes of *Xanthomonas axonopodis* [12], *Xanthomonas campestris* [12], *Pseudomonas putida*

[22], *Escherichia coli* [23] and *Arabidopsis thaliana* [24] were downloaded from GenBank (13/08/2003). The *Xanthomonas axonopodis* proteins were searched against the proteins from the remaining four organisms using BLASTp [25]. CLUSTALW [26] was used to generate multiple sequence alignments and Neighbour-Joining phylogenetic trees from 4307 sets of five proteins consisting of one protein from each of the five organisms. *Xanthomonas axonopodis* proteins with greater than 25% identity to their *Arabidopsis thaliana* homologues that clustered with the *Arabidopsis thaliana* homologue on a phylogenetic tree were retained for further analysis. Each of these proteins was searched against the GenBank non-redundant protein database. Homologous sequences were downloaded and phylogenetic trees were constructed using the Neighbor-Joining method [26].

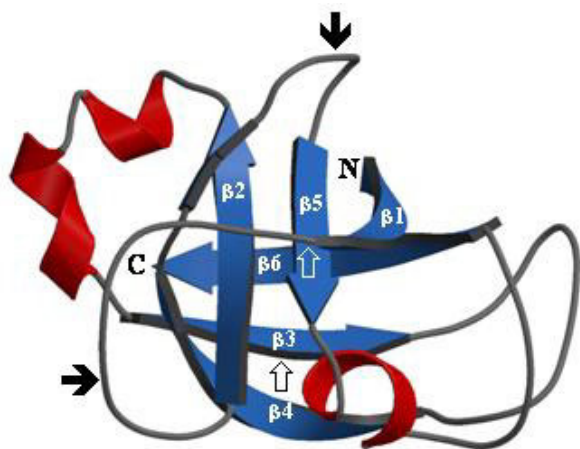


Figure 3

Modelled fold of a PNP-like molecule showing the six stranded double-psi β barrel structure. Fold recognition methods predict with certainty (Z score: >5) that AtPNP-A and the *Xanthomonas axonopodis* PNP-like molecule both adopt this fold. The N- and C-terminus of the protein are indicated, the α -helices are in red, the 6 β -strands are in blue and the two protruding psi loops are marked with a solid arrow (\uparrow). The open arrows (\uparrow) delineate the 33 amino acid long domain critical and sufficient for biological activity [5]. The N-terminal signal peptide that is not required for biological function outside the cell [5] was not included in the model. The model was generated using the software MOLSCRIPT [34].

This approach was used to determine the number of *Xanthomonas axonopodis* genes that showed evidence of horizontal acquisition from plants. The initial screen with the five completely sequenced organisms returned seven cases of putative horizontal transfers (Table 1). However, only two of the proteins (Table 1), NP_642965.1, the subject of this study, and NP_643621.1 had no significant bacterial homologs in the NCBI database using the default E-value cut-off 10. The remaining proteins had bacterial homologs that suggested that they were not likely to have been acquired through horizontal transfer. The search indicated that horizontal transfer of genes between plants and the plant pathogen *Xanthomonas axonopodis*, if it has indeed occurred, has been rare.

Recently, another example of a pathogen mimicking an extracellular plant molecule has been reported [27]. This protein (GrEXP1), a molecule with cell wall loosening (expansin) activity previously seen in plants [28] and other organisms with cell walls only [29], was found in the plant-parasitic roundworm *Globodera rostochiensis*. The infective juvenile nematodes express and secrete GrEXP1

in the subventral oesophageal glands [27] using this 'typical plant' protein to their advantage when invading the host root system.

Finally, if the PNP-like gene was indeed horizontally transferred from a plant to *Xanthomonas axonopodis* it is also consistent with the complexity theory of gene transfer [30] which postulates that a major factor in the more frequent horizontal transfer of operational genes such as *expansins* and PNPs as compared to informational genes is that they are structurally and functionally less complex. This bias is explained by the increased chance of transfer of a functional unit advantageous to the recipient. It would also appear that particularly in the case of a pathogen, extracellular signals, transporters or surface components perceived by the host can cause systemic host responses that give the pathogen a significant advantage. A point in case are eukaryotic genes found in *Mycobacterium tuberculosis* many of which directly modulate host responses and have a role in the specific pathogenesis induced by the bacterium [31].

The experimental test of the hypothesis of molecular mimicry of the *Xanthomonas axonopodis* PNP-like molecule will require two types of investigations. In the first, a recombinant *Xanthomonas axonopodis* protein must be obtained and tested for effects on (host) plant tissue. Molecular mimicry would require that net H_2O uptake is increased and ion transport is affected in the host tissue in response to the recombinant peptide. In a second experiment, a *Xanthomonas axonopodis* mutant with a knocked-out PNP-like gene must be obtained. If the mutant induces altered host symptoms and in particular an absence of watery edges of the lesions, then the hypothesis can be considered proven.

Implications

If the hypothesis is true, then the bacterial PNP-like protein plays a role in manipulating the homeostatic balance of the host. Such mimicry could at least in part explain why the citrus pathogen *Xanthomonas campestris* that does not contain a PNP-like gene produces dry corky lesions while the closely related *Xanthomonas axonopodis* forms wet lesions [32]. Furthermore, the hypothesis suggests that the presence of "typical" and functional host genes in pathogens can explain key aspects of host-pathogen interactions in general and can help elucidate the specific molecular and cellular interactions between hosts and pathogens.

Authors' contributions

VN and CS carried out the bioinformatics and phylogenetic analyses, MS performed the structural analysis and CG advised on the biological function of PNPs and drafted the manuscript. All authors contributed to the

Table 1: Analysis of putative laterally transferred genes obtained from the whole genome analyses.

<i>X. axonopodis</i>	Proteins: <i>A. thaliana</i>	Function:	Bacterial homologs:
NP_640439	NP_564216	short-chain dehydrogenase	yes
NP_641062	NP_196873	N-acetylglucosaminidase	yes
NP_642289	NP_196225	3-oxoacyl-[ACP] reductase	yes
NP_642965	NP_194767	hypothetical protein	no
NP_643053	NP_568712	amine oxidase related	yes
NP_643621	NP_173748	expressed protein	no
NP_644089	NP_182049	methionine aminopeptidase	yes

editing of the manuscript and approved of the final version.

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