A plant-responsive bacterial-signaling system senses an ethanolamine derivative

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Certain plant-associated Proteobacteria sense their host environment by detecting an unknown plant signal recognized by a member of a LuxR subfamily of transcription factors. This interkingdom communication is important for both mutualistic and pathogenic interactions. The *Populus* root endophyte *Pseudomonas* sp. GM79 possesses such a regulator, named PipR. In a previous study we reported that PipR activates an adjacent gene (*pipA*) coding for a proline iminopeptidase in response to *Populus* leaf macerates and peptides and that this activation is dependent on a putative ABC-type transporter [Schaefer AL, et al. (2016) mBio 7:e01101-16]. In this study we identify a chemical derived from ethanolamine that induces PipR activity at picomolar concentrations, and we present evidence that this is the active inducer present in plant leaf macerates. First, a screen of more than 750 compounds indicated ethanolamine was a potent inducer for the PipR-sensing system; however, ethanolamine failed to bind to the periplasmic-binding protein (PBP) required for the signal response. This led us to discover that a specific ethanolamine derivative, *N*-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide (HEHEAA), binds to the PBP and serves as a potent PipR-dependent inducer. We also show that a compound, which coelutes with HEHEAA in HPLC and induces *pipA* gene expression in a PipR-dependent manner, can be found in *Populus* leaf macerates. This work sheds light on how plant-associated bacteria can sense their environment and on the nature of inducers for a family of plant-responsive LuxR-like transcription factors found in plant-associated bacteria.

ethanolamine | LuxR homolog | plant–microbe interactions | transcription activator | quorum sensing

A variety of plant-associated bacteria sense plants by recognizing an unknown signal that binds to one of a family of bacterial transcriptional regulators called plant-associated bacteria LuxR homologs (1–5). These regulators are thought to have evolved from acyl-homoserine lactone (acyl-HSL)-responsive LuxR homologs involved in quorum sensing, which is the population-wide regulation of gene expression in a cell density-dependent manner (6). However, instead of responding to self-produced molecules, plant-associated bacteria LuxR homologs respond to a plant signal (7, 8).

Interkingdom-signaling systems involving plant-derived molecules and bacterial protein receptors have been well studied in the cases of legumes and nitrogen-fixing rhizobial bacteria as well as the Rhizobiaceae genus *Agrobacterium* and its plant hosts. Examples include the rhizobial transcriptional regulator NodD that senses flavonoids produced by legumes to activate nodulation genes (9, 10) and the two-component system VirA/VirG from *Agrobacterium* that is involved in sensing plant-derived phenolic compounds to activate virulence genes (11). Whereas these systems are confined to Rhizobiaceae, the putative plant-associated bacteria LuxR homologs are more widespread and can be found in a number of genera belonging to the alpha-, beta- and gammaproteobacteria classes. Of the "Top 10 Plant Pathogenic Bacteria", five (*Pseudomonas syringae* pathovars, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas campestris* pathovars, *Xanthomonas axonopodis* pathovars, and *Dickeya dadantii* and *solani*) (12) possess putative plant-associated bacteria *luxR* homologs. These *luxR* genes are usually in close proximity to genes annotated as coding for a proline iminopeptidase (*pip*), and the LuxR protein has been shown to bind to specific DNA sequences to activate *pip* expression (4, 13). This system is important for both plant pathogenesis and mutualism. Pip proteins and the LuxR homologs that control their expression are important for virulence of *Xanthomonas* spp. and *Pseudomonas syringae* pv. *actinidiae* (1, 2, 4, 14). Additionally, plant-associated bacteria LuxR homologs have been shown to be important for biocontrol of wheat by *Pseudomonas protegens* (3) and for competition for nodulation of *Medicago sativa* by *Sinorhizobium meliloti* (15). Although our understanding of plant-associated bacteria LuxR homologs has improved in recent years, progress has been limited because an actual plant signal has not yet been identified.

We recently described a plant-associated bacteria LuxR homolog, called PipR, from the *Populus deltoides* root endophyte *Pseudomonas* sp. GM79 (16). Like its homologs in other plant-associated bacteria, PipR activates a downstream *pip* gene (called *pipA* in strain GM79) in response to plant leaf macerates. Genes divergently transcribed from *pipR* are annotated as a peptidase (named *aapA*) and an ABC-type transporter (Fig. 1*A*). PipA and AapA were purified and shown to cleave N-terminal amino acids. PipA was most active as a prolyl iminopeptidase, whereas AapA was most active as an alanyl aminopeptidase (16). Strain GM79 Δ*pipA* and Δ*aapA* mutants had elevated PipR-dependent responses to leaf macerates, consistent with the hypothesis that they degrade the plant signal as part of a negative feedback loop. In contrast, a mutation in the ABC transporter gene *aapB* abolished the ability of PipR to activate *pipA* in the

Significance

A number of plant-associated bacteria possess transcription factors homologous to LuxR that respond to compounds found in plant tissue. These LuxR homologs are known to regulate virulence or symbiosis. Although the first of these proteins were described more than a decade ago, the factors they respond to remain unknown. Here we identify a compound that functions with PipR, a plant-responsive LuxR homolog from a tree root endopytic *Pseudomonas* isolate. The inducer is an ethanolamine derivative. Ethanolamine is a building block for plant membrane phospholipids and signaling molecules. This discovery connects at least one of the plant-responsive LuxR homolog systems to a growing understanding of ethanolamine chemistry and responses of bacterial cells to ethanolamine and ethanolamine derivatives.

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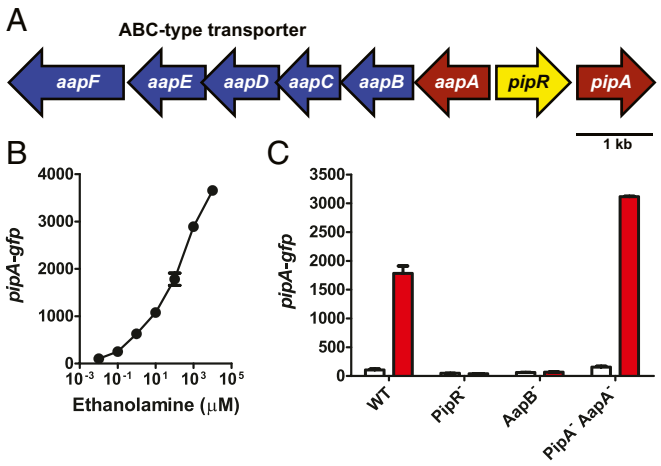

Fig. 1. The *pipR* gene region in *Pseudomonas* GM79 and PipR-dependent ethanolamine activation of P*pipA-gfp*. (*A*) The *pipR* (yellow) genomic region. Genes coding for the predicted ABC-type transporter (blue) and genes coding for the peptidases (red). (*B*) Activation of *gfp* expression in strain GM79 containing pP_{pipA}-*gfp* by ethanolamine. (*C*) Influence of mutations in ethanolamine (100 μM) activation of *gfp* expression in strains carrying pP_{pipA}-*gfp*. AapB$^-$, AapB transporter mutant; PipA$^-$ AapA$^-$, PipA, AapA double-peptidase mutant; PipR$^-$, PipR mutant; WT, wild type. Data are GFP fluorescence in cells grown without additions (white bars) or cells grown with 100 μM ethanolamine (red bars). *B* and *C* show mean relative fluorescence units (RFU) per optical density (OD_{600}) × 10 of four biological replicates, and the bars represent the SDs.

presence of leaf macerates, indicating that the ABC transporter is required for uptake of the plant signal. The involvement of peptidases in the PipR system suggested that the plant signal might be peptide-like, which led to our report that PipR responds to specific small peptides (16). However, the response to peptides was relatively weak and required high peptide concentrations.

Here we show that *N*-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide (HEHEAA), a compound that forms spontaneously from ethanolamine, induces *pipA* expression at extremely low concentrations (10 pM), and PipR is required for this activation. Our findings suggest that ethanolamine and its derivatives, which serve as important intermediates in plant cell membrane biogenesis and can serve as plant hormones, play a role in plant–bacteria interactions.

Results

PipR Responds to a Compound Derived from Ethanolamine. In a previous work (16), we screened a small library of compounds (268 dipeptides and 14 tripeptides) for signals capable of serving as PipR coactivators of *pipA*. In the screen we used *Pseudomonas* GM79 containing a plasmid with the *pipA* promoter fused to *gfp* (pP_{pipA}-*gfp*). We discovered that the tripeptide SHS induced *gfp* expression, but only at relatively high concentrations (1 mM). To identify a more active PipR coinducer of *pipA* expression we performed a screen by using a *Pseudomonas* GM79 with Δ*pipA* Δ*aapA* mutations containing pP_{pipA}-*gfp*. The response of the double-peptidase mutant to SHS is much more robust than the response in wild-type *Pseudomonas* GM79 (16). We screened the 760 compounds available in Biolog plates PM1-8. These plates contain compounds that are potential carbon, nitrogen, phosphorous, or sulfur sources and nutrient supplements (17). Consistent with the prior publication (16) the screen showed several peptides served as relatively weak PipR coinducers of GFP. The fluorescence generated by them was on the order of 2- to 17-fold greater than controls without an added compound (*SI Appendix*, Table S1). By far the greatest response was to ethanolamine (nearly 50 times that of control fluorescence).

We tested high-purity ethanolamine (99%) to confirm the results of the screen. Ethanolamine at concentrations as low as 100 nM induced GFP fluorescence in the wild-type strain GM79 (Fig. 1*B*). Moreover, this activity was dependent on PipR and the ABC-type transporter, and ethanolamine induction of GFP was greater in the peptidase double mutant than in the wild type (Fig. 1*C*). These results are consistent with our previous results with plant macerates and peptides (16). The fact that the mutant analysis showed that the putative ABC-type transporter was necessary for ethanolamine induction of GFP was surprising because strain GM79 has an ethanolamine transporter gene (*PMI36_01922*) homologous to the *eat* transporter gene (*PA4023*) of *Pseudomonas aeruginosa*, and the GM79 gene is linked to ethanolamine catabolism genes *eutBC* (*PMI36_01923* and *PMI36_01924*). Ethanolamine can also diffuse into cells, and in *Salmonella enterica*, its active import is only necessary when its concentration is less than 25 μM (18, 19). That the transporter was required for the response to 100 μM ethanolamine suggested that ethanolamine itself did not serve as a PipR coinducer of *pipA* transcription, but rather the inducer was a low-level contaminant in the ethanolamine solution. To test this hypothesis we fractionated a solution of ethanolamine by hydrophilic interaction liquid chromatography (HILIC) and found two active peaks (Fig. 2*A*). One active peak coeluted with ethanolamine, as revealed by high-resolution mass spectrometry (HRMS). Unfortunately, there was not enough material in the other peak for mass spectrometry. That there was activity in the HILIC ethanolamine fraction led us to hypothesize that the active compound was spontaneously formed from ethanolamine. Support for this hypothesis came from the following experiment: we subjected the HILIC-purified ethanolamine fraction to a second round of HPLC, and bioactive material was again found in the ethanolamine fraction and in the second fraction (Fig. 2*B*). We also subjected the second fraction to another round of HILIC and found that all of the active material eluted at the position of the second activity peak, with no detectable activity where ethanolamine elutes (Fig. 2*C*).

Use of AapF to Trap the Active *pipA-gfp* Inducer. To purify enough of the active spontaneously forming ethanolamine derivative we decided to make use of the periplasmic-binding protein (PBP) of the ABC-type transporter required for the induction of *pipA* transcription. PBPs, also referred to as substrate-binding proteins, are a common feature of ABC-type transporters and are involved in the initial binding of the substrate and its delivery to the membrane-bound subunits that use ATP hydrolysis to catalyze uptake into cells (20). To confirm that the PBP is important for induction of *pipA* transcription, we made a deletion of its coding gene, *aapF*. An *aapF* mutation abolished the responses to ethanolamine solutions and to leaf macerates (Fig. 3*A*). Thus, we conclude that the PipR coactivating ligand requires initial binding to the PBP before its transport into the cell.

To collect sufficient amounts of the inducer for HRMS we purified His-tagged AapF from recombinant *Escherichia coli* grown in glucose minimal medium plus ethanolamine and denatured the purified His-tagged AapF to release bound molecules. As shown in Fig. 3*B*, active material was released from AapF isolated from *E. coli* grown in the presence of ethanolamine, but active material was not recovered from protein purified from recombinant *E. coli* grown without added ethanolamine. Moreover, separation of the active AapF-released material by HILIC showed the presence of only one active peak, with no detectable activity where ethanolamine should elute (Fig. 3*C*). As expected, AapF binds to the active PipR coactivator and not to ethanolamine.

Purification and High-Resolution Mass Spectrometry of the PipR-Dependent *pipA* Inducer. We obtained material trapped in 500 mg of recombinant His-tagged AapF by denaturing the protein purified from *E. coli* grown in glucose minimal medium plus ethanolamine and, separately, from *E. coli* grown in glucose minimal medium plus ^{15}N-labeled ethanolamine. The active material released into the buffer by denaturation was further purified by HILIC. The $[M+1]^+$ of the purified material derived from ethanolamine- and ^{15}N-labeled ethanolamine-grown *E. coli* were 163.1071 and 165.0997, respectively. This result indicates the

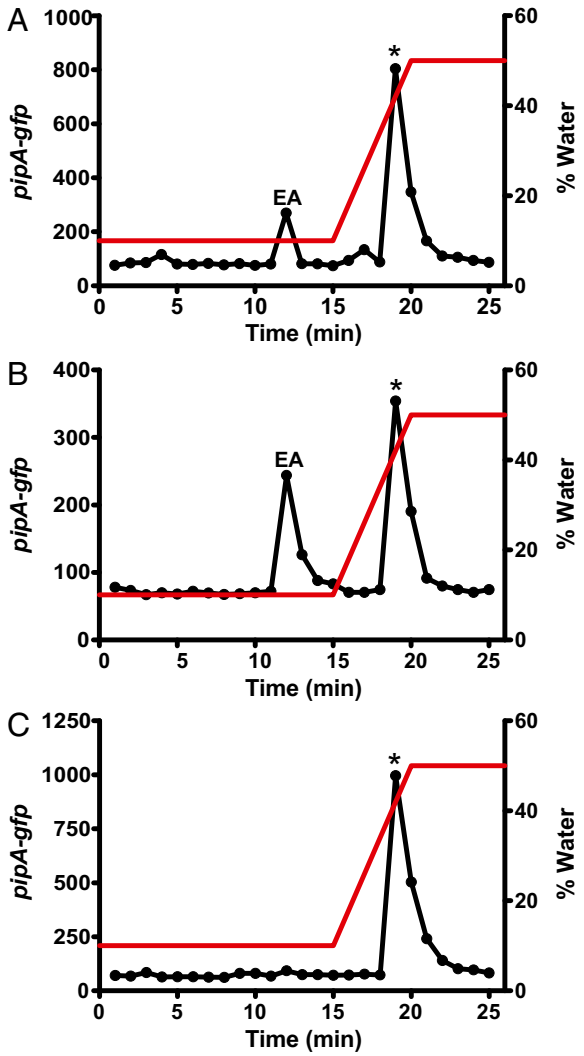

Fig. 2. A compound formed from ethanolamine activates P_{pipA}-*gfp* expression. (*A*) Fractions from HILIC-column chromatography of ethanolamine (12 μg) were tested by using the *Pseudomonas* GM79 (pP_{pipA}-*gfp*) bioassay. Ethanolamine (EA) was eluted in fraction 12, as confirmed by MS, and a second peak of activity was eluted in fractions 19 and 20. (*B*) The material from fraction 12 from *A* was subjected to a second round of HILIC, and fractions were tested with the bioassay as in *A*. (*C*) The material from fraction 19 from *A* was subjected to a second round of HILIC, and fractions were tested with the bioassay, as in *A*. The red line shows the gradient of water. The asterisks indicates the fraction where synthetic HEHEAA elutes.

inducer contains two nitrogen atoms. Based on the molecular mass of the inducer and the indication that it contains two nitrogen atoms we predicted a chemical formula of $C_6H_{14}N_2O_3$. The chemical engineering literature on compounds formed spontaneously from ethanolamine is extensive (21), and the molecular weight of the purified inducer is consistent with that of *N*-(2-hydroxyethyl)-2-(2-hydroxyethylamino)acetamide (HEHEAA). HEHEAA is formed by the condensation of ethanolamine and *N*-(2-hydroxyethyl)glycine (HeGly), another common spontaneously derived product of ethanolamine, through the formation of an amide bond and release of water (Fig. 4*A*) (21–23).

HEHEAA Is a PipR-Dependent Inducer of *pipA* Expression in *Pseudomonas* GM79. Our analysis of the material purified by binding to AapF and subsequent HILIC indicated that the *pipA* inducer is HEHEAA. Thus, we asked whether chemically synthesized HEHEAA purchased commercially served as an inducer. Indeed, fluorescence of *Pseudomonas* GM79 carrying the *pipA* promoter-*gfp* plasmid showed a dose-dependent increase (Fig. 4*B*). Cells responded to HEHEAA at concentrations of this inducer as low as 10 pM, and saturation of the response was achieved at about 1 μM HEHEAA (Fig. 4*B*). Thus, synthetic HEHEAA has a potency about 10,000 times that of ethanolamine solutions, suggesting that HEHEAA is present at a level of about 0.01% in solutions of ethanolamine. As expected, the HEHEAA response was dependent on PipR and on the ABC transporter (Fig. 4*C*). Furthermore, the elution profile

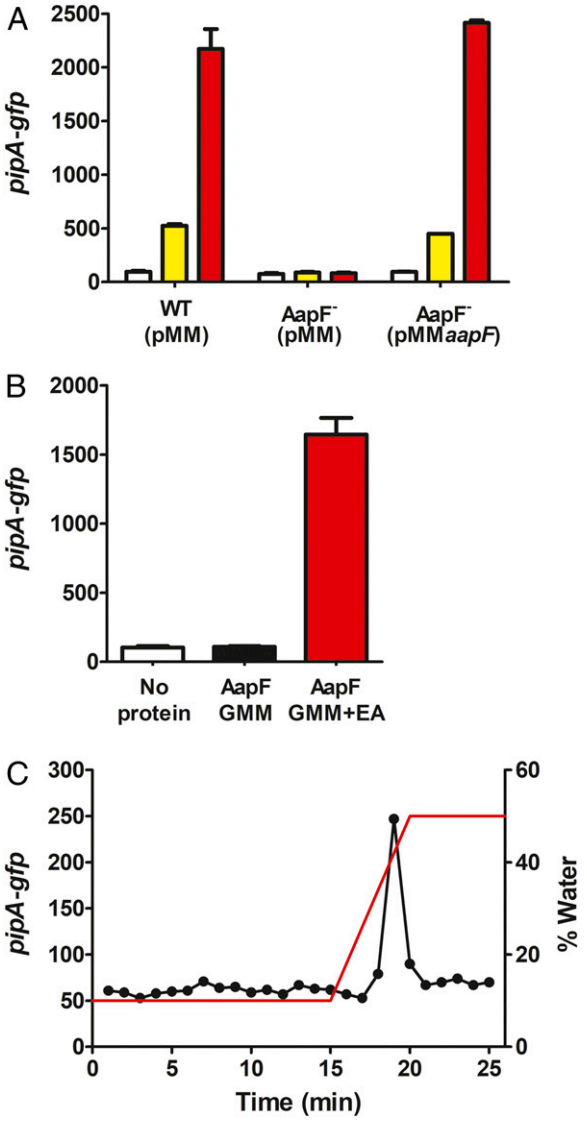

Fig. 3. The PBP encoded by *aapF* is required for activation of the pP_{pipA}-*gfp* reporter by leaf macerates and ethanolamine, and it can be used to purify the active molecule present in ethanolamine solutions. (*A*) Strains are wild type (WT) and the AapF mutant ($AapF^-$) carrying pP_{pipA}-*gfp* and either a pMMB67EH-TetRA vector control (pMM) or the *aapF* expression vector pMM*aapF*. Data are the activities of the pP_{pipA}-*gfp* reporter grown with water control (white bars), 0.5% leaf macerates (yellow bars), or 100 μM ethanolamine (red bars). (*B*) The *aapF* gene was expressed in *E. coli* grown in glucose minimal medium (GMM) ± 10 mM ethanolamine (EA). Purified protein (5 μg) was denatured to release bound molecules. The supernatant fluid containing the released molecules was processed as described in *Materials and Methods*, and the bioassay was used to measure inducer activity of the released material. *A* and *B* show the mean RFU per OD_{600} × 10 of four biological replicates, and the bars represent SDs. (*C*) Material released from AapF was separated by HILIC, and fractions were tested with the bioassay. The red line shows the gradient of water.

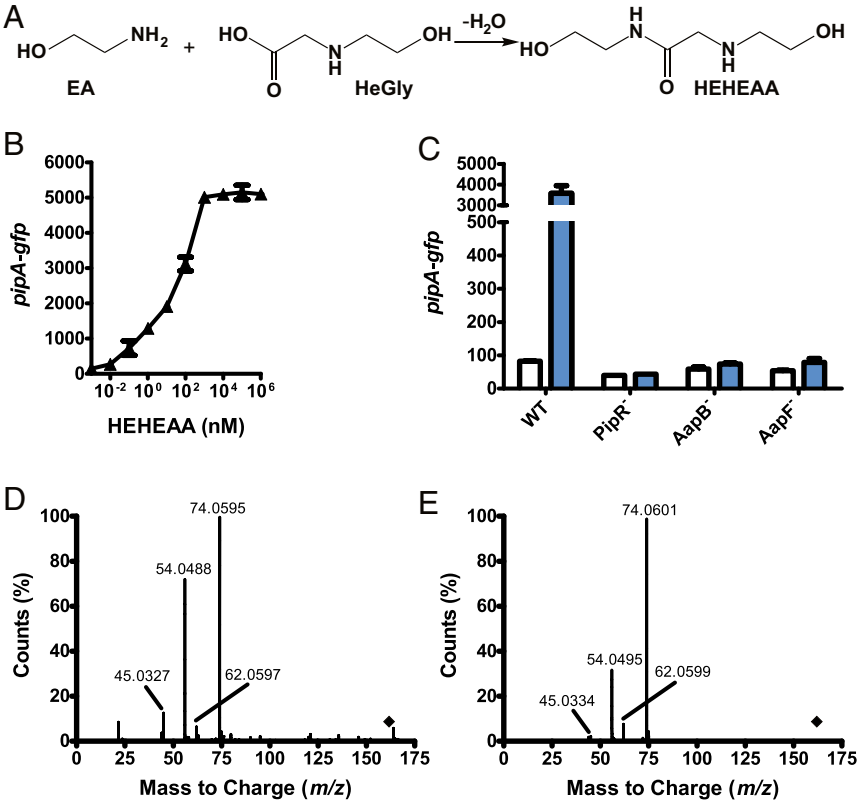

Fig. 4. Picomolar levels of HEHEAA, a compound spontaneously formed from ethanolamine, induce *gfp* expression in *Pseudomonas* GM79 (pP_{pipA}-*gfp*). (*A*) HEHEAA formation from ethanolamine and HeGly (21–23). (*B*) Activation of *gfp* expression in the *Pseudomonas* GM79 (pP_{pipA}-*gfp*) bioassay by HEHEAA. (*C*) Influence of mutations in the genes flanking *pipR* on HEHEAA activation of P_{pipA}-*gfp*. The strains are AapB$^-$, AapB transporter mutant; AapF, AapF PBP mutant; PipR$^-$, PipR mutant; WT, wild type. Data are GFP fluorescence of cells grown with (blue bars) or without (white bars) 1 μM HEHEAA. *B* and *C* show mean RFU per OD_{600} × 10 of four biological replicates, and the bars represent the SDs. (*D*) The MS2 fragmentation spectrum of active material purified from ethanolamine, $[M+H]^+$ 163.1071 *m/z*, at 8.18 min (indicated by the diamond). (*E*) The MS2 fragmentation spectrum of chemically synthesized HEHEAA, $[M+H]^+$ 163.1101 *m/z*, at 8.14 min.

(Fig. 2) and the tandem mass spectrum (Fig. 4 *D* and *E*) were indistinguishable from those of the material purified from ethanolamine. We note that the commercial preparation of HEHEAA contained a synthetic precursor, HeGly, at about 20% based on ^{1}H NMR. Therefore, we tested HeGly and found it did not serve as a PipR coinducer of *pipA* over a wide range of concentrations.

Active Inducer from *Populus* Tissue Coelutes with HEHEAA. Does the active material isolated from *Populus* tissue have HEHEAA characteristics? It is possible that the *Populus*-derived coinducer is HEHEAA or an entirely different small water-soluble compound. Ethanolamine is an important plant metabolite for both the synthesis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC), two major phospholipids in plant membranes (24), and for the production of the plant-signaling lipids *N*-acylethanolamines that modulate plant physiological processes (25). Plants are able to directly generate ethanolamine by serine decarboxylation, and free ethanolamine levels are estimated to be 50–250 nmol per gram of fresh weight in plant tissues (26, 27). Because HEHEAA is formed spontaneously from ethanolamine, we hypothesized that this molecule would also be present in plants. To test this hypothesis we separated *Populus* leaf-derived material by HILIC and tested fractions using *Pseudomonas* GM79 with the *pipA-gfp* reporter plasmid. We found that the active molecule in leaf macerates coelutes with HEHEAA (Fig. 5). This result is evidence in favor of the hypothesis that the PipR-dependent *pipA* inducer in plant tissue is HEHEAA.

Discussion

We identified an ethanolamine-derived small molecule, HEHEAA, as a PipR coinducer of *pipA* in the plant root endophytic bacterium *Pseudomonas* GM79. HEHEAA forms spontaneously from ethanolamine and is present in ethanolamine solutions. Our evidence indicates HEHEAA interacts with AapF, a periplasmic-binding protein, which delivers the inducer to an ABC-type transporter. Based on the finding that PipR is required for HEHEAA activity we propose that this LuxR-like protein binds to HEHEAA, and HEHEAA-bound PipR serves to activate *pipA* expression. Although there is no direct evidence that HEHEAA binds to PipR, our identification of HEHEAA as a potent PipR coactivator of *pipA* makes it possible to begin to study PipR activity. An important step toward identification of HEHEAA was the use of the PBP, AapF, as "bait" for HEHEAA. This innovation was inspired by previous use of a PBP required for activity of the *Vibrio harveyi* quorum-sensing signal AI-2. The AI-2 structure was elucidated in the crystal structure of the PBP (28).

HEHEAA induced PipR-dependent transcription of *pipA* at concentrations as low as 10 pM with a maximal response at about 1 μM HEHEAA. The level of sensitivity is greater than that for most acyl-HSL–responsive LuxR homologs, which function in the range of 10–500 nM with two exceptions of sensitive acyl-HSL systems, BjaR and BraR from *Bradyrhizobium* spp (29, 30).

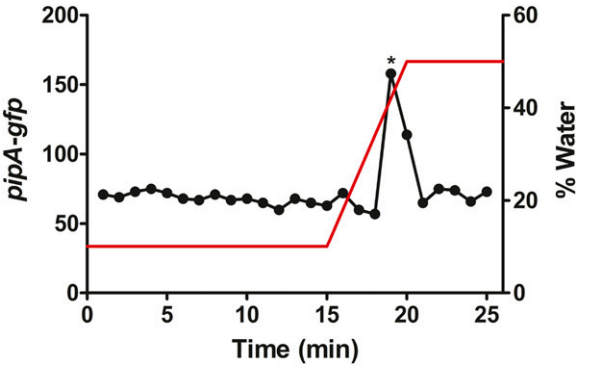

Fig. 5. Fractionation of *Populus* leaf macerates by HILIC suggests that HEHEAA is the active compound in plants. HILIC-fractionated *Populus* leaf macerates (50 μL of 5% plant macerate wt/vol) were tested by using the bioassay. The red line shows the gradient of water. The asterisk indicates the fraction where synthetic HEHEAA elutes.

In the case of the HEHEAA-responsive system in *Pseudomonas* GM79, active transport of HEHEAA presumably serves to concentrate the signal in cells, and if PipR is in fact the HEHEAA receptor it might have a HEHEAA affinity similar to typical acyl-HSL–responsive LuxR homologs.

An important question is whether HEHEAA is the *Pseudomonas* GM79 *pipA* inducer *in planta*. Ethanolamine is an important metabolite required for synthesis of the membrane phospholipids PE and PC (24), and it can be formed either directly or indirectly from serine (26, 31, 32). Plants are able to directly generate ethanolamine by serine decarboxylation (26, 27). Plants also use ethanolamine in the production of a variety of *N*-acylethanolamines. These molecules modulate several plant physiological processes such as seed germination, plant–pathogen interactions, chloroplast development, and flowering (25). Our results indicate that HEHEAA is present in *Populus* tissue (Fig. 5). Based on the activity of *pipA*-inducing water-soluble material in plant leaves we estimate HEHEAA is at a concentration of about 10 pmol per gram of plant tissue. We know that the material obtained from leaf macerates requires AapF to induce the *pipA* promoter, coelutes with authentic HEHEAA, and activates the *pipA* promoter in a PipR-dependent fashion. We have not been able to confirm the molecular mass of the plant material because we have not collected a sufficient amount of inducer for mass spectrometry.

Plant-responsive LuxR homologs, which activate genes coding for putative proline iminopeptidases, occur in a variety of Proteobacteria (7, 33). These genes have been studied in some detail in certain plant pathogens and mutualistic symbionts. In both rice and cabbage pathogenic species of the genus *Xanthomonas* the PipR homolog responds to plant material by activating the linked proline iminopeptidase gene and other genes, and mutations of the *pipR* homologs attenuate virulence (1, 2, 4). There have been unsuccessful efforts to identify the inducers for the xanthomonads, but we know that the inducers for these pathogens are water-soluble and low molecular weight (13, 34). Perhaps the activator of some of these related systems is HEHEAA. We believe a sensitive response to HEHEAA would require a HEHEAA transport system like that encoded by genes linked to *pipR*. In fact the arrangement of *pipR*-like, *pipA*-like, and transport genes in a number of bacteria is similar to that in *Pseudomonas* GM79 (35), but in other bacteria with linked *pipR*-like, *pipA-like* genes there is either a set of different putative transporter genes or there are no linked transporter genes. We note that there are dozens of small molecules that form spontaneously from ethanolamine (21). Perhaps some of these compounds will serve as ligands for PipR-like proteins in other bacterial species.

Also of interest is that ethanolamine catabolism by some animal and plant pathogens is important for virulence (36). For instance, ethanolamine utilization promotes survival of *Listeria monocytogenes* in both intestinal epithelial cells and in bloodstream infection (37, 38), and a mutant of the plant pathogen *Erwinia chrysanthemi* blocked in ethanolamine metabolism was unable to cause systematic infection in plants (39). Ethanolamine has also been reported to be a signal or cue involved in responses of bacteria to mammalian hosts. For example, this molecule has been reported to activate expression of genes important for virulence of enterohemorrhagic *E. coli* O157:H7 (EHEC) at concentrations that do not support growth of the bacterium (40). Our findings raise the possibility that the *E. coli* response might not be to ethanolamine itself but rather to one of the many small molecules formed spontaneously in ethanolamine solutions.

The results presented here substantially advance the understanding of an important group of non–acyl-HSL–responsive LuxR homologs and lend insight into how these transcription factors might be responding to their plant host environments. The work also connects at least one of the plant-responsive systems to a growing understanding of ethanolamine chemistry and responses of bacterial cells to ethanolamine and ethanolamine derivatives.

Methods

Bacterial Strains and Growth Conditions. Bacterial strains and plasmids are described in *SI Appendix*, Table S2. *Pseudomonas* sp. GM79 and its derived strains were grown in LB broth (41) or M9 minimal medium (42) with 10 mM succinate (succinate minimal medium) at 30 °C with shaking, unless otherwise indicated. *E. coli* strains were grown in LB broth or in M9 minimal medium with 20 mM glucose, 1 μg/mL biotin, and 1 μg/mL thiamine (glucose minimal medium) at 37 °C with shaking. Antibiotics were used as appropriate at the following concentrations: 50 μg/mL (*E. coli*) or 25 μg/mL (*Pseudomonas* GM79) kanamycin (Km), 20 μg/mL (*E. coli*) or 50 μg/mL (*Pseudomonas* GM79) gentamicin (Gm), and 10 μg/mL tetracycline (Tc). For plating we used media solidified with 1.5% agar.

Chemicals. Ethanolamine (99% pure) was purchased from Acros Organics, and ^{15}N-ethanolamine (98% pure) was purchased from Sigma-Aldrich. HEHEAA (95% pure) was purchased from Chiron AS; however, our own analysis determined that the compound was only 75% pure, and most of the impurity (20% of the total sample) was identified as HeGly. HeGly (90% pure) was purchased from Santa Cruz Biotechnology, Inc. Partially purified *Populus* leaf macerates were prepared as described before (16).

Reporter Assays. Bioassays were performed by growing bacteria containing the plasmid pP_{pipA}-*gfp* in succinate minimal medium with Km, as described previously (16). Briefly, inocula from 18-h cultures were used to inoculate fresh medium, and the inoculum size was 5%. The inoculated medium was then dispensed into wells of a 384-well microtiter dish (70 μL per well) containing the material to be tested and incubated at room temperature for 8 h. GFP fluorescence (excitation 485 nm, emission 535 nm) and growth (optical density at 600 nm) were assessed by using a Synergy H1 (BioTek) plate reader, and data are reported as relative fluorescence units (RFU) per optical density (OD) × 10.

Screening a Small-Molecule Library in Biolog Plates. We screened 760 small molecules available in Phenotype Microarray Plates PM1-8 (Biolog, Inc.) by using *Pseudomonas* GM79 Δ*pipA* Δ*aapA* (pP_{pipA}-*gfp*) as a reporter. Plates were inoculated with 100 μL of cultures prepared as in the previous section and incubated for 18 h, after which GFP fluorescence and culture density were measured. Fluorescence per OD compared with that without added test compounds was used to assess whether a compound could serve as a *pipA-gfp* activator.

Mutant and Plasmid Construction. All plasmids and primer sequences are described in *SI Appendix*, Tables S2 and S3, respectively. To construct the *aapF* null mutant, we PCR-amplified sequences upstream (642 bp) and downstream (615 bp) of the intended deletion. The upstream fragment was amplified from strain GM79 genomic DNA using the primer pair aapFmut_1F and aapFmut_1R, and the downstream fragment was amplified with the primer pair, aapFmut_2F and aapFmut_2R. Overlap extension PCR was used to combine the fragments with the primers aapFmut_1F and aapFmut_2R. The resulting amplicon was cloned into EcoRI-BamHI–digested pEX19-Gm (43). This suicide vector was then introduced into *Pseudomonas* GM79 by conjugal mating, and colonies with single crossovers were selected by plating on succinate minimal agar containing Gm. To screen for double-crossover mutants we streaked Gm-resistant isolates on LB agar containing 10% sucrose and screened for loss of Gm resistance. To evaluate PipR activation of the *pipA* promoter in the AapF mutant we introduced pP_{pipA}-*gfp* (16) into the mutant by conjugation.

To complement the *aapF* mutation, a DNA fragment containing *aapF* was PCR-amplified by using *Pseudomonas* GM79 genomic DNA as a template with the primer pair aapFcomp_F and aapFcomp_R. The PCR product was cloned into EcoRI-HindIII–digested pMMB67EH-TetRA. The complementing plasmid (or pMMB67EH-TetRA vector control) was introduced into the *aapF* mutant harboring the pP_{pipA}-*gfp* reporter by conjugation. All mutant and plasmid constructs were confirmed by DNA sequencing.

Purification of His_6-Tagged AapF. A cytoplasmic version of AapF for overexpression in *E. coli* was created by cloning the *aapF* gene with the secretion signal peptide omitted (nucleotides 1–78 of the *aapF* ORF) into the His_6-tag protein expression vector pQE-30 to generate pQEaapF (*SI Appendix*, Tables S2 and S3). The signal peptide was identified computationally by using SignalIP (44). To obtain purified His_6-AapF, *E. coli* M15 pRep4 containing pQEaapF was grown at 37 °C in glucose minimal medium plus antibiotics to an optical density of 0.6 at 600 nm. We then added 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated cultures at 18 °C for 16 h, after which cells were pelleted by centrifugation. Cell lysates were prepared by suspending the pelleted cells in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl,

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10 mM imidazole, pH 8), lysing them by using a French Pressure Cell, and removing cell debris by centrifugation for 20 min at 14,000 × *g*. The His_6-tagged AapF was purified from the cell lysate by nickel resin column chromatography (Qiagen). To obtain purified His_6-$AapF_{cyto}$ bound to the ligand we added 10 mM of either ethanolamine or ^{15}N-labeled ethanolamine to the *E. coli* M15 pRep4-containing pQEaapF culture together with the IPTG and then proceeded to purify AapF as described above.

HILIC Fractionation. Materials were fractionated by using a CORTECS HILIC column (4.6 × 100 mm, 2.7 μm particle size; Waters) and the following solvents: A. 10:90 acetonitrile:water 10 mM ammonium formate pH 4 and B. 90:10 acetonitrile:water 10 mM ammonium formate pH 4. Runs were started at 100% solvent B for 15 min, after which a 5-min gradient to 50:50 A:B was executed, and the A:B 50:50 mixture was maintained for an additional 5 min. The flow rate was 1.2 mL per min, and 1-min fractions were collected. Fractions were concentrated under a gentle stream of nitrogen gas at 70 °C and tested for bioactivity as described above.

Purification and Identification of AapF-Released Compounds. His_6-Aap was purified from *E. coli* as described above, and the buffer was exchanged with 100 mM ammonium acetate pH 7.5 by using an Amicon Ultra-15 filter with a 30,000 nominal molecular weight limit. The protein was then denatured at 70 °C for 20 min, centrifuged at 5,000 × *g* to separate the protein pellet from the released material, and the supernatant fluid was filtered with an Amicon Ultra-15 filter (3,000 nominal molecular weight limit) to remove any larger-mass, nonactive compounds. The material released from His_6-AapF denaturation was dried by a rotary evaporation and suspended in liquid chromatography-mass spectometry (LC/MS)-grade water (Fischer Scientific). The active material was separated by HILIC, and fractions were tested for bioactivity as described above. The bioactive molecule was eluted in fractions 19 and 20 (Fig. 3*C*). The LC/MS analysis was performed on an Agilent 6530 LC-q-TOF mass spectrometer equipped with an ultrahigh performance liquid chromatography system using a Kinetex 2.6-μm HILIC (100 Å-pore size 100 × 2.1 mm) column under the same gradient described above for purification but with a 0.5 mL/min flow rate. The active metabolite was eluted predominately between 7–8 min.

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