A LuxR Homolog in a Cottonwood Tree Endophyte That Activates Gene Expression in Response to a Plant Signal or Specific Peptides

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ABSTRACT Homologs of the LuxR acyl-homoserine lactone (AHL) quorum-sensing signal receptor are prevalent in Proteobacteria isolated from roots of the Eastern cottonwood tree, Populus deltoides. Many of these isolates possess an orphan LuxR homolog, closely related to OryR from the rice pathogen Xanthomonas oryzae. OryR does not respond to AHL signals but, instead, responds to an unknown plant compound. We discovered an OryR homolog, PipR, in the cottonwood endophyte Pseudomonas sp. strain GM79. The genes adjacent to pipR encode a predicted ATP-binding cassette (ABC) peptide transporter and peptidases. We purified the putative peptidases, PipA and AapA, and confirmed their predicted activities. A transcriptional pipA-gfp reporter was responsive to PipR in the presence of plant leaf macerates, but it was not influenced by AHLs, similar to findings with OryR. We found that PipR also responded to protein hydrolysates to activate pipA-gfp expression. Among many peptides tested, the tripeptide Ser-His-Ser showed inducer activity but at relatively high concentrations. An ABC peptide transporter mutant failed to respond to leaf macerates, peptone, or Ser-His-Ser, while peptidase mutants expressed higher-than-wild-type levels of pipA-gfp in response to any of these signals. Our studies are consistent with a model where active transport of a peptidomimetic signal is required for the signal to interact with PipR, which then activates peptidase gene expression. The identification of a peptide ligand for PipR sets the stage to identify plant-derived signals for the OryR family of orphan LuxR proteins.

IMPORTANT We describe the transcription factor PipR from a Pseudomonas strain isolated as a cottonwood tree endophyte. PipR is a member of the LuxR family of transcriptional factors. LuxR family members are generally thought of as quorum-sensing signal receptors, but PipR is one of an emerging subfamily of LuxR family members that respond to compounds produced by plants. We found that PipR responds to a peptidomimetic compound, and we present a model for Pip system signal transduction. A better understanding of plant-responsive LuxR homologs and the compounds to which they respond is of general importance, as they occur in dozens of bacterial species that are associated with economically important plants and, as we report here, they also occur in members of certain root endophyte communities.
plant-associated compounds that serve as their ligands have yet to be identified. The best-studied examples are from plant-pathogenic members of the genus Xanthomonas (14–17), but similar systems are found in other plant-associated bacteria (11–13), including plant symbionts (18) and biocontrol agents (12). LuxR homologs from several of these bacteria have been shown to activate the transcription of adjacent genes annotated as encoding proline iminopeptidases (pip genes). The pip genes have been implicated as virulence factors in some bacteria (14, 15). To distinguish the plant-responsive LuxR homologs from the AHL-responsive LuxR homologs, we refer to this subfamily of regulators as OryR regulators, because X. oryzae OryR was one of the earliest described plant-responsive LuxR homologs (16).

Here, we describe an OryR regulator that we name PipR, encoded in the Populus root endophyte Pseudomonas sp. strain GM79 (2), a member of the Pseudomonas fluorescens subfamily (19, 20). The genes flanking pipR are predicted to encode peptidases and an ATP-binding cassette (ABC) peptide transporter. We show that, similar to X. oryzae OryR, PipR activates the transcription of a flanking peptidase gene in response to plant leaf macerates but not in response to AHLs. PipR also responded to protein hydrolyses and a specific peptide (Ser-His-Ser) to activate the expression of the flanking peptidase gene. We show that the PipR response requires the ABC transporter and is modulated by the adjacent peptidase enzymes, perhaps forming a feedback loop. We propose that because we have identified a specific signal molecule, the Pseudomonas sp. GM79 PipR system can serve as a model for molecular analyses of the plant-responsive OryR family of signaling systems, which are found in a large number of diverse, plant-associated bacteria.

RESULTS

GM79 possesses an oryR homolog, which is flanked by peptidase genes. The genome of Pseudomonas sp. GM79 (21) contains two orphan luxR homologs (2), PMI36_01833 and PMI36_04623. The polypeptide encoded by PMI36_01833 is a homolog of the PpoR orphan from Pseudomonas putida, which responds to the AHL signal, 3-oxo-hexanoyl-3-homoserine lactone (3-oxo-C6-HSL) (2, 22). The other luxR homolog, PMI36_04623, is predicted to be a member of the OryR subfamily of plant-responsive LuxR homologs, based on its amino acid sequence and the context of neighboring pip genes (2, 12). Like other OryR-type polypeptides, PMI36_04623 has a tryptophan in place of a tyrosine that is conserved in the AHL-responsive LuxR homologs, but unlike the Xanthomonas and Ensifer OryR homologs, a conserved tryptophan residue remains unchanged (see Fig. S1 in the supplemental material) (reviewed in reference 12).

All known oryR homologs are flanked by at least one gene annotated as a proline iminopeptidase gene (pip) (15). In GM79, the oryR homolog is flanked by two genes predicted to encode proline iminopeptidases (http://img.jgi.doe.gov) (21) (Fig. 1), in a genomic arrangement similar to that of the oryR homolog (nesR) in Ensifer melliloti (18). To confirm whether the genes flanking the GM79 oryR homolog actually code for peptidases, both enzymes were purified as hexahistidine-tagged fusion proteins and assayed for their ability to cleave N-terminal amino acid residues from a

### TABLE 1 Substrate specificities of purified His<sub>6</sub>-PipA and His<sub>6</sub>-AapA enzymes

| Substrate                        | His<sub>6</sub>-PipA   | His<sub>6</sub>-AapA |
|----------------------------------|-----------------------|---------------------|
| L-Proline-β-naphthylamide        | 100.0 ± 13.4          | 9.7 ± 0.8           |
| L-Alanine-β-naphthylamide        | 79.4 ± 12.5           | 331.1 ± 39.9        |
| L-Hydroxy-proline-β-naphthylamide | 30.4 ± 1.3        | 23.5 ± 2.6          |
| L-Serine-β-naphthylamide         | 21.5 ± 2.6            | 12.0 ± 0.5          |
| L-Leucine-β-naphthylamide        | 7.7 ± 1.8             | 2.1 ± 1.0           |
| L-Histidine-β-naphthylamide      | ND                    | 2.9 ± 0.8           |
| L-Glutamic acid-β-naphthylamide  | ND                    | ND                  |
| L-Proline-p-nitroanilide         | 0.72 ± 0.01           | 0.006 ± 0.001       |
| L-Methionine-p-nitroanilide      | 0.17 ± 0.02           | 0.160 ± 0.021       |
| L-Lysine-p-nitroanilide          | ND                    | ND                  |

*Enzyme (PipA [PMI36_04624] and AapA [PMI36_04622]) purification and assay conditions are described in Materials and Methods; the results are the mean activities from 4 to 8 assays. Naphthylamide substrate results were measured as relative fluorescence units (RFU) per min per mg of protein and normalized to the activity exhibited by His<sub>6</sub>-PipA with L-proline-β-naphthylamide as the substrate. Nitroanilide substrate results are reported as micromoles cleaved per min per mg of protein. ND, not detected (not above the background of the no-added-enzyme control).
variety of fluorescent (β-naphthylamide) and chromogenic (p-nitroanilide) substrates (Table 1). The PMI36_04622 enzyme was most active in cleaving an N-terminal alanine, while the PMI36_04624 enzyme exhibited good activity in cleaving N-terminal proline and, to a slightly smaller degree, alanine. Both enzymes had moderate activity with hydroxy-proline-, serine-, and methionine-linked substrates, while little-to-no peptidase activity was observed with histidine-, glutamic acid-, and lysine-linked substrates (Table 1). Based on the substrate specificities exhibited by the purified GM79 enzymes, we propose naming PMI36_04622 and PMI36_04624 aapA for alanine aminopeptidase and pipA for proline iminopeptidase, respectively.

A bioassay for the plant-derived signal. To aid in the identification of the predicted plant-derived signal for Pseudomonas sp. GM79, we required a promoter that uses the PMI36_04623 OryR homolog for activation. In other systems, the pip gene adjacent to the oryR-type gene is often under OryR control (14–16). In the presence of the plant-derived ligands, the OryR homologs are believed to bind inverted repeat DNA elements (23) and activate gene transcription. The gene encoding the Pseudomonas sp. GM79 OryR homolog is also upstream from a proline iminopeptidase gene (pipA), and thus, we have named it pipR (Fig. 1). Previously, we reported that an inverted repeat sequence centered −71.5 bp upstream from the translational start site of the GM79 pipA gene matched the published DNA-binding site for X. oryzae OryR in 13 of 20 bases (2). We created the reporter plasmid pP_{pipA-gfp} (see Materials and Methods; see also Table S1 in the supplemental material), which contains a transcriptional fusion of the GM79 pipA promoter with the green fluorescent protein gene (gfp) (Fig. 2a). We hypothesized that the GM79 pipA promoter would be active when GM79 (pP_{pipA-gfp}) was grown in the presence of plant macerates but not when grown with AHLs (16). For these experiments, we grew the GM79 (pP_{pipA-gfp}) strain in minimal medium (see Materials and Methods) to avoid the potential activation of the PipR system, as has been reported for OryR when X. oryzae is grown in rich medium even in the absence of rice macerates (24). We tested six AHL signals (see Materials and Methods) with various side-chain lengths and substitutions and found that, even at relatively high concentrations (1 μM), pP_{pipA-gfp} expression was not higher than in the controls with only water added. Our initial experiments using Populus leaf macerates were unsuccessful, as the growth of our reporter strain was inhibited. Populus leaves are known to contain high concentrations of phenolics (25), which can be toxic to bacteria. Therefore, we utilized a protocol to remove the growth inhibition activity from the Populus leaf macerates (see Materials and Methods). The partially purified leaf macerates, referred to hereinafter as leaf macerates (Fig. 2b). These results are quantitatively similar to those observed with X. oryzae (24).

PipR can respond to protein hydrolysates and specific triptides. Because the genes flanking pipR are involved in peptide metabolism, we hypothesized that the plant signal may be peptide-like. We tested a variety of peptide-rich hydrolysates and found several that could activate the expression of the pP_{pipA-gfp} gene fusion (Fig. 3a). Enzymatic digests of animal tissue (Bacto-peptone), soybean meal (Bacto-soytone), and pancreatic digest of casein (Bacto-trypetone) each activated pP_{pipA-gfp} expression.

Because protein hydrolysates are rich in small peptides (26), we screened a small library of compounds (268 dipeptides and 14 triptides) that are available as part of the Biolog phenotype microarrays for microbial cells for the ability to activate pP_{pipA-gfp}. Five dipeptides induced GFP above background levels: Gly-Cys, His-Gly, His-Pro, His-Ser, and Ser-Pro. Small amounts (1 mg) of His-Ser, His-Pro, and Ser-Pro are available for purchase (AnaSpec), so we retested these dipeptides using known concentrations, but only His-Ser had appreciable pP_{pipA-gfp} reporter activity (data not shown). We purchased a larger amount (100 mg) of His-Ser from another vendor (Sigma-Aldrich) but were surprised to find that this material failed to activate our reporter. Mass spectrometry analysis confirmed that the primary species (100% relative abundance) found in both samples was His-Ser (M + H = 243.1090, 0 ppm); however, a minor species (~5% relative abundance) with a mass consistent with a tripeptide compound con-
taining one histidine and two serine residues ($M + H = 330.1407$, 0 ppm) was found only in the active sample (AnaSpec). To test the hypothesis that this minor tripeptide species was responsible for the pP<sub>pipA-gfp</sub> reporter activation, we tested all three possible tripeptide variations (SSH, SHS, and HSS) (Fig. 3b). Two of the tripeptides, SSH and HSS, had little to no activity (Fig. 3b, black and blue circles) even at the highest concentration tested (16.5 mg/ml or 50 mM). However, the SHS tripeptide showed a moderate level of pP<sub>pipA-gfp</sub> reporter expression (Fig. 3b, red circles), but only at relatively high concentrations ($\approx 0.33$ mg/ml or 1 mM). We suspect that the signal(s) present in the leaf macerate is not the SHS tripeptide, as LuxR homologs usually respond to nM (or lower) levels of their ligand (27): at 1 mM concentrations, SHS would be easily detected by mass spectrometry of plant macerates, and we cannot find it there. However, the pP<sub>pipA-gfp</sub> reporter expression with the specific SHS tripeptide is further evidence that the native ligand may be peptidelike.

The PipR protein is the receptor for the response to plant macerates and the transcription activator of pipA expression. Leaf macerate, peptone, and the SHS tripeptide all failed to activate the expression of the pP<sub>pipA-gfp</sub> reporter in a pipR deletion mutant, thus implicating the PipR protein as the signal receptor (Fig. 4). To confirm whether the DNA region of dyad symmetry predicted to bind the PipR protein was required for pP<sub>pipA-gfp</sub> activation, we mutated two conserved bases known to be important for binding of LuxR homologs (28) to create pP<sub>pipA-mut-gfp</sub> (see Table S1 and Fig. S2a in the supplemental material) and found that PipR protein-dependent transcription from the pipA pro-
moter was abolished (Fig. 2b). The pipR mutation was complemented by expressing pipR from a plasmid—although overexpression of pipR on a multicopy plasmid resulted in high GFP expression levels even in the absence of signal (see Fig. S2a).

There is also a potential PipR-binding site centered −91.5 bases upstream from the ATG start of the aapA gene (2), although this sequence overlaps the 5′ coding region of the pipR gene (Fig. 2a). To test whether the aapA gene was also under control of PipR, we created an aapA promoter reporter plasmid, pP_{aapA-gfp} (see Table S1 and Fig. S2a in the supplemental material). The basal gfp expression levels of pP_{aapA-gfp} were about five times higher than those of pP_{pipA-gfp} in wild-type cells. The addition of leaf macerates had a very small effect, but peptone stimulated pP_{aapA-gfp} expression by about 1.5-fold (Fig. 2b). The expression of pP_{aapA-gfp} in a PipR deletion strain was reduced in cells grown in the presence of peptone (Fig. 2b). These results indicate that PipR strongly controls downstream pipA expression and has a small but measurable effect on aapA expression.

A mutation in the putative ABC transporter gene aapB abolishes induction of pP_{pipA-gfp} by plant macerates, peptone, and SHS tripeptide. The aapA gene and the downstream ABC-type transporter genes, now named aapB, -C, -D, -E, and -F, are likely cotranscribed as an operon (the aapA-F operon), as there is little intergenic sequence between them (Fig. 1). The transmembrane domain (TMD) polypeptides (encoded by PMI36\_04621 and _04620; aapBC) are predicted to have six transmembrane domains each (http://www.cbs.dtu.dk/services/TMHMM-2.0/), placing this transporter in the type 1 family of ABC importers (29, 30). Because a similarly annotated ABC-type peptide transporter is adjacent to the pipR homolog in E. meliloti (18) (as well as several bacterial isolates from Populus roots [2, 21, 31]) and because PipR responds to the tripeptide SHS, we wondered whether the putative transporter was required for the PipR signal(s) to enter the cell. To assess the role of aapB-F in pipA activation, we created an in-frame deletion mutation in aapB. This AapB mutant did not respond to leaf macerates, peptone, or the SHS tripeptide (Fig. 4). The aapB mutation could be complemented with an aapB expression plasmid (see Fig. S2b in the supplemental material). These data are consistent with the idea that the PipR signal is taken up by cells via the aap operon-encoded ABC-type transporter.

Peptidase mutants exhibit an enhanced pipA-gfp response. We showed as described above that aapA and pipA encode peptidases capable of cleaving several different N-terminal amino acid residues (Table 1). We investigated whether peptidase gene inactivation had an effect on PipR signaling and found that pP_{pipA-gfp} expression was much higher in the peptidase mutants than in the wild-type GM79 when grown with leaf macerate or peptone. When grown with leaf macerates, pP_{pipA-gfp} expression in the peptidase single mutants and the pipA aapA double mutant was about twofold and sixfold higher, respectively, than in the wild type (Fig. 4a). These levels were even higher when cells were grown with peptone (2- to 5-fold higher for the single peptidase mutants and 14-fold higher in the pipA aapA double mutant relative to the levels in the wild type) (Fig. 4b). The higher pP_{pipA-gfp} activities in the single aapA and pipA peptidase mutants were complemented to nearly wild-type levels by the expression of the respective peptidase gene (see Fig. S2c and d in the supplemental material). The AapA and PipA enzymes of GM79 are both predicted to localize to the cytoplasm (32). Our results are consistent with a model where the transported plant or peptone signals are degraded by the enzymatic activities of AapA and/or PipA (Fig. 5). However, we cannot exclude the possibility that the imported signal is modified by GM79 and that this modified form of the signal is a substrate for the peptidases or that the peptidases target other components of the PipR system.

DISCUSSION
We show here that, as in several plant-associated bacteria (14–16, 18, 33), the Populus tree endophyte Pseudomonas sp. GM79 possesses a LuxR homolog that does not respond to AHL signals but instead recognizes an unknown compound in Populus leaf macerates. We call this LuxR homolog PipR. Our work demonstrates that PipR binds to a specific DNA sequence to activate the expression of its downstream proline iminopeptidase gene (pipA) in response to an unknown plant signal (Fig. 2b and 3). These results are similar to those found previously in X. oryzae (16, 24).

To extend our work in Pseudomonas sp. GM79 beyond what is known about the homologous Xanthomonas systems (14–17), we examined whether the genes surrounding pipR contribute to its activity. These flanking genes are annotated as being involved in peptide degradation and transport, leading us to hypothesize that PipR could respond to peptidolike compounds. Indeed, we found that a variety of peptidase-rich peptones (including Bacto-peptone) and a specific tripeptide (SHS) could activate a PipR-dependent reporter.

A strain with a mutation in a transmembrane domain (TMD)
protein gene (aapB) of the ABC transporter near pipR (Fig. 1) did not respond to plant leaf macerates, peptone, or the SHS tripeptide (Fig. 4), suggesting that these signals (s) enter cells by active transport. Transporters are not required for entry of AHL signals into cells, as AHLs can diffuse into and out of bacterial cells (34, 35). However, ABC-type transporters are used in many of the Gram-positive quorum-sensing systems for the import of peptide pheromone signals (reviewed in Cook and Federle [36]). There are no ABC-type transporters genetically linked to the oryR homologs in Xanthomonas species (http://img.jgi.doe.gov); however, upstream from the oryR-type genes is a gene annotated as a member of the amino acid/polyamine/organocation (APC) transporter superfamily (TC 2.A.3); interestingly this transporter gene is highly expressed (12-fold higher than in the wild type) in an X. axonopodis strain overexpressing an OryR (XagR) homolog (14). One could imagine that this APC transporter may play a role in Xanthomonas species similar to that of the GM79 ABC transporter: import of the OryR-responsive plant signal(s).

Strains with mutations in the flanking peptidease genes showed elevated expression of pP_pipa-gfp compared to the level in the wild type when grown in the presence of leaf macerates and peptone (Fig. 4a and b). A similar result, increased pip expression compared to the level in the wild type, was reported for an X. campesiris Pip- mutant (15).

One interpretation of these results is that the peptideases enzymatically degrade the PipR signal(s) and in the peptidease mutants, less signal degradation occurs, resulting in higher PipR-dependent gene activation. A model of the PipR system consistent with these data is depicted in Fig. 5. Signal(s) enter the cell via the ABC-type transporter and activate PipR-dependent transcription of pipA. Although the Pip activity from X. campesiris has been reported as localized to the periplasm (15), both AapA and PipA of Pseudomonas sp. GM79 are predicted to be cytoplasmic (32). For Pseudomonas sp. GM79, our data suggest that AapA and PipA can utilize a transported PipR ligand as a substrate, although we cannot exclude the possibility that they act on a compound derived from the ligand or on some other component of the PipR signaling system. This arrangement constitutes a negative-feedback loop for the system, which would ensure a rapid inactivation of pipA transcription when the signal becomes limited.

There is increasing evidence that not all orphan LuxR homologs sense AHLs. In addition to the plant-responsive OryR-type transcription factors discussed here, the LuxR homologs CarR (Serratia sp. strain 39006) (37) and MalR (Burkholderia thailandensis) (38), which both retain all of the conserved amino acid residues in the AHL-binding domain of LuxR homologs, do not require an AHL for activity. There are also examples of orphan LuxR homologs that utilize endogenous non-AHL compounds as signal ligands, including PluR (Photorhabdus luminescens) (9) and PauR (Photorhabdus asymbiotica) (10), which respond to α-pyrones and dialkylresorcinols, respectively. In addition, activators of AHL-responsive LuxR homologs have been identified which bear little resemblance to the native AHL signal ligand (39). Our work suggests that the GM79 PipR ligand is peptidelike. It will be interesting to purify and elucidate the structures of the PipR signals from both the plant macerate and peptone material. We predict that the plant and peptone signals will be structurally similar but not necessarily identical.

We are curious to test whether the PipR system mutants created here are also impaired in Populus host interactions, as is the case with PipR homologs in several plant pathogens (14–17) and mutualists (12, 18). We are also interested to know which GM79 genes, other than the peptidease genes, are under the control of PipR. In other bacteria, PipR homologs regulate not only proline iminopeptidase gene expression but additional traits, including those important for colonization of and movement through the plant host (motility [40] and biosurfactant and adhesin production [14]), accumulation of osmoprotectants (14), and synthesis of antifungal compounds (12).

PipR homologs are encoded in the genomes of several plant-associated bacterial genera, including Xanthomonas, Dickeya, Agrobacterium, Rhizobium, Ensifer, and Pseudomonas (reviewed in references 5, 11, and 12), and whether or not all these transcription factors respond to the same plant signal or different but related compounds is not known. The plant- responsive OryRs are of general importance, as they appear to play a role in the health of economically important plants (14–17). We believe Pseudomonas GM79 is a useful model to begin to understand the chemistry of what may prove to be a new family of interkingdom signals, or cues, involved in plant-bacterium interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are described in Table S1 in the supplemental material. Pseudomonas sp. GM79 and its derived strains were grown in R2A or M9 minimal medium (41) with 10 mM succinate (M9-suc) at 30°C. E. coli strains were grown in LB broth (42) and incubated at 37°C with shaking. Antibiotics were used when required at the following concentrations: 50 μg/ml (Escherichia coli) or 25 μg/ml (GM79) kanamycin, 100 μg/ml ampicillin, 20 μg/ml (E. coli) or 50 μg/ml (GM79) gentamicin, and 10 μg/ml tetracycline.

Chemicals. AHL signals were tested at 1 μM concentrations and included N-butanoyl-γ-homoserine lactone (C4-HSL); 3-oxo-hexanoyl-γ-HSL (3-oxo-C6-HSL), 3-oxo-octanoyl-γ-HSL (3-oxo-C8-HSL), 3-oxo-hexanoylanol-γ-HSL (3-oxo-C6-HSL), 3-oxo-decanoylanol-γ-HSL (3-oxo-C12-HSL), and (N-p-coumaryl)-γ-HSL (p-coumaroyl-HSL) (purchased from Sigma-Aldrich, St. Louis, MO, or the University of Nottingham, Nottingham, United Kingdom). The β-naphthylamide and β-nitroanilide amino acid substrates were purchased from Sigma-Aldrich. Bacto-peptone, Bacto-tryptone, and Bacto-trypsin were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). The HS dipeptide was purchased from both AnaSpec (Fremont, CA) and Sigma-Aldrich. The tripeptides HSH, SHS, and SSH were custom synthesized by Peptide 2.0 (Chantilly, VA).

Reporters, mutants, and plasmids. All plasmids and primer sequences are described in Tables S1 and S2, respectively, in the supplemental material. We created the reporter plasmids pP_pipa-gfp and pP_pipa-gfp by PCR amplifying 263-bp DNA fragments containing the intergenic promoter regions, using GM79 genomic DNA as the template, and cloning the PCR products into HindIII-BamHI-digested pPROBE-NT (43). To create pP_pipa-gfp, we ordered a gBlock gene fragment (Integrated DNA Technologies, Coralville, IA) containing the exact promoter sequence that was cloned into pP_pipa-gfp, except that the CT nucleotides present in the predicted PipR-binding site were changed to TA. Mutant constructions were performed similarly: DNA sequences of about 500 bp from both up- and downstream of the desired in-frame deletion locations were either created by two-step overlap extension PCR amplification (ΔpipA mutation) or synthesized as a single DNA fragment of about 1 kb (Eurofins Genomics, Huntsville, AL) and cloned into EcoRI-BamHI-digested suicide vector pEX19-Gm (44). The knockout suicide vector was introduced into Pseudomonas GM79 strains by conjugal mating, and single-crossover mutants were selected by plating on M9-suc agar containing gentamicin. Double-crossover mutants were selected by streaking onto R2A agar containing 5% sucrose and screened for loss of Gm.

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For complementation of the pipR mutant, we PCR amplified a DNA fragment containing 250 bp of the pipR promoter sequence, the pipR gene, and the intergenic region between pipR and pipA and cloned the PCR product into HindIII-BamHI-digested pPROBE-NT (43). For complementation of the pipA mutant, the pipA gene and 254 bp of its promoter sequence were PCR amplified by using GM79 genomic DNA as the template, and the product was cloned into the BamHI-HindIII sites of pMMB67EH-TetRA. The plasmid for aapA complementation was constructed similarly except that only 190 bp of its promoter sequence was included. Because the aapB gene likely shares a promoter with the upstream aapA gene, we used the same forward primer as was used for complementation of the aapA mutant (Aap-CompEOR) plus a reverse primer for the 3’ end of the TMD gene (TspT-CompREV) and used genomic DNA from the aapA mutant (79ΔAapA strain; see Table S1 in the supplemental material) as a PCR template. The PCR product was cloned into BamHI-HindIII-digested pMMB67EH-TetRA. Complementing plasmids (or pMMB67EH-TetRA vector controls) were introduced into the appropriate mutant strains harboring the pPlb_pipC:GFP reporter by conjugal mating. All mutant and plasmid constructs were confirmed by DNA sequencing.

Purification of His6-tagged proteins. To obtain purified PipA and AapA, the genes were cloned into the His6-tagged protein expression vector pQE-30, creating plasmids pQE-pipA and pQE-aapA, respectively (see Tables S1 and S2 in the supplemental material). *E. coli* M15 pREP4 containing either pQE-pipA or pQE-aapA was grown at 30°C in 500 ml of LB plus antibiotics to an optical density at 600 nm of 0.6 (OD600). The production of His-tagged protein was then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued at 16°C overnight, after which cells were pelleted, resuspended in buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8), broken by French pressure cell, and centrifuged for 20 min at 14,000 × g. The His6-tagged proteins were purified from clarified cell extracts by cobalt resin column chromatography (Qiagen, Valencia, CA).

Peptidase assays. Enzyme assays were performed in 0.1-ml volumes containing 50 mM Tris, pH 7.4, 10 mM MnCl2, 0.75 mM amino acid substrate, and 0.6 μg His-tagged protein. Reaction mixtures were incubated for 20 min at 30°C and stopped by equivalence addition of 0.1 M acetic acid. Substrate cleavage was assessed by measuring either fluorescence (excitation at 355 nm and emission at 415 nm) for the β-naphthylamidine-linked substrates or color ([410 nm, molar extinction coefficient (M⁻¹ cm⁻¹) = 8.000] for the p-nitroaniline-linked substrates.

Reporter assays. Bioassays were performed in M9-suc for two reasons. (i) OryR accumulated in *X. oryzae* when grown in rich medium (peptone-yeast extract-salts) in the absence of plant macerates (24), suggesting that something in complex medium can induce the system. Therefore, we decided to use a minimal medium so as not to confound our results. (ii) Sucinate was chosen as the carbon and energy source in the minimal medium because there were no significant growth rate differences between the wild-type and pipR mutant strains in this medium. Strains containing pPlb_pipC:GFP were incubated overnight (24 h) in M9-suc plus kanamycin at 30°C with shaking. Cells were diluted 1:100 into fresh medium, 150-μl aliquots were added to individual wells of a 96-well microtiter dish containing 7.5 μl (except as indicated in Fig. 3) of material to be tested (leaf macerates, peptone, peptides, or AHLS), and the plates were sealed with Breathe-Easy sealing membrane (Research Products International, Mount Prospect, IL) and incubated at room temperature for 24 h. GFP fluorescence (excitation at 485 nm and emission at 535 nm) and growth (OD595) were assessed using a Tecan Genios pro plate reader, and data were plotted as relative fluorescence units (RFU) per OD unit.

Preparation of partially purified *Populus* leaf macerates and peptone material. Because various additions to the bioassay strain culture showed both inhibitory (leaf macerates) and stimulatory (Bacto-peptone) growth effects, we developed a two-step cleanup protocol to produce the partially purified material used in all of our experiments. For leaf macerates, 5 g of *P. deltoides* WV94 leaves (greenhouse grown) were frozen in liquid nitrogen, macerated with a mortar and pestle, and added to 100 ml of Milli-Q water (5% weight/vol), sterilized by autoclaving, and then filtered to remove plant tissue (as described in reference 24). Peptone was prepared in Milli-Q water at a concentration of 10 g/100 ml (10% wt/vol). Both leaf and peptone material were then passed over a C18 reverse-phase (RP) solid-phase extraction (SPE) cartridge (Waters Corp., Milford, MA). The C18-RP cartridge did not bind the active material but did retain a large amount of nonactive material (including the bacterial-growth-inhibiting activity in the leaf macerates). The flowthrough fraction was passed through an Amicon ultra-15 filter with a nominal molecular weight limit of 3,000 (Merck Millipore, Cork, Ireland) to remove any higher-mass, nonactive compounds. Partially purified material was concentrated, re-suspended in Milli-Q water to its original concentration, and filter sterilized with a 0.2-μm syringe filter.

**Peptide screening with Biolog plates.** Biolog phenotype microarray plates for nitrogen utilization assays (PM6, PM7, and PM8) were used (Biolog, Inc., Hayward, CA). GM79 (pPpipC:GFP) cells in M9-suc medium were incubated in the Biolog plates for 18 h, and then GFP fluorescence (excitation at 485 nm and emission at 535 nm) and growth (OD595) were determined. As a control for PipR activity, 1% peptone was added to the 1-glutamine positive control present on every Biolog plate.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01101-16/-/DCSupplemental.

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