Development of Chemical and Metabolite Sensors for *Rhodococcus opacus* PD630

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Abstract

Rhodococcus opacus PD630 is a non-model, gram positive bacterium that possesses desirable traits for biomass conversion, including consumption capabilities for lignocellulose-based sugars and toxic lignin-derived aromatic compounds, significant triacylglycerol accumulation, relatively rapid growth rate, and genetic tractability. However, few genetic elements have been directly characterized in *R. opacus,* limiting its application for lignocellulose bioconversion. Here, we report the characterization and development of genetic tools for tunable gene expression in *R. opacus*, including: 1) six fluorescent reporters for quantifying promoter output, 2) three chemically inducible promoters for variable gene expression, and 3) two classes of metabolite sensors derived from native *R. opacus* promoters that detect nitrogen levels or aromatic compounds. Using these tools, we also provide insights into native aromatic consumption pathways in *R. opacus*. Overall, this work expands the ability to control and characterize gene expression in *R. opacus* for future lignocellulose-based fuel and chemical production.

Keywords: *Rhodococcus opacus*, lignin, promoter, sensor, nitrogen, aromatic

The Actinomycetales bacterium *Rhodococcus opacus* PD630 (hereafter *R. opacus*) has many desirable traits for the microbial valorization of lignocellulosic biomass. The depolymerization of lignocellulose, particularly lignin, generates a spectrum of growth inhibiting compounds.[1-3] *R. opacus* has previously demonstrated a high natural tolerance towards such products, including biomass pretreatment by-products (e.g., furfural and hydroxymethylfurfural), polychlorinated biphenyls, and aromatic and halogenated compounds.[4-6] This tolerance is partially attributed to numerous enzymatic consumption pathways that funnel aromatics into central metabolism via its high flux β-ketoadipate pathway.[7, 8] Intermediates in the β-ketoadipate pathway can be diverted into high-value products, such as muconic acid.[9-11] *R. opacus* can also concurrently consume glucose (via Entner–Doudoroff pathway), acetate (via glyoxylate shunt), and phenolic compounds (via β-ketoadipate pathway), improving productivity.[12, 13] Its high osmotic tolerance also allows for a high loading of sugars and high product titers.[14] *R. opacus* is primarily known for its ability to produce significant amounts of the biodiesel precursor triacylglycerol (TAG; up to ~78% of its cellular dry weight when grown on gluconate).[6] Both alkali lignin and biomass gasification wastewater have successfully been converted into TAG-based biofuels using *R. opacus*.[15, 16] These traits make *R. opacus* an ideal microbial biorefinery for the conversion of lignocellulosic material, particularly lignin, into fuels and chemicals.

R. opacus has been engineered to consume other lignocellulose components, including xylose, arabinose, and cellobiose, but the modifications were achieved using borrowed and generally uncharacterized genetic elements from related Actinomycetales.[13, 17-19] Optimization of gene expression and dynamic regulation have both been demonstrated to significantly improve product titers, yields and productivity, but this process requires well-characterized genetic tools to precisely control gene expression.[20-22] Promoters and dynamic sensors, often taken for granted in

model organisms, are limited in *R. opacus,* but this work expands the available genetic toolbox for future engineering efforts.

Developing synthetic biology tools in *R. opacus*, such as promoters and systems of gene control, requires easily discernable reporters. To compare fluorescence outputs relative to the background fluorescence (empty vector control strain), six reporter proteins with three distinct wavelength ranges (CFP, RFP, mCherry, GFP+, sfGFP, and EYFP) were tested under the same constitutive promoter and ribosome binding site (RBS; Figure 1A). mCherry demonstrated the highest level of fluorescence relative to the empty vector control (~452-fold), and cells were visibly red (Supplementary Figure 1). We have demonstrated the outputs of multiple fluorescent reporters that span a wide range of excitation and emission wavelengths for quantifying expression (Supplementary Table 8).

The ability to precisely regulate gene expression is critical to synthetic control systems and metabolic engineering. Chemically inducible promoters allow an external, non-feedstock signal to modulate the transcription level. Only one chemically inducible promoter has been previously described in *R. opacus*: the thiostrepton inducible TipA promoter.[(23)] Thiostrepton is an antibiotic that inhibits growth of *R. opacus*, and thus this promoter also requires a thiostrepton resistance marker. Here, we provide three additional chemically inducible promoters for *R. opacus*: an arabinose inducible promoter from *E. coli* (pBAD), an acetamide inducible promoter from *Mycobacterium smegmatis* (pAcet), and an anhydrotetracycline (aTc) inducible promoter from *E. coli* (pTet).

The pBAD promoter was placed in front of EYFP and induced with arabinose (0 to 250 mM).[(24)] We observed a ~59-fold increase in fluorescence (Figure 1B) and found that arabinose had no effect on cell growth (measured by final culture absorbance at 600 nm; Supplementary

Figure 2). These results confirm that arabinose does not support or inhibit *R. opacus* growth at these concentrations, which is consistent with the previous report.[18] pAcet has been previously utilized as a constitutive promoter in *R. opacus*.[19] To further characterize this promoter, the *amiACDS* genes were expressed in *R. opacus* with GFP+ under the pAcet control. When acetamide was added to the culture (0 to 1000 nM), we observed a ~5-fold increase in fluorescence (Figure 1B). Acetamide at these concentrations was found to have no effect on final culture absorbance at 600 nm of the empty vector control strain, but led to a minor growth reduction in the strain harboring the pAcet construct at higher inducer concentrations (Supplementary Figure 3). This result suggests that acetamide does not directly affect *R. opacus* growth, but that the expression of GFP+ and the transcriptional regulators/transporter (AmiACDS) imposes burdens on the cells at higher induction levels. Ehrt et al. (2005) and Rock et al. (2017) developed pTet promoters for the Actinomycetales *M. smegmatis* and *M. tuberculosis*.[25, 26] Both of these promoters were screened in *R. opacus*, but they exhibited low inducibility and either leakiness or over-repression (data not shown). To optimize an inducible pTet system for *R. opacus*, the pTet from Rock et al. (2017) was used as a starting point for the creation of a mutagenesis library.[26] Due to a significant growth defect and the inability to achieve appreciable induction (~1.5-fold), it was hypothesized that the TetR repressor was being too highly expressed. The -10 region of the constitutive TetR promoter (TATAAT) underwent saturation mutagenesis to reduce transcription. After screening colonies for improvements in growth and dynamic range, the best candidate (-10 region: ACCTCT) exhibited no growth defect relative to the empty vector control and demonstrated a ~67-fold increase in fluorescence when aTc was added to the culture (0.05 to 100 ng/mL; Figure 1B). aTc at these concentrations was found to have no effect on final culture absorbance at 600 nm, suggesting that aTc does not support or inhibit growth at these concentrations (Supplementary Figure 4).

Chemically inducible promoters can precisely tune gene expression, but their use is limited in industrial scale fermentations due to prohibitive inducer costs. In contrast, metabolite sensors can dynamically regulate gene expression based on changing concentrations of metabolites. Dynamic pathway regulation has previously been demonstrated to improve final product titers, yields, and productivity.[21] One of *R. opacus*' potential products is triacylglycerol (TAG), a biodiesel precursor, and its production is increased when cells are grown in low nitrogen conditions. Lipid biosynthesis pathways can also be engineered to synthesize fatty acids of different chain lengths, saturation degrees, or chain branching types by introducing heterologous genes.[27] A nitrogen sensor would allow such heterologous genes to be expressed only under lipid production conditions, thus conserving cellular resources and improving product titers and productivity.

We tested seven *R. opacus* promoters with the hope of identifying a nitrogen responsive promoter (Figure 1C). Although the corresponding seven genes were found to be highly transcribed under low nitrogen conditions (from RNA-sequencing),[7] RNA-sequencing was not performed under high nitrogen conditions in that study, requiring further tests. To this end, the upstream regions of these seven genes were placed in front of GFP+ and screened with either 0.05 or 1.0 g/L ammonium sulfate (Figure 1C). The promoter region of LPD03031 (pLPD03031), which encodes a putative nitrite extrusion protein, was found to be induced ~18-fold in fluorescence under low nitrogen stress. To further characterize this promoter, a range of initial ammonium sulfate concentrations (0.05 to 0.8 g/L) with 10 g/L glucose were used to examine expression over time (Figure 1D). The response time of the promoter (time to reach the half maximum fluorescence) was linearly correlated ($R^2 = 0.99$; Figure 1E) with the initial ammonium sulfate concentration. In this way, the nitrogen sensor can be used as a cellular timer based on how much ammonium sulfate is initially added to the culture. As the cells consume the available

nitrogen, the promoter will eventually be induced once nitrogen stress occurs. Interestingly, pLPD03031 was not repressed by high initial concentrations of sodium nitrate (0.64 and 3.22 g/L), implying that pLPD03031 may be an ammonium-specific sensor rather than a general nitrogen state sensor (Supplementary Figure 5). High concentrations of casamino acids (1.04 and 5.18 g/L), which may be a source of ammonium via deamination, led to modest pLPD03031 repression at an earlier growth phase (5 hrs) but activation at a later phase (12 hrs). A saturating amount of ammonium sulfate (1.5 g/L) prevented induction within the tested time-frame, demonstrating that the promoter is indeed responsive to low ammonium levels and not to growth phase (Supplementary Figure 6). Future work may delve into the molecular mechanism for specificity of this sensor.

Our previous transcriptomic analysis found that genes associated with aromatic transport and consumption were upregulated in the presence of phenol when compared to a glucose control.[7] To further investigate this upregulation of phenol-related consumption pathways, the upstream regions of four of these genes were placed in front of GFP+ (LPD06568 [putative catechol 1,2-dioxygenase], LPD06575 [putative small subunit of two-component phenol hydroxylase, copy 1], LPD06699 [putative shikimate transporter], and LPD06740 [putative small subunit of two-component phenol hydroxylase, copy 2]). The fluorescence was measured with the addition of 1 g/L glucose and either 0, 0.1, 0.3, or 0.75 g/L phenol (Figure 1F). Fold inductions (0.75 g/L phenol vs. 0 g/L phenol) were 80-fold (pLPD06568), 39-fold (pLPD06699), and 247-fold (pLPD06740). The fluorescence output of pLPD06575 in the glucose-only condition was “completely off”, meaning that it was indistinguishable from the background fluorescence (within one standard deviation), and thus a fold change relative to the “off” state could not be calculated.

The mechanisms of regulation of these phenol sensors are not well understood. Aromatic metabolic pathways and related genes (Figure 2A) in Actinobacteria are in general regulated by numerous transcriptional regulators (TRs) that interact directly with the aromatic compounds and the DNA.[28, 29] These regulators frequently occur in close genomic context to their primary targets (Figure 2B). It was demonstrated in *Rhodococcus erythropolis* CCM2595 that the promoter of the two-component phenol hydroxylase operon (*pheA2* [small subunit] and *pheA1* [large subunit]) is activated by the AraC family regulator PheR when it binds to phenol.[29] Both LPD06575/LPD06740 (copy 1 and 2 of putative small subunit of two-component phenol hydroxylases) and LPD06574/LPD06739 (copy 1 and 2 of putative AraC family regulators) show high nucleotide and amino acid sequence similarity to *pheA2* and *pheR*, respectively (Figure 2B and Supplementary Table 6). The promoters pLPD06575 and pLPD06740 also share high sequence conservation with the *pheA2* upstream region (Supplementary Table 6). It was also demonstrated that the three gene *catABC* operon is repressed by the adjacent IclR family regulator CatR in *R. erythropolis* CCM2595,[29] which all exhibit high nucleotide and amino acid sequence similarity to the four gene cluster LPD06566 to LPD06569 (Figure 2B and Supplementary Table 6). The promoter pLPD06568 also shares high sequence conservation with the *catABC* operon upstream region (Supplementary Table 6). The regulatory mechanism of LPD06699 (shikimate transporter) is unknown, but an IclR family regulator (LPD06698) is located adjacent to it.

We sought to examine how a diverse set of aromatic monomers, typically found in depolymerized lignin, would affect each of these promoters (Figure 2). Each construct was tested in the presence of either of 0.3 g/L phenol (PHE) or an equimolar amount of protocatechuic acid (PCA), sodium benzoate (BEN), 4-hydroxybenzoic acid (HBA), vanillic acid (VAN), or guaiacol (GUA). All conditions also contained 1 g/L glucose (GLU). Interestingly, the tested promoters

exhibited differential expression based on the aromatic added, and the response generally corresponds to the annotated gene function. For example, both phenol hydroxylase promoters (pLPD06575 and pLPD06740) were substantially induced only in response to phenol. Comparatively, the promoter for the shikimate transporter (pLPD06699), which is expected to aid in general aromatic transport, was upregulated in the presence of all tested compounds. The catechol degradation operon promoter (pLPD06568) was most substantially upregulated by compounds known to be metabolized thorough the catechol branch of the β-ketoadipate pathway (PHE, BEN, and GUA), but minimally affected by compounds (PCA, HBA, and VAN) that are degraded via the parallel PCA branch.[30-32] This work represents the first demonstration of compound-specific, inducible aromatic consumption pathways in *R. opacus*.

R. opacus is an ideal candidate for conversion of lignocellulose, particularly lignin, due to its natively high tolerance to depolymerization products and ability to consume diverse aromatic compounds.[4-7] However, the tool to express genes in *R. opacus* has been limited to constitutive promoters or a thiostrepton inducible promoter.[13, 23] In this work, we characterized and optimized three chemically inducible sensors, which had been developed in other organisms, and newly developed two classes of metabolite sensors in *R. opacus*. Additionally, our aromatic sensors were applied to provide insights into native aromatic consumption pathways in *R. opacus*. The tools developed herein set a foundation for more controlled expression of genes for metabolic engineering in *R. opacus*.

Supporting Information

Complete Materials and Methods, Supplementary Tables 1-8, and Supplementary Figures 1-7.

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Conflict of Interest

The authors declare no competing financial interest.

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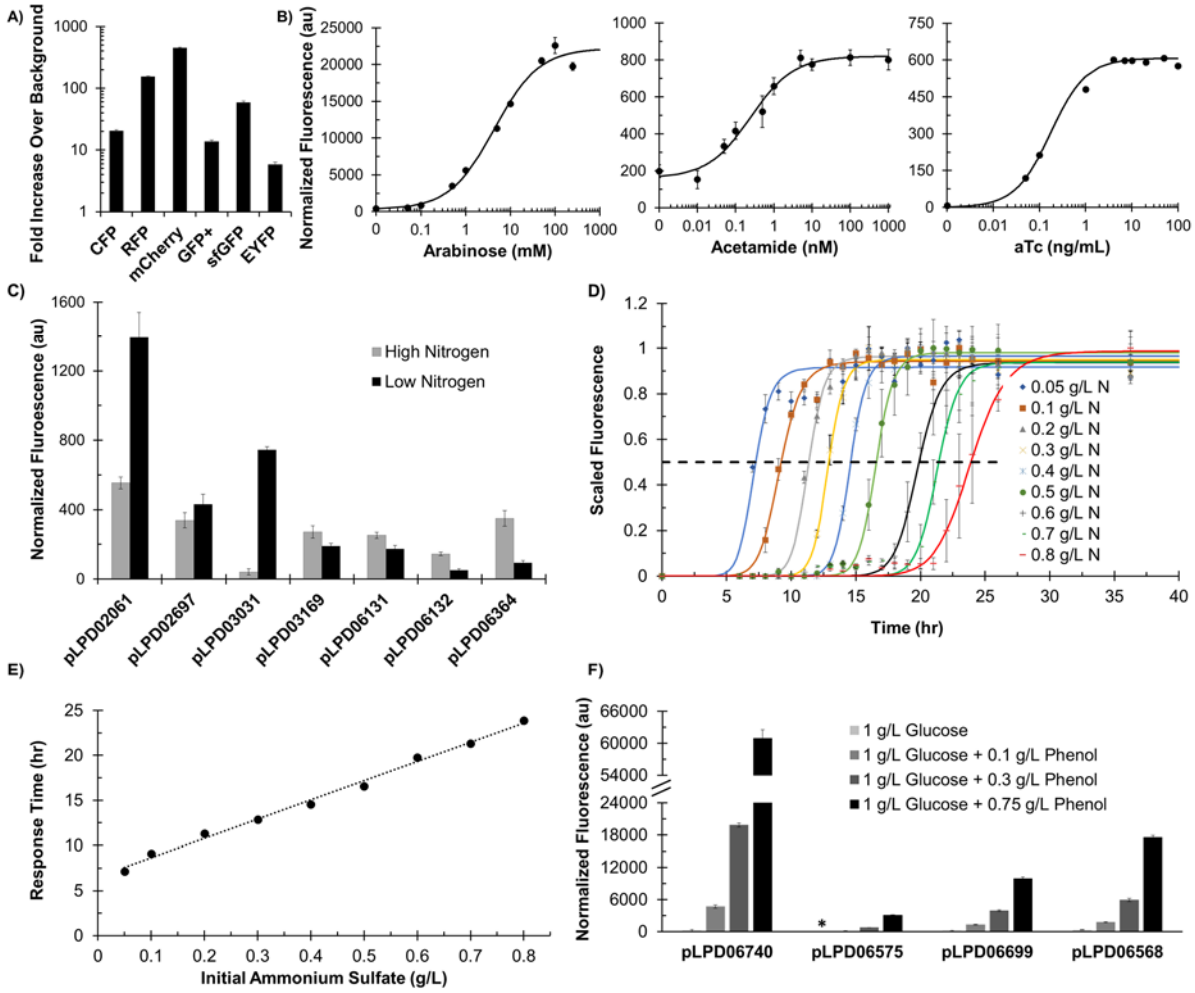

Figure 1. Chemical and metabolite sensors developed in *R. opacus*. **A)** Six fluorescent reporters expressed under the same promoter and ribosome binding site (RBS). Fold increase over background represents the absorbance-normalized fluorescence of the reporter strain ($Fluo_{sample}$ / $Abs600_{sample}$) divided by the absorbance-normalized fluorescence of the control strain containing the empty vector ($Fluo_{control}$ / $Abs600_{control}$) (see Supplementary Materials and Methods). Values represent the average of three replicates grown in minimal media A, and error bars represent one standard deviation. **B)** Three chemically inducible promoters. Transfer curves are shown for the arabinose inducible pBAD promoter driving EYFP production (~59-fold),[24] the acetamide inducible pAcet promoter driving GFP+ production (~5-fold),[33] and the optimized aTc inducible pTet promoter driving mCherry production (~67-fold).[26] Values represent the average of three replicates grown in minimal media A with 1 g/L glucose (pBAD), minimal media A with 2 g/L glucose (pAcet), or minimal media B with 2 g/L glucose (pTet), and error bars represent one standard deviation. Lines represent the fitted transfer functions (see Supplementary Table 4 for fitted parameters). **C)** Nitrogen sensor candidate promoters. The promoters of seven genes that were found to be highly transcribed under low nitrogen conditions (using previously published RNA-Seq data) were placed in front of GFP+.[7] Cultures were grown in minimal media A with 2 g/L glucose and either 0.05 g/L (low nitrogen) or 1.0 g/L (high nitrogen) ammonium sulfate.

Fluorescence was measured after 48 hrs. pLPD03031 was found to be induced ~18-fold between low and high nitrogen conditions. Values represent the average of three replicates, and error bars represent one standard deviation. **D)** Dynamics of pLPD03031 at different initial ammonium sulfate concentrations. Cultures were grown with 10 g/L glucose and either 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, or 0.8 g/L ammonium sulfate (N). The normalized fluorescence was scaled to the maximum value for each respective condition (scale 0 to 1). Values represent the average of three replicates grown in minimal media B, and error bars represent one standard deviation. Continuous lines represent the fitted transfer functions (see Supplementary Table 4 for fitted parameters). **E)** The response time (time required to reach the half-maximal fluorescence; dashed line in 1D) is directly correlated to the initial ammonium sulfate concentration (R^2 = 0.99; the dotted line represents linear fit). **F)** Four phenol sensors characterized using GFP+. The strains were grown with 1 g/L glucose and either 0, 0.1, 0.3, or 0.75 g/L phenol. Because increasing concentrations of phenol led to lengthening of the cell culture lag phase, early stationary phase fluorescence was measured at different time points (see Supplementary Table 5 and Supplementary Figure 7). Fold inductions (0.75 g/L phenol vs. 0 g/L phenol) were 80-fold (LPD06568), 39-fold (LPD06699), and 247-fold (LPD06740). The fluorescence value of pLPD06575 at 1 g/L glucose was indistinguishable from the background level (*, within one standard deviation) and a fold change could not be calculated. Values represent the average of three replicates grown in minimal media A, and error bars represent one standard deviation.

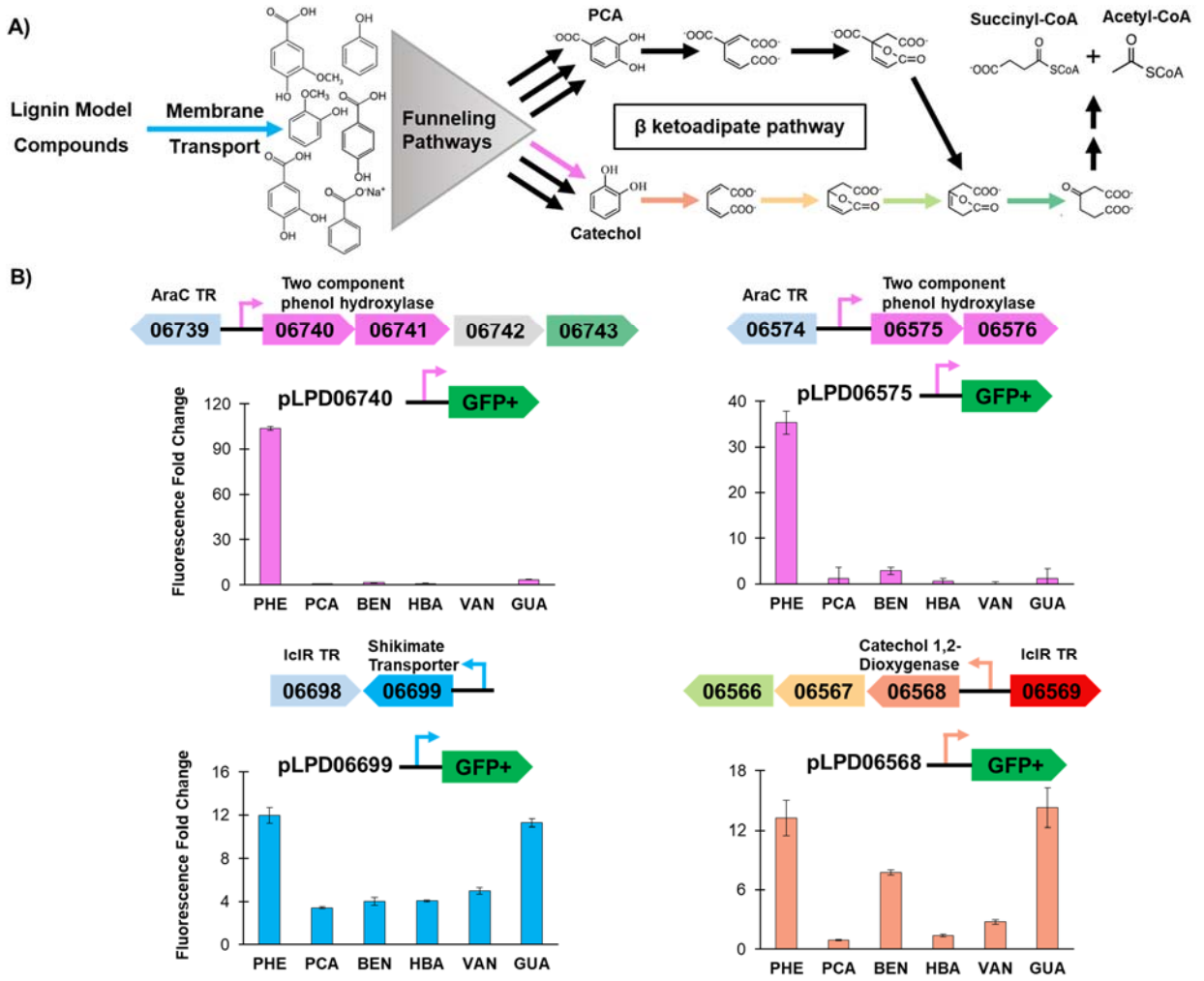

Figure 2. Differential response of native *R. opacus* promoters to distinct aromatic monomers. **A)** Schematic of lignin model compound conversion to central metabolites via aromatic funneling enzymes and the central β-ketoadipate pathway. Arrow colors correspond to those of genes and promoters in 2B, and black arrows are for conversion steps whose genes are not shown in 2B. **B)** Native genomic context of aromatic degradation and transporter operons with annotated transcriptional regulators (TR). Promoters are represented as small arrows. Genes are represented as large arrows and are annotated with LPD gene numbers from the NCBI database (Refseq, NZ_CP003949.1). The upstream regions of LPD06568, LPD06575, LPD06699, and LPD06740 were cloned in front of GFP+. All cultures contained 1 g/L glucose (GLU) in addition to any additional aromatic monomers as a carbon source. The fluorescence fold change in the presence of 0.3 g/L phenol (PHE) or an equimolar amount of protocatechuic acid (PCA), sodium benzoate (BEN), 4-hydroxybenzoic acid (HBA), vanillic acid (VAN), or guaiacol (GUA) was determined at early stationary phase (See Supplementary Table 7 and Supplementary Figure 7) relative to the glucose only condition (GLU). Bar chart colors correspond to functional steps in 2A and promoters in 2B. Values represent the average of three replicates grown in minimal media B, and error bars represent one standard deviation.