A dual selection system for directed evolution to identify allosteric transcription factor PobR variants responsive to different aromatic compounds

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Abstract:

Biosensors based on allosteric transcription factor are widely used in synthetic biology for metabolite detection, directed evolution, and dynamic control of metabolic pathways. The Acinetobacter sp. ADP1 transcription factor PobR activates the PpobA promoter when binding to its natural ligand, 4-hydroxybenzoic acid (4HB). In this study, we developed a dual selection system in *Escherichia coli* to screen for PobR mutants responsive to aromatic analogs of 4HB. This system consisted of both a positive selection to enrich PobR mutants recognizing a desired ligand, and a negative selection to eliminate PobR mutants still responsive to 4HB, or with constitutive transcriptional activity. Directed evolution of a PobR library generated a series of mutants that responded to desired aromatic compounds. Among them, a mutant $PobR^{W177R}$ displayed 5.1-fold increase of responsiveness to 4-hydroxyphenylpyruvate compared to $PobR^{WT}$. Overall, we developed a useful dual selection system to be used in selecting novel allosteric transcription factor variants.

Key words:

dual selection system, aromatic compound, directed evolution, PobR, ligand specificity

Introduction

Biosensors based on allosteric transcription factors (aTFs) have emerged as valuable tools to quantitatively detect changes of small molecules through initiating downstream genetic circuits as outputs[(1)]. The amounts of intracellularly accumulated compounds are converted into machine-readable outputs, such as fluorescent or colorimetric changes, cell density alterations, etc.[(2)]. Thus, aTF-based biosensors with innately high ligand specificity are widely used in molecule detection, enzyme-directed

evolution, dynamic control of metabolic pathways and adaptive laboratory evolution[3-6]. Despite promising applications in synthetic biology, only a handful of TFs have been developed as biosensors due to a limited number of reported ligand compounds and their effector aTFs[7]. Therefore, the ligand-binding specificity of several allosteric aTFs have been modified through rational mutagenesis or directed evolution to generate customized biosensors for the detection of various compounds of interest. Taking the aTF AraC as an example, its ligand specificity has been evolved from L-arabinose to D-arabinose, mevalonate, and triacetic acid lactone[8-10]. An obvious dilemma for the applications of these adapted biosensors is that mutations frequently change aTFs' original ligand docking pockets leading to relaxed ligand specificity that hampers their use in larger-scale screening campaigns. In order to obtain a highly specific mutant specific to a new ligand, a dual selection system was applied in previous studies; prior to ligand-responsiveness screening, the mutants responding to the original compound were wiped out by a negative selection[11,12]. Using this strategy, desirable aTFs can be enriched and identified, and novel biosensors can be developed.

PobR, a member of the iclR superfamily, is an aTF responsive to 4-hydroxybenzoic acid (4HB). In the Acinetobacter strain ADP1, when PobR activates the pobA promoter (PpobA) involved in 4HB metabolism[11], low 4HB levels trigger pobA gene expression[6,11,13-16]. Jha et al. used rational structure-based mutagenesis combined with flow cytometry to screen for PobR mutants sensitive to 3,4-dihydroxybenzoate and p-nitrophenol[14,16]. Another study established a high-throughput screening method to alleviate 4HB-mediated inhibition of choroidal pyruvate lyase (UbiC). A positive feedback gene circuit was constructed by expressing ubiC and gfp driven by PpobA UbiC variants with reduced 4HB inhibition, and thus increased 4HB production, which subsequently activated PpobA expression and enhanced UbiC and gfp expression[6,13]. Last year, our team employed error-prone PCR to develop a PobR-based biosensor responsive to a 4HB analogue, 4-hydroxymandelate (HMA). Our data implicated that PobR possesses the potential to be modified as biosensors for a variety of aromatic compounds.

In the current study, we developed a dual selection system searching for PobR mutants responsive to a series of aromatic compounds. A PobR mutant library including 21,000 mutants was established based on random mutagenesis. Mutants with transcriptional activity in the absence of inducer or in the presence of 4HB were eliminated by a negative selection. Multiple aromatic compounds were tested for their responsive PobR mutants. After discovering a PobR mutant responsive to 4-hydroxyphenylpyruvate (HPP), protein structure and ligand docking were used to decipher the alteration of ligand specificity. Overall, our work developed a robust selection system for new biosensors.

Results

Design of a Dual Selection System.

As an aTF, PobR (or $PobR^{WT}$) that drives the promoter P_{pobA} is highly specific to its original effector 4HB, and very difficult to be engineered using any rational approach based on available literature. Therefore, we used the random mutagenesis approach to modify its ligand specificity, aiming to reduce its sensitivity to 4HB, but increase the responsiveness to other aromatic compounds. To eliminate the PobR mutants that were either still responsive to 4HB or constitutively active in driving the P_{pobA} independent of any ligand, we designed a dual selection system (Figure 1a). In this system, we first constructed a negative selection system consisting of the promoter P_{pobA} and the cytosine deaminase (*codA*), to exclude PobR mutants that retained the ability to respond 4HB, as well as pseudo-positive mutants. $PobR^{WT}$ activates the P_{pobA} through binding to the cis-acting element (Oi), which activates the expression of downstream *codA* genes[17]. Its product (CDase) converts exogenously added 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), leading to cell death (Figure 1b). Thus, the 5-FC sensitivity excluded the pseudo-positive strains that produced PobR mutants capable of activating the *codA* gene driven by P_{pobA} either in the absence of any ligand or through binding to any unknown molecule that activates the P_{pobA}. Similarly, bacteria containing PobR mutants that still recognized 4HB perished. However, the *E. coli* genome has the *codA* gene that can produce endogenous CDase. Therefore, we knocked tis gene out by pCas9 in the BW25113 strain, and generated a new *E. coli* strain named BW∆*codA* .

In addition, we also placed the chloramphenicol (Cm) resistant gene (*cmr*) under the control of the P_{pobA} and used it to select the PobR mutants with the desired ligand specificity. The Cm-resistant of generated recombinant *E. coli* strain harboring P_{pobA}-codA-cmr and $PobR^{WT}$ depends on the ability of 4HB responsiveness. Since no 5-FC was added in the medium during incubation, the growth of the strain was not impacted regardless of CDase expression. Thus, after we added Cm to the LB agar medium containing an aromatic compound as a candidate ligand, and cultivated PobR mutants from the library, strains responsive to the compound could grow predominantly, representing a positive selection (Figure 1c).

An additional reporter gene for the red fluorescent protein *mCherry* was also added into the selection system (Figure 1). The reporters would be expressed if the PobR protein underwent allosteric changes when binding to a ligand, and then activated the P_{pobA}[13]. The red fluorescence intensity of the mCherry protein would be proportional to the ligand levels in the medium, and could therefore be used to determine ligand binding affinity. Using this dual selection system, PobR mutants that effectively recognized an aromatic compound as the most preferable ligand could be enriched for further characterization.

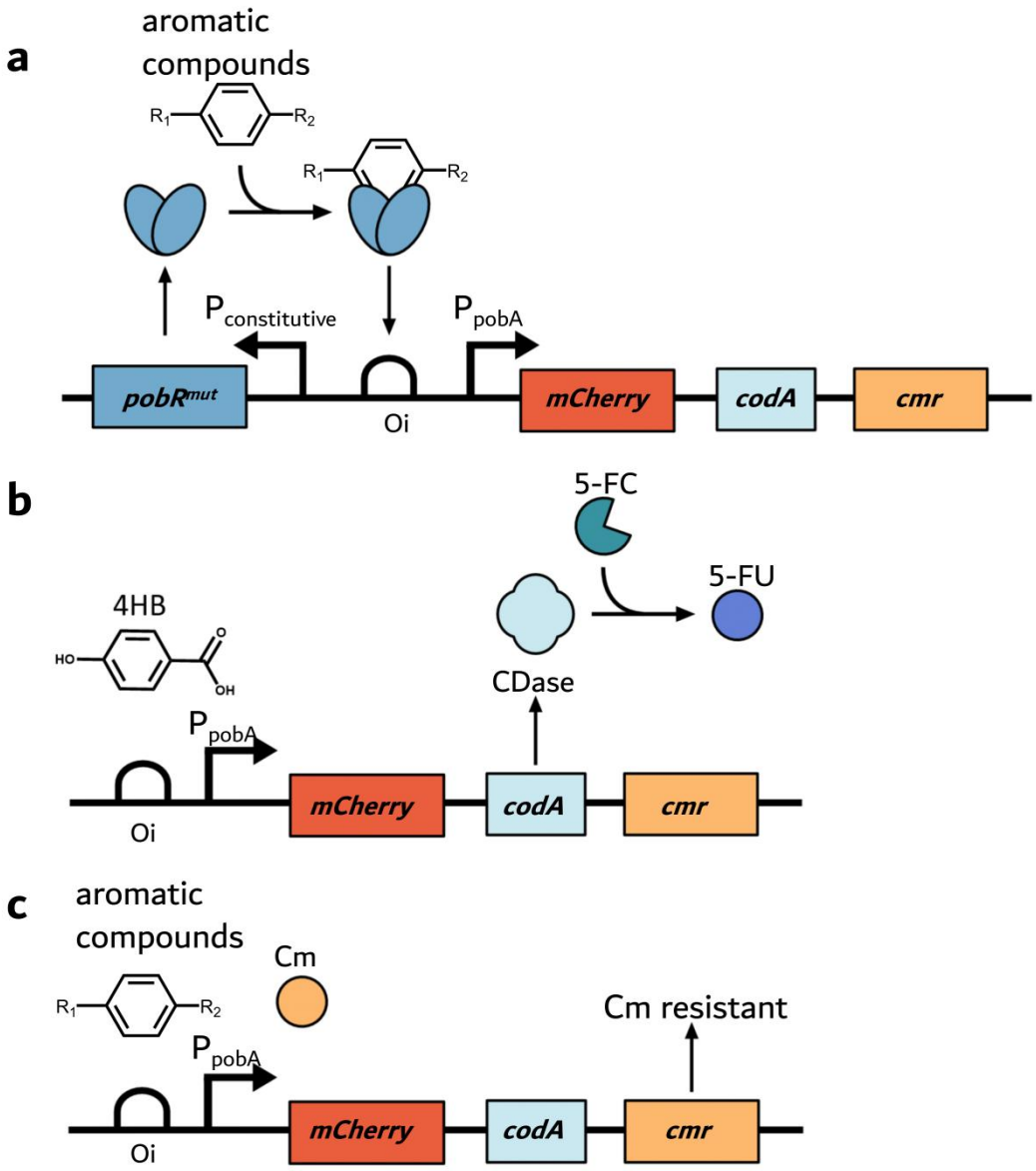

Figure 1. Schematic diagram of the dual selection system. (a) The transcription factor PobR activates the pobA promoter (P_{pobA}), when it binds to a ligand. (b) When the P_{pobA} is allosterically activated by its native ligand 4HB, CDase is produced and converts the exogenously added nontoxic 5-FC to toxic 5-FU. (c) When the P_{pobA} is activated by another aromatic compound, the mutant becomes Cm-resistant.

Based on the aforementioned design, we constructed the plasmid gYB2a-pobRWT-mCherry-codA-cmr, the $PobR^{WT}$ biosensor, to test the experimental conditions. $PobR^{WT}$ exhibited a high degree of ligand specificity with a narrow dynamic range (0.03-0.5 g/L, Supplementary Figure 1). Thus, we used 0.5 g/L 4HB to enable the expression of downstream reporter genes. In the presence of 4HB, the *E. coli* BW Δ *codA* harboring the $PobR^{WT}$ biosensor was resistant to chloramphenicol (Figure 2a), but sensitive to 5-FC (Figure 2b). Meanwhile, mCherry expression in the

bacteria could be detected by a high fluorescence reading. With the addition of chloramphenicol, the growth of the bacteria in the presence of 4HB was significantly better than that without it. In the experiments simulating the negative selection, the growth of the bacteria was repressed in medium containing 5 mg/L 5-FC and 0.5 g/L 4HB. Moreover, the bacterial strains were generally able to grow normally in the presence of either 4HB or 5-FC alone. With these experimental data, we verified the effectiveness of the dual selection system.

In this study, seven valuable aromatic compounds with structural similarity and similar functional groups to 4HB, including phenylethanol (2-PE), mandelate (MA), 4-hydroxymandelate (HMA), phenylpyruvate (PPA), 4-hydroxyphenylpyruvate (HPP), phenylacetaldehyde (PAld) and p-Coumaric acid, were selected to assess their candidacy as allosteric ligands of PobR mutants. The following experiments were conducted to evaluate whether any PobR mutant could allosterically respond to these compounds.

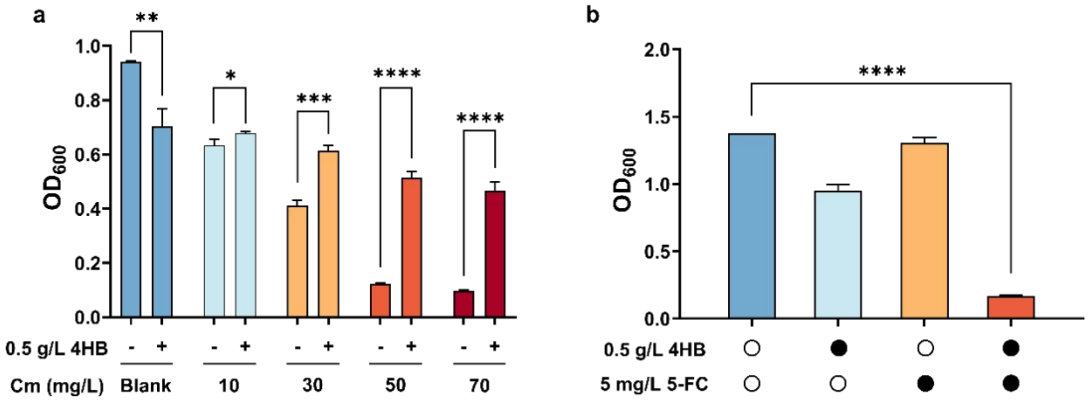

Figure 2. Model experiments of negative and positive selections. (a) Growth of bacteria harboring the PobR^{WT} biosensor in medium containing different concentrations of chloramphenicol and supplied with 0.5 g/L 4HB. (b) The growth of bacteria in medium containing either 0.5 g/L 4HB or 5 mg/L 5-FC alone, or both. The OD_{600} was measured after 12h of cultivation. Each value represents the mean ± standard deviation from 3 biological replicates.

Directed evolution of PobR.

We constructed a random mutagenesis PobR library by the error-prone PCR approach as described in the Methods section. The PobR mutants were subcloned into the vector harboring the $P_{pobA,}$ *mCherry*, *codA* and *cmr,* through the Golden Gate Assembly system, and then transformed into the BW Δ *codA* strain. The storage capacity of the PobR mutagenesis library was determined as approximate 21,000 clones with an average mutation rate of about 0.36%.

In the negative selection, the obtained bacteria were cultivated in liquid culture

supplemented by 4HB and 5-FC. In the initial negative selection, we used a constant 4HB concentration of a relatively high level, 0.5 g/L, and then tested different concentrations of 5-FC to inhibit both the pseudo-positive and 4HB-responsive strains. In this selection step, we first used 50 mg/L 5-FC, and observed insufficient inhibition of the bacterial growth. Thus, we increased the 5-FC concentration to 200 mg/L, improving the selection effectiveness. In the positive selection, the bacterial cultivation was conducted in the negative selection liquid medium supplied with different aromatic compounds, as well as chloramphenicol. The selection capacity for each compound was more than 900,000 clones (with at least four plates about 225,000 clones per plate.) (Supplementary Figure 2) We controlled the selection pressure intensity by adjusting chloramphenicol levels, transferred PobR mutants post negative selection into LB media containing ampicillin and chloramphenicol, and tested each ligand at a concentration of 0.5 g/L for initial positive selection and single colony isolation. Then, we picked colonies for liquid cultivation and obtained ligand-responsive PobR mutants for further characterization of the responsiveness to any aromatic compound through evaluating the induction of downstream genes. (Figure 3)

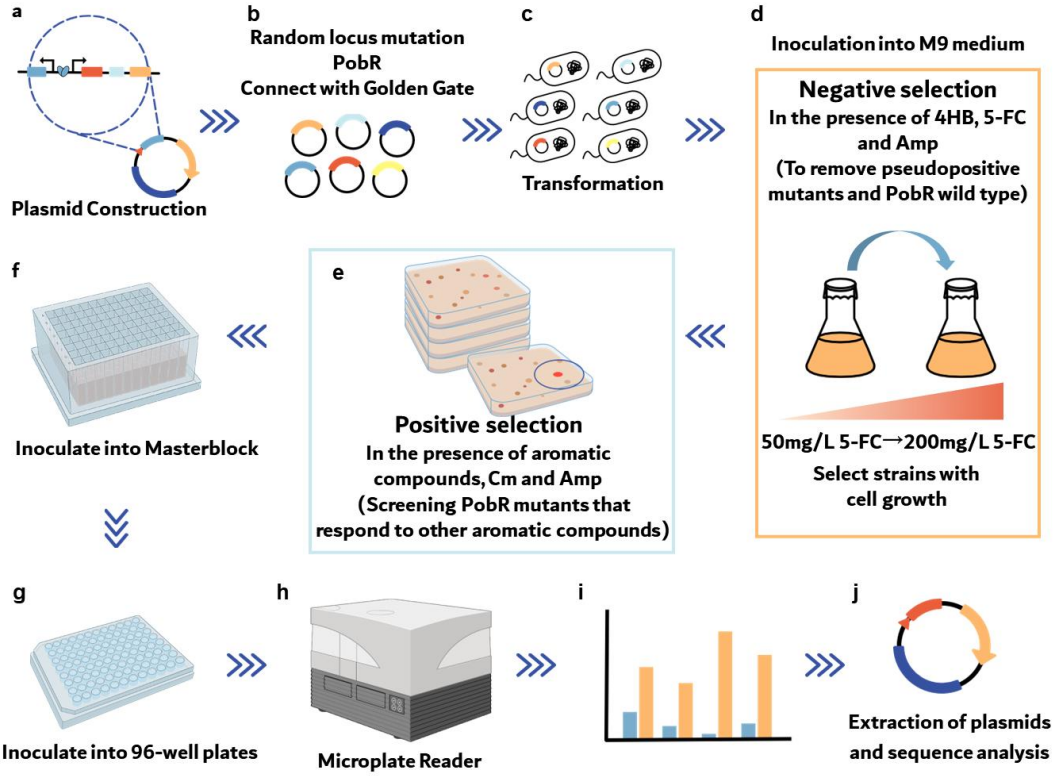

Figure 3. Schematic diagram of the selection procedures for ligand-responsive PobR mutants through eliminating false positive clones, functional characterization and sequence identification. (a), (b) and (c) Construction of a random PobR mutagenesis library generated by the Golden Gate Assembly system and transformed in the *E. coli* BWΔ*codA*. (d) Negative selection of bacteria in the liquid medium supplemented with 4HB and 5-FC. (e) Positive selection of the bacteria was obtained by the step b in

liquid medium containing different aromatic compounds, including 2-PE, MA, HMA, PAld, HPP, and PPA, as well as Cm. (f) and (g) Inoculation of selected bacteria into Masterblock followed by transfer into 96 well plates. (h), (i) and (j) Determination of fluorescence intensity and altered sequences of the PobR mutants.

The specificity and detection range of PobR mutants responsive to new ligands.

As these mutants underwent two rounds of negative selections, when tested with increasing 4HB concentration, the obtained PobR mutants exhibited significantly reduced basal expression and robustly attenuated 4HB responsiveness compared to that of $PobR^{WT}$ (Supplementary Figure 3). Meanwhile, a negative control in the absence of any candidate ligand was used, and almost no pseudo-positive mutant strains were discovered. Furthermore, since the expression of mCherry reporter positively correlated with the responsiveness of a tested PobR mutant, only pink colonies needed to be picked and subsequently evaluated by 0.5 g/L of a candidate ligand for initial characterization. In positive selection experiments of PobR mutants using different ligands, we obtained several responsive strains, of which the fluorescence intensity was 1.5-fold higher than that of the negative control (Supplementary Figure 4). To further evaluate the PobR mutants obtained above, the second round of characterization experiments were carried out to individually examine their responsiveness to each candidate ligand. For each ligand, we selected a mutant strain with the highest responsive profiles and plotted the curve for their ligand response. In total, 9 potential biosensors were isolated after the second round of characterization, and all these variants were sequenced to determine the mutations in their primary sequences. Amino acids at positions 163, 177 and 234 are located near the ligand binding pocket of PobR, and amino acid at position 40 is located in the DNA binding domain. The remaining mutant sites (S114, Y145, L183, L198, L201, L202, V225, Q250, and V255) are undetermined, and it is speculated that amino acid changes at these sites may either indirectly alter the conformation of the ligand-binding domain or affect PobR dimerization. We were surprised to find that one-third of the twelve variants all owning the same point mutation of S114A which indicated that the conversion from S to A in the position 144 affected the PobR ligand specificity to a great extent. These results demonstrating the effectiveness of our dual selection system (Figure 5, and Supplementary Figure 5).

Among these PobR mutants, the best responsive sensor was the PobR mutant clone 0914-A8-1 with a point mutation of W177R, which showed 5.1-fold increase of mCherry fluorescence in response to HPP. The $PobR^{W177R}$ displayed a significant change of specificity and a broader dynamic range of 0.01-1.0 g/L, indicating a reduction of ligand binding affinity. We assessed the specificity of $PobR^{W177R}$ in detecting different aromatic compounds, and observed that 4HB could still trigger its activity in driving *mCherry* expression (Supplementary Figure 6). Nevertheless, compared with $PobR^{WT}$, the sensitivity of $PobR^{W177R}$ to 4HB significantly reduced. Additionally, at 0.5 g/L of PPA, another clone, $PobR^{R40C}$ exhibited over 2 times of

maximal induction to PPA compared to the condition without the ligand. Importantly, this PobR mutant showed marginal basal transcriptional activity.

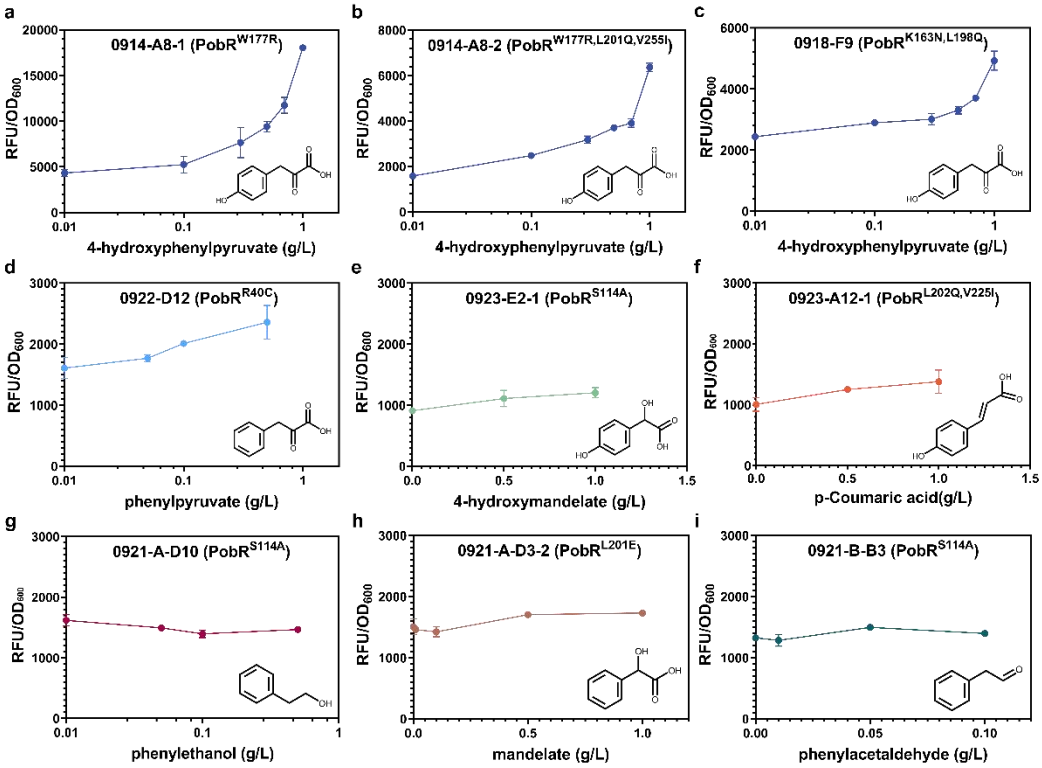

Figure 4. Characterization of the PobR mutants' responsiveness to different aromatic compounds. (a) to (i). The fluorescent gene expression normalized to cell density (RFU/OD_{600}) was plotted. Each value represents the mean ± standard deviation from 3 biological replicates.

Chemical compound	Number	Fold	DNA binding domain	Ligand binding pocket			Unidentified alterations								
			R40	K163	W177	T234	S114	Y145	L183	L198	L201	L202	V225	Q250	V255
4-hydroxyphenylpyruvate	0914-A8-1	5.12			R										
	0914-A8-2	4.21			R						Q				I
	0918-F9	2.38		N						Q					
mandelate	0921-D3-2	1.15									E				
phenylacetaldehyde	0921-B-B3	1.05					A								
	0921-A-A8	1.08						F							
phenylpyruvate	0922-D12	2.01	C												
	0922-F9-1	1.22					A								
p-coumaric acid	0923-B7-1	1.26				A			Q					L	
	0923-A12-1	1.37										Q	I		
phenylethanol	0921-A-D10	0.92					A								
4-hydroxymandelate	0923-E2-1	1.32					A								

Figure 5. Amino acid substitutions in the transcriptional factor PobR mutants observed in the isolated biosensor candidates.

Structure modeling and ligand docking.

To evaluate whether structural changes could contribute to altered allosteric

response, we employed the Alphafold2 and SWISS-MODEL[18] to predict the structures, and generate homology modeling of PobR^{WT} and its mutant PobR^{W177R}. Autodock was used to dock 4HB and HPP molecules to the proteins, and the ligand-protein interactions were analyzed using PyMOL and Ligplus. The PobR^{W177R} protein showed a 10.4-fold higher fluorescence intensity than PobR^{WT}. During the docking of HPP and PobR^{W177R}, HPP could form hydrogen bonds with I235 and R177, and hydrophobic interactions with L169, L165, L122 and V121. In the surface model, R177 was spatially close to the HPP binding pocket of PobR^{W177R}, while W177 likely closer α helix than that in PobR^{WT} (Figure 6a,b). In this mutant, R177 formed a hydrogen bond with HPP, while W177 in PobR^{WT} showed no interaction with HPP (Figure 6c,d). Overall, the W177R mutation allowed PobR to gain the affinity to HPP, and subsequently activated the expression of the PpobA.

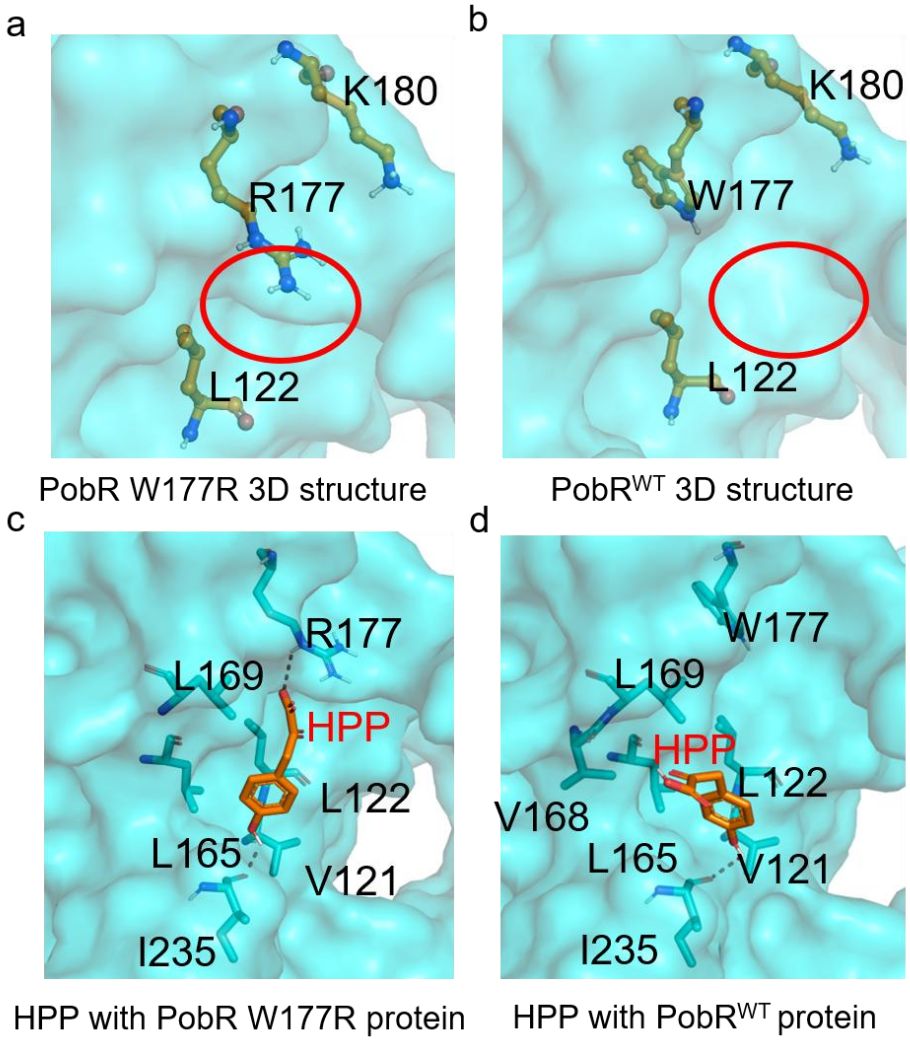

Figure 6. Structural analyses of PobR^{W177R} and PobR^{WT} using the Autodock and PyMOL. Docking analyses generate binding simulation between HPP and the residues in the PobR proteins. (a) The surface model showed that R177 in PobR^{W177R} is spatially close to the HPP interaction to the PobR ligand binding domain. Red cycles showed different protein conformations that PobR^{W177R} and PobR^{WT} in binding pocket. (b) W177 in PobR^{WT} tends to be closer to α helix than that in the PobR^{W177R}. (c)

In the PobR^{W177R}, R177 forms a hydrogen bond with HPP. (d) In PobR^{WT}, W177 does not interact with HPP.

Discussion

In the past decades, various methods have been developed for the directed evolution of aTFs to alter their compound-binding specificity. PobR is an aTF that responds specifically to 4HB. In our previous study, we developed two biosensors responsive to HMA through screening for a library of random PobR mutants. However, in addition to HMA responsiveness, they also responded to their native ligand, 4HB, and other aromatic compounds, indicating the high potential of PobR variants to become biosensors of various aromatic compounds. Based on these findings, we designed a dual selection system in this study to search for PobR mutants with highly specific responsiveness to different aromatic compounds.

In the negative selection of the system, the *codA*, a conditional lethal gene, can convert 5-FC to cytotoxic 5-FU in the presence of exogenous 5-FC and high 4HB levels, resulting in the elimination of pseudo-positive strains and those highly responsive to 4HB. By increasing 5-FC concentration, we successfully increased the effectiveness of the selection from 89% to 98%. Thus, after two rounds of negative selection, we obtained the mutants with over 98% lacking constitutive expression and irresponsive to 4HB.

We next used antibiotics as a positive selection marker. According to our design, some mutants could have changes in promoter binding and become constitutively transcriptional active, which made the bacteria survive on solid media containing both chloramphenicol and aromatic compound. Using our dual selection system, we have increased the efficiency of a single round of screening to 225,000 monoclonal clones/plate, greatly improving the selection speed. Compared to other high-throughput selection or screening methods, such as flow cytometry, our method is equally efficient, but more economic and easier to operate. We finally obtained several mutants that responded to HPP and PPA with best response multiples of 5.12 and 2.01, respectively, which is the proof of concept of our system. Notably, the PobR^{W177R} mutant retained 5.5-fold response to 4HB, and its affinity for 4HB was significantly lower than that of PobR^{WT} (28-fold). In addition, we also tested multiple additional aromatic compounds, including HMA, MA, p-coumarins, etc., in the positive selection, and observed weak response to the compounds by some mutants. All these results demonstrated the effectiveness of our system.

Sequencing of the PobR^{W177R} mutant showed a single amino acid substitution of W177 by arginine near its binding pocket. In our previous studies, mutants responsive to HPP and HMA exhibited low ligand specificity, which could be due to the attenuated allostery and/or distorted ligand binding pocket caused by the multiple-site mutations.

Clearly, PobR^{W177R} has better ligand specificity. This suggests that ligand specificity alterations from a single mutation are likely more stable.

In summary, we have designed a dual selection system that can successfully select for PobR mutants responsive to HPP and PPA. The system has great potential for the development of various aromatic biosensors. These biosensors with low detection thresholds and wide dynamic ranges may be of high value for quantitative measurements of valuable aromatic compounds.

Methods

Bacterial strains, media, chemicals and other materials

The bacterial strains and plasmids used in this study are listed in **Supplementary Table 1**. *E. coli* DB3.1 was used for the construction of the original plasmid gYB2a-pobR-mCherry-codA-cmr. In the negative selection using the *codA* gene, the BWΔ*codA* was used for the construction and screening of a random mutation library. *E. coli* bacteria were cultured in the Luria-Bertani (LB) medium for propagation. LB liquid medium was prepared by dissolving 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 litter of deionized water, while LB solid medium contained 15 g/liter of agar. In the construction of the libraries and screening, *E. coli* bacteria were grown in the M9 medium (17 g Na_2HPO_4 x7H_2O, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 4 g/liter glucose). The bacteria were cultured in a shaking incubator at 37°C and 200 rpm. In particular, when cultured in 96-well plates, the culture conditions were 37°C, 850 rpm. Ampicillin was the conventional antibiotic of choice at a concentration of 100 mg/L. Primers for plasmid construction and PobR random mutagenesis listed in **Supplementary Table 2** were synthesized by Ruibiotech (Harbin, China). All chemicals, including 4HB and other aromatic compounds, were purchased from Aladdin.

Construction of the gYB2a-pobR-mCherry-codA-cmr plasmid

The gYB2a-pobR-mCherry-codA-cmr contains the PobR coding sequence, an engineered operon consisting of two repetitive P_{pobA} promoters, and three coding sequence, the *mCherry* CDS, the cytosine deaminase (*codA),* and the chloramphenicol resistant gene *cmr*. PobR coding sequence and P_{pobA} were derived from a previous study, where the PobR coding sequence had the codons optimized for *E. coli* preferences. In the original construct, a DNA fragment containing P_{pobA} promoters, mCherry and Sucrose-fructanase encoding gene (*sacB)* was amplified using the plasmid pYP1a-pobR-P_{pobA}*2-mCherry-sacB from the laboratory stock as a template, and primers P_{pobA}*2-mc-0311-F and Primer2-0311-R. This fragment has homologous arms to gYB2a at both ends. The linearized vector was obtained by the digestion of gYB2a-ccdB using *Eco*RI and Kpn I. The fragment and vector were mixed with the ClonExpress^{TM} II recombinant system (Vazyme), followed by transformation into *E. coli* DB3.1 competent cells to obtain the gYB2a-P_{pobA}*2-mCherry-sacB. The

generated gYB2a-P_{pobA}*2-mCherry-sacB was next digested using *Eco*RI. Amplification of the *cmr* fragment was performed using the pYB1a-eGFP-cmr as a template with the primers Cmr-Gibson-0317-F/R. Both fragments were also assembled using the Gibson Assembly System, and resulted in the construction of the gYB2a-P_{pobA}*2-mCherry-sacB-cmr. In the subsequent experiments, as *sacB* performed poorly as a negative selection marker, it was replaced with the cytosine deaminase (*codA*) gene. The replacement method was performed as follows: the pUAM-RE-CD was used as the template and the CD-Gibson-0425 F/R were used as primers to amplify *codA*. Using the gYb2a-P_{pobA}*2-mcherry-SacB-cmr as the template, the upstream primer cmr-Gibson-F with a homologous arm downstream of the *codA* fragment and the downstream primer Mc-Gibson-F with a homologous arm upstream of the *codA* fragment were used together to reversely amplify the vector fragment followed by Gibson assembly. The Gibson Assembly approach employed in this study was performed using the ClonExpress™ II recombinant system (Vazyme). In the final step, the gYb2a-P_{pobA}*2-mCherry-SacB-cmr was used as the vector for the wild type *pobR* as the target fragment to be ligated into a complete plasmid by the Golden Gate Assembly.

Design and construction of the PobR mutant library

To generate PobR mutants, we developed a library through random mutagenesis of PobR using error-prone PCR amplifications. Primers PobR-P1-BsaI-F and PobR-P2-BsaI-R covering the PobR CDS were used with pLB1s-PobR as a template. The purified PCR products containing various PobR mutants were used as the donor in the following Golden Gate Assembly, while the gYB2a-mCherry-codA-cmr was used as the receptors. The donor, receptors and the restriction endonuclease BsaI used to generate the sticky ends were mixed together BSA, T4 DNA ligase and the corresponding buffer were added followed by the golden gate assembly. The generated library with highly random PobR mutations was transformed into *E. coli* BW∆*codA* to obtain transformants containing mutant plasmids. The PobR mutant library was transformed into BW∆*codA* competent cells and transferred to M9 medium for culture in shaking flasks. A fraction of the grown cultures was spread on LB agar plates for colony counting. The experimental library size is 2.5×10^4. Ten clones were randomly picked to sequence their PobR CDS regions for the quality control, which revealed diverse mutations with an average mutation rate of about 0.36%.

Counter-selection via CDase

The library generated above was inoculated into M9 medium containing 0.5 g/L 4HB and 50 mg/L 5-FC, and cultured for 12 h to reach the optical density at 600 nm (OD_{600}). Then, the overnight cultured bacteria were inoculated (1% v/v) into M9 medium for a second round of counter selection. In this round of selection, the M9 medium was supplemented with 0.5 g/L 4HB and 200 mg/L 5-FC, and cultured for 24 h to OD_{600} ~0.5. A fraction of the cultured medium was transferred onto LB agar plates to isolate monoclonal colonies. Forty-five colonies were picked from the plate and inoculated into LB medium containing ampicillin. After 8-10 hours of culturing, 2 μL of

bacteria from each well were transferred into 200 μL of M9 medium containing ampicillin and 0.5 g/L 4HB. Strains cultured in M9 medium without 4HB was used as a negative control. Strains containing wild-type PobR CDS were used as positive controls under the same culture conditions. After 12 h of incubation in 96-well plates, OD_{600} and red fluorescence were measured (with 552 nm as the excitation wavelength and 600 nm as the emission wavelength).

Positive-screening via Cm

Cultures after the first step counter selection were transferred into fresh LB agar plates, each of which contained 0.5 g/L of various aromatic compounds (HPP, PPA, etc.), with different concentrations (60, 90 and 120 mg/L) of chloramphenicol. As a result, approximately 30 single clones could be screened per solid medium. The colonies were picked from the plates and inoculated into 600 μL of LB medium with ampicillin in deep-well microplates, followed by culturing in a shaking incubator at 850 rpm and 37°C for 8-10 h. Then, 2 μL of cultured bacteria from each well was taken and inoculated into 96 microplates. Each well had 200 μL of M9 medium containing ampicillin and 0.5 g/L of the corresponding aromatic compounds. As a negative control, the same bacteria were added to the M9 medium without any aromatic compound, while wild-type PobR grown in culture in the presence 4HB was used as a positive control. Finally, 200 μL of the cultured bacteria from each well was collected to measure the OD_{600} and red fluorescence.

Modeling and docking

The Homologous Model website SWISS-MODEL was employed to construct the PobR mutant model using the PobR wild-type which was simulated by Alphafold2 as a template.

The structure files of the ligands 4HB, 2-PE, MA, HMA, PAld, HPP, and PPA were obtained from the organic small molecule database Pubchem. (https://pubchem.ncbi.nlm.nih.gov/).

Autodock is used to simulate molecular docking. Autogrid search space coordinates were set as center_x = -4.672, center_y = 3.331 and center_z = -2.213. Dimensions of the search space were set as size_x = 40, size_y = 40 and size_z = 40, and exhaustiveness was set at 15. The 15 conformational conditions in a score based on the lowest binding energy were listed as the docking results. The interaction between a small molecule and predicted protein receptors was examined by Ligplus to evaluate the accuracy of the docking. The three-dimensional schematics of the protein and its ligand were portrayed using PyMol Version 2.2.0.

Statistical analysis

All statistical analyses were performed using the Prism 8.3.0 software (GraphPad, La Jolla, CA). All data were derived from at least three independent experiments. Results were presented as a mean with either standard deviation (SD) or standard error of mean (SEM), and sample numbers are indicated unless otherwise noted in the figure legends. Statistical significance calculations comparing two conditions were

performed using a two-tailed unpaired Student's t-test. The criterion of statistical significance level was denoted as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

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