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Multilevel engineering of the upstream module of aromatic amino acid biosynthesis in *Saccharomyces cerevisiae* for high production of polymer and drug precursors

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ABSTRACT

A multilevel approach was implemented in *Saccharomyces cerevisiae* to optimize the precursor module of the aromatic amino acid biosynthesis pathway, which is a rich resource for synthesizing a great variety of chemicals ranging from polymer precursor, to nutraceuticals and pain-relief drugs. To facilitate the discovery of novel targets to enhance the pathway flux, we incorporated the computational tool YEASTRACT for predicting novel transcriptional repressors and OptForce strain-design for identifying non-intuitive pathway interventions. The multilevel approach consisted of (i) relieving the pathway from strong transcriptional repression, (ii) removing competing pathways to ensure high carbon capture, and (iii) rewiring precursor pathways to increase the carbon funneling to the desired target. The combination of these interventions led to the establishment of a *S. cerevisiae* strain with shikimic acid (SA) titer reaching as high as 2.5 g L^{-1}, 7-fold higher than the base strain. Further expansion of the platform led to the titer of 2.7 g L^{-1} of muconic acid (MA) and its intermediate protocatechuic acid (PCA) together. Both the SA and MA production platforms demonstrated increases in titer and yield nearly 300% from the previously reported, highest-producing *S. cerevisiae* strains. Further examination elucidated the diverged impacts of disrupting the oxidative branch (*ZWF1*) of the pentose phosphate pathway on the titers of desired products belonging to different portions of the pathway. The investigation of other non-intuitive interventions like the deletion of the Pho13 enzyme also revealed the important role of the transaldolase in determining the fate of the carbon flux in the pathways of study. This integrative approach identified novel determinants at both transcriptional and metabolic levels that constrain the flux entering the aromatic amino acid pathway. In the future, this platform can be readily used for engineering the downstream modules toward the production of important plant-sourced aromatic secondary metabolites..

1. Introduction

Engineering microbial factories for the production of valuable chemicals often requires the optimization of long metabolic pathways. Splitting the pathways into modules can allow for faster optimization, leading to higher overall yields and titers. Grouping genetic elements permits a more precise analysis of bottlenecks, rate-limiting steps, and metabolic imbalances (Biggs et al., 2014). Moreover, studying metabolic pathways as independent modules facilitates the analysis of transcriptional regulators that may act on the genes within distinct modules, hence enabling a multilevel approach encompassing engineering at both transcriptional and metabolic levels.

Modular approaches for engineering microbes have been implemented in a variety of cases. In *Escherichia coli*, for example, implementing a modular design strategy led to significant increases in the production of total fatty acids (Xu et al., 2013). In *Saccharomyces cerevisiae*, a recent example was observed for the *de novo* production of benzylisoquinoline alkaloids (BIAs), which required the coordinated overexpression of more than 20 endogenous and heterologous genes (Galanie et al., 2015). Partitioning the pathway into several modules

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allowed efficient optimization of precursor and cofactor availability, and reduction of pathway bottlenecks. In fact, the aromatic amino acid pathway from which BIAs are originated is a source of a great variety of chemicals that range from polymer precursors to nutraceuticals and pain-relief drugs (Suástegui and Shao, 2016). Previous metabolic engineering efforts in *S. cerevisiae* have enabled the production of chemicals derived from this pathway including, but not limited to, shikimic acid (SA) (Suástegui et al., 2016a), muconic acid (MA) (Curran et al., 2012; Suástegui et al., 2016b), vanillin (Brochado et al., 2010), L-tyrosine (L-tyr) (Gold et al., 2015), coumaric acid (Rodriguez et al., 2015), and secondary metabolites from the flavonoid and stilbenoid families, as well as BIAs (Galanie et al., 2015; Jiang et al., 2005; Koopman et al., 2012; Li et al., 2015; Trantas et al., 2009; Yan et al., 2005).

One major metabolic engineering strategy employed to unlock the production of derivatives spun off the aromatic amino acid biosynthetic pathway is the overexpression of the feedback insensitive mutated enzymes 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, $aro4_{K229L}$, and chorismate mutase, $aro7_{G141S}$, catalyzing the first committed step in the pathway, and the branching point towards the production of L-tyr and phenylalanine (L-phe), respectively (Brown and Dawes, 1990; Luttik et al., 2008). As shown in Fig. 1, other strategies include rewiring the pentose phosphate pathway (PPP) for increasing the pool of erythrose-4-phosphate (E4P), and the overexpression of mutant versions of the pentafunctional protein Aro1 to halt the activity at specific subunits for accumulating the desired intermediates (Suástegui and Shao, 2016). Despite the implementation of these strategies, the yields of the compounds based on carbon sources in *S. cerevisiae* still remain low, suggesting that higher carbon fluxes into the pathway are constrained by other unknown factors. Therefore, a more comprehensive investigation needs to be prosecuted to determine novel transcriptional and metabolic targets, which will benefit the design of microbial cell factories for producing many high-value compounds from such a resourceful pathway.

Herein, we have focused the attention on engineering the precursor module of the aromatic amino acid pathway using SA as a reporter product. The target metabolic module is composed of the genes in the glycolytic pathway (up to the production of phosphoenolpyruvate, PEP), the PPP (leading to the production of erythrose-4-phosphate, E4P), and the genes in the SA pathway including *ARO2*, *ARO3*, *ARO4*, and the pentafunctional *ARO1* (Fig. 1). Besides implementing a comprehensive engineering strategy at the metabolic level (*i.e.* deletion or overexpression of structural genes), we identified that the protein Ric1, involved in regulating the efficient localization of *trans*-Golgi network proteins (Bensen et al., 2001), can act as a transcriptional repressor of multiple genes in the aromatic amino acid pathway. Furthermore, the incorporation of *in silico* modeling and pathway analysis led to the discovery of a novel genetic target. Overexpression of ribose-5-phosphate ketol-isomerase, *RKI1*, in addition to other tested interventions, facilitated PPP flux redirection into the aromatic amino acid pathway through E4P. The combination of these novel, multilevel interventions led to high production of SA, MA, and its intermediate. Such a multilevel intervention strategy thoroughly removes the constraints in the upstream precursor module of the aromatic amino acid pathway, maximizes the carbon flux flowing to the downstream, and therefore paves the way for synthesizing high-value molecules from the downstream branches.

2. Results

2.1. Identification of a novel transcriptional regulator

SA was selected as the reporter molecule to track the entrance of carbon into the precursor module. Initially, to study the transcriptional regulation upon this module, we focused the attention to the *ARO1* promoter as it controls the expression of the pentafunctional gene *ARO1*, the main core of the module. Table 1 shows the plasmid design based on the previously reported platform in *S. cerevisiae* for production of SA (Suástegui et al., 2016a). The promoter controlling the expression of the mutant gene $aro1_{D920A}$ was switched from the strong constitutive *GPD1* promoter (*GPD1* encodes glycerol-3-phosphate dehydrogenase, strain SA2) to the native *ARO1* promoter, yielding plasmid pRS413-lowAA (strain SA1, Table 2 and Fig. 2a). The titer after 3 days of fermentation with strain SA1 reached $176.27 \pm 0.50\ mg\ L^{-1}$ of SA (Fig. 2b), a reduction of almost 46% compared to strain SA2. This indicated that, indeed, the two promoters had different strengths, presumably due to distinct responses to transcriptional regulatory elements. Hence we proceeded to investigate which specific transcription factors (TFs) could be involved in the regulation of the upstream precursor module of the aromatic amino acid biosynthesis, including *ARO1*, *ARO2*, *ARO3*, and *ARO4* (Fig. 1), as a direct approach to discerning the cause of the repressive regulation of carbon flux into the pathway.

A list of TFs with reported evidence of acting as repressors was retrieved from the Yeast Search for Transcriptional Regulators and Consensus Tracking (YEASTRACT) website (Teixeira et al., 2014). YEASTRACT is a repository of over 200,000 regulatory associations between transcription factors and target genes in *S. cerevisiae*, developed based on more than 1300 references. The list contained 66 unique TFs from which 21 were selected for further analysis according to the criteria described in the Materials and methods section (Supplementary Table 3).

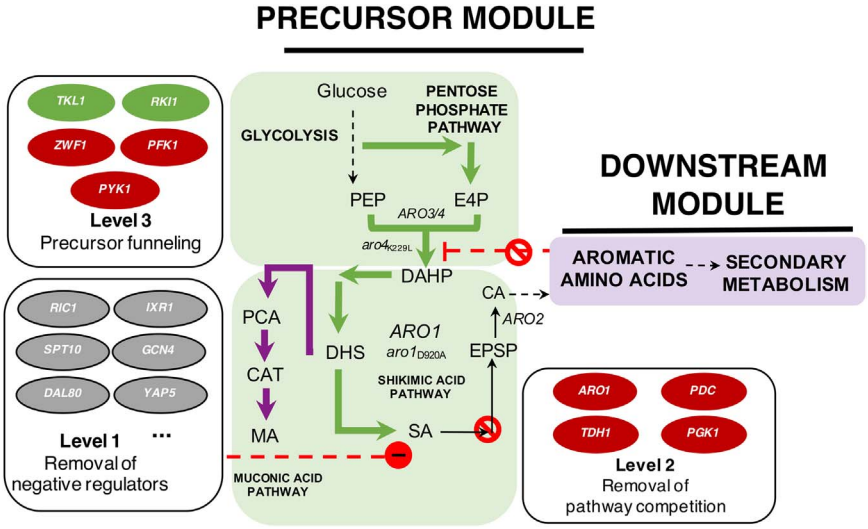

Fig. 1. Schematic representation of the metabolic engineering rationale for enhancing the carbon flux into the aromatic amino acid biosynthetic pathway. To establish the boundaries, the pathway was visualized in two main modules, namely the precursor module, and the downstream module. The multilevel engineering strategy consisted of removing pathway repressors (level 1), removing pathway competition (level 2), and increasing precursor funneling (level 3). Color codes: green, gene overexpression; red, gene knockout; purple, heterologous pathway. Metabolite abbreviations: PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHS, 3-dehydroshikimic acid; SA, shikimic acid; EPSP, 5-enolpyruvyl-3-shikimate phosphate; CA, chorismic acid. Gene abbreviations: *TKL1*, transketolase; *RKI1*, ribose-5-phosphate ketol-isomerase; *ZWF1*, glucose-6-phosphate dehydrogenase; *PFK1*, phosphofructokinase; *ARO1*, pentafunctional aromatic enzyme; $aro1_{D920A}$, mutant version of *ARO1* with disrupted shikimate kinase activity; *ARO2*, chorismate synthase; *ARO3/4*, DAHP synthase; $aro4_{K229L}$, feedback-insensitive DAHP synthase; *PDC*, pyruvate decarboxylase; *PGK1*, 3-phophoglycerate kinase; *TDHI1*, glyceraldehyde-3-phosphate dehydrogenase. See Supplementary Table 3 for the full list of negative regulators studied.