Synthetic Core Promoters for *Pichia pastoris*

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Supporting Information

**ABSTRACT:** Synthetic promoters are commonly used tools for circuit design or high level protein production. Promoter engineering efforts in yeasts, such as *S. cerevisiae* and *Pichia pastoris* have mostly been focused on altering upstream regulatory sequences such as transcription factor binding sites. In higher eukaryotes synthetic core promoters, directly needed for transcription initiation by RNA Polymerase II, have been successfully designed. Here we report the first synthetic yeast core promoter for *P. pastoris*, based on natural yeast core promoters. Furthermore we used this synthetic core promoter sequence to engineer the core promoter of the natural *AOX1* promoter, thereby creating a set of core promoters providing a range of different expression levels. As opposed to engineering strategies of the significantly longer entire promoter, such short core promoters can directly be added on a PCR primer facilitating library generation and are sufficient to obtain variable expression yields.

**KEYWORDS:** synthetic promoters, core promoter construction, promoter library, transcriptional fine-tuning, *Pichia pastoris*

Engineered promoters are commonly applied DNA parts for circuit design and used to increase titers of heterologous proteins and metabolites. In eukaryotes, where promoters are longer and more complex than in prokaryotes, the core or minimal promoter is the crucial region providing binding sites for general transcription factors and RNA polymerase II. In addition, transcript stability and efficient translation initiation depend on the 5′ untranslated region (UTR). In higher eukaryotes the design of synthetic core promoters has led to strongly improved synthetic variants. In lower eukaryotes such as yeasts and fungi, promoter engineering has mostly concentrated on upstream regulatory sequences (URS), their combination with natural core promoters and/or random mutagenesis approaches of selected core promoter regions. In the model yeast *Saccharomyces cerevisiae*, large scale studies have been used to analyze the sequence/function relationship of natural core promoters.

*Pichia pastoris* is a commonly used expression system for the production of biocatalysts and biopharmaceuticals, as it allows secretion of pure heterologous proteins to the culture broth and grows to high cell densities. The most commonly used, exceptionally strong, and tightly regulated methanol inducible promoter of the *alcohol oxidase 1* gene (*pAOXI*) has been studied in terms of regulatory sequences and factors. It was also used for the design of synthetic promoter variants providing a range of expression levels and altered regulatory profiles. These engineering efforts have focused on URS, random mutagenesis of core promoter sections or the 5′ UTR. Here we aimed for the first time to design a fully synthetic core promoter and 5′ UTR for *P. pastoris* and to employ such artificial sequences for *pAOXI* core promoter engineering to obtain a library of sequence-diversified promoters with different properties.

**CONSTRUCTION OF SYNTHETIC CORE PROMOTER PCORE11**

While promoters in bacteria can be rationally engineered considering conserved regions and spacing (e.g., conserved −35, −10 regions), yeast core promoters have only been engineered by random mutagenesis methods. We designed a synthetic yeast core promoter by using a consensus sequence of natural core promoters that was refined by incorporating common transcription factor binding site (TFBS) motifs (Figure 1A). The core promoter sequences of four natural, differently regulated promoters (*pAOXI*, *pGAP*, *pHIS4*, and *pScADH2*) were aligned using MultAlin to identify a general minimal consensus (Figure 1B). Detailed information on the promoter choices and exact sequence selection is provided in Supplementary Figures S 1, Supporting Information. This first core promoter sequence (PCore1) was successively re-engineered on the basis of an *in silico* analysis for putative TFBSs (Figure 1C, Supplementary Figures S 1, and Supporting File 1, Supporting Information). We aimed to identify common sequence motifs of the natural promoters and to integrate them into pCore1. Therefore the natural promoters used for the consensus design were analyzed for putative TFBSs using Matisnspc. TFBSs predicted in several natural promoters were incorporated into the pCore1 sequence, while superfluous
sites of pCore1 were removed, resulting in pCore11 (Figure 1C,D). Detailed information on the putative TFBSs found and the engineering thereof are provided in Supporting File 1 and Supplementary Figures S 1, Supporting Information. In P. pastoris, these predicted TFBSs are not necessarily bound by homologues of the factors identified by the MatInspector analysis, as these factors bind usually further upstream to URSs (except TATA binding protein, a crucial core promoter factor). As the MatInspector matrix-based model of TFBSs tolerates also variations in binding motifs, the motifs found may also

Figure 1. Design steps of the synthetic core promoter pCore11. (A) Schematic diagram of the design strategy. Four natural yeast core promoters were aligned resulting in a consensus sequence/pCore1. TFBSs predicted in at least two out of the four core promoters were incorporated into pCore1 resulting in pCore11 (Supplementary Figures S 1 and Supporting File 1, Supporting Information). Sequence elements and positions are not drawn to scale. (B) Sequence alignment of the core promoters used for the generation of a consensus sequence. Detailed information on the sequence selection is provided in Supplementary Figures S 1, Supporting Information. (C) The consensus sequence of the alignment was the basis for pCore1 generation. The four natural core promoters and pCore1 were analyzed for putative TFBSs using MatInspector (Supporting File 1, Supporting Information); common TFBSs were incorporated as described in Supplementary Figures S 1, Supporting Information, resulting in pCore11. The synthetic promoters fused to the EcoRI site (underlined), Kozak sequence (in italics) and the start codon (lower case) are shown (see also Supplementary Figures S 2, Supporting Information). (D) TFBS prediction of the engineered pCore11 (for detailed information and analysis of the other core promoters, see Supplementary Figures S 1 and Supporting Figure 1, Supporting Information).
constitute degenerate determinants for the binding of general transcription factors or RNA polymerase II.

As core promoters alone provide either no or only low basal transcription, we fused pCore1 and pCore11 to the upstream region of pAOX1. These fusions were subsequently assayed with a GFP reporter, established for yeast promoter studies. While pCore1 fluorescence only marginally surpassed background fluorescence, the re-engineered pCore11 showed tight repression on glucose and upon methanol induction reached about 10% of the wildtype pCoreAOX1 (Figure 2B). These results show that functional synthetic yeast core promoters can in principle be obtained by complementation of a core sequence elements with additional nucleotides. This approach is similar to prokaryotic promoter engineering, although conserved, tightly localized sequence motifs such as the −10 and −35 region of prokaryotic promoters are not obvious (except the TATA box).

Identification of Variable Sequence Stretches of pCoreAOX1

Previous studies of the pAOX1 URS focused either on systematic deletions, putative TFBSs, or the identification of TFBSs by DNA/protein interaction studies (see Supplementary Note, Supporting Information). However, while URS can be positioned variably over the promoter sequence (e.g., TFBSs of the methanol master regulator Mxr1 in pAOX1 and pDAS2, reviewed in the literature), core promoter recognition by RNA Polymerase II requires tight interaction with the DNA over more than 60 bp, and deletions in the core promoter may interfere with the spacing of regulatory sequences. Therefore we replaced putative regulatory sequences in pCoreAOX1 with elements of the functional synthetic pCore11. As opposed to randomized sequences, such sequence transfer promised functional engineered AOX1 core promoters variants. Since pCore11 is shorter than pCoreAOX1 also insertion variants have been made. The exact sequence selections for the synthetic core promoters (Sync1 to Sync6ins) are illustrated in Figure 2A and described in detail in Supplementary Figures S2, Supporting Information. As the natural pCoreAOX1 sequence, all synthetic core promoters linked to the upstream region of pAOX1 were repressed on glucose (Figure 2B). GFP fluorescence upon methanol induction of the synthetic variants, including pCore11, ranged from 10 to 117% percent of the wildtype promoter. Several variants showed only moderate changes in expression levels, even though key regions close to the TSS were changed (Sync2, Sync3, Sync3ins). Sync4 and Sync4ins show the strongest decrease of expression (30−40% of the wildtype promoter activity), suggesting an important region downstream the TSS in the beginning of the 5′ UTR. In general the length correction by insertions did not cause significant differences.

Our results show that the construction of diversified synthetic core promoters and 5′ UTRs is also possible in yeast. A first generation synthetic core promoter sequence

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**Figure 2.** Engineered pAOX1 core promoter variants exhibit a range of expression levels. (A) Design schematic of the variants. Regions from pCore11 were incorporated into pCoreAOX1. Insertions for length corrections are indicated in yellow. Detailed information on the selection process and sequences is provided in Supplementary Figures S2, Supporting Information. Numbering is relative to the translational start (+1). (B) Expression levels of the variants. The variants Sync1 to Sync6ins and controls (wildtype pCoreAOX1, pCore1, pCore11) were cloned upstream of a GFP reporter (N.C.: negative control of untransformed wildtype strain). The strains were grown on glucose and induced with methanol, and fluorescence was measured as outlined in the methods section in the Supporting Information.
showing at least some functionality can be used to recruit suitable sequence stretches for the design and construction of a next generation library of fully functional core promoters with varying sequences and strength. By testing a moderate number of variants, we achieved a range of expression levels, and these variants can be applied to fine-tune gene expression. Compared to engineering by random mutagenesis (e.g., error prone PCR), much fewer constructs need to be tested. However, screening a higher number of randomly mutated sequences of pCoreAOX1 recently also led to considerably improved variants, while our more rational approach resulted only in moderately improved variants so far (Sync5 and Sync6). Both kinds of core promoters might be combined with URS variants of other studies to achieve synergistic effects. It remains to be shown whether the observed effects are a result of changed transcription or mRNA stability, or if the translation initiation was influenced. However, the replacement of parts of natural core promoter sequences also provides a tool for sequence diversification while maintaining natural expression levels and regulation. Engineering of URS may also interfere with regulation of the promoter. For our synthetic core promoter/pAOX1 URS fusions, the mode of regulation remained untouched. Therefore a similar strategy as in prokaryotes, where ribosome binding sites are modified to fine-tune strong natural promoters, is feasible by engineering eukaryotic core promoters and UTRs.

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