Engineered bidirectional promoters enable rapid multi-gene co-expression optimization

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Numerous synthetic biology endeavors require well-tuned co-expression of functional components for success. Classically, monodirectional promoters (MDPs) have been used for such applications, but MDPs are limited in terms of multi-gene co-expression capabilities. Consequently, there is a pressing need for new tools with improved flexibility in terms of genetic circuit design, metabolic pathway assembly, and optimization. Here, motivated by nature’s use of bidirectional promoters (BDPs) as a solution for efficient gene co-expression, we generate a library of 168 synthetic BDPs in the yeast Komagataella phaffii (syn. Pichia pastoris), leveraging naturally occurring BDPs as a parts repository. This library of synthetic BDPs allows for rapid screening of diverse expression profiles and ratios to optimize gene co-expression, including for metabolic pathways (taxadiene, β-carotene). The modular design strategies applied for creating the BDP library could be relevant in other eukaryotic hosts, enabling a myriad of metabolic engineering and synthetic biology applications.
Efﬁcient and well-tuned co-expression of multiple genes is a common challenge in metabolic engineering and synthetic biology, wherein protein components must be optimized in terms of cumulative expression, expression ratios, and regulation. When co-expressing multiple proteins, not only their ratios to each other but also their total (cumulative) amounts summed together matter. Too excessive loads of heterologous proteins may overburden the cellular machinery of recombinant expression hosts. Hence, in addition to balancing the proteins relative to each other, their total (cumulative) expression strength needs to be adjusted. Else, burdensome overexpression of proteins or accumulation of toxic intermediate metabolites may prove detrimental to the cellular host and undermine engineering goals. One remedy has been to restrict protein overexpression to only certain times through dynamic or regulated transcription (inducibility). A second is to balance pathway expression to prevent toxic metabolite accumulation, mimicking natural pathways’ balanced protein stoichiometries.

Though effective, these methods’ ability to improve pathway performance by controlling gene expression is constrained to the tools available. To date, and especially in the context of eukaryotic performance by controlling gene expression is constrained to the tools available. To date, and especially in the context of eukaryotic microbes, this has primarily been restricted to monodirectional expression hosts. Hence, in addition to balancing the proteins relative to each other, their total (cumulative) expression strength needs to be adjusted. Else, burdensome overexpression of proteins or accumulation of toxic intermediate metabolites may prove detrimental to the cellular host and undermine engineering goals. One remedy has been to restrict protein overexpression to only certain times through dynamic or regulated transcription (inducibility). A second is to balance pathway expression to prevent toxic metabolite accumulation, mimicking natural pathways’ balanced protein stoichiometries.

Natural BDPs (nBDPs) and divergent transcription have been characterized in many model organisms, with RNAseq studies even indicating that eukaryotic promoters are intrinsically bidirectional. Moreover, nBDPs with non-cryptic expression in both orientations frequently co-regulate functionally related genes. Inspired by these circuits, biological engineers have recently utilized BDPs to improve designs for gene co-expression in Escherichia coli. Saccharomyces cerevisiae, plants, and mammals. These studies offer promise, but larger sets of readily available BDPs remain limited, and the reported strategies have lacked generalizability. To our knowledge, S. cerevisiae’s less than dozen BDPs represent the largest collection and do not provide the desired spectrum of different expression ratios or constitutive induction.

BDPs offer the ability to dramatically improve pathway design, with applicability in numerous and even emerging hosts. In contrast to monodirectional expression cassettes in tandem, bidirectional cloning offers a simple and quick solution to identify optimal promoter contributions for co-expression in a single cloning expression screening experiment. But, for BDPs to be fully utilized a much larger set must be engineered, with the ideal library representing different expression levels and regulatory profiles varied per expression direction. Such a library could halve cloning junctions compared to conventional MDPs, facilitating rapid assembly of combinatorial libraries that efficiently explore broad expression landscapes. In addition, development of tools such as these could help to unlock the use of emerging hosts, such as Pichia pastoris (syn. Komagataella phaffii), which have the potential not only for industrial and pharmaceutical enzyme production but also food and dairy protein production and as chemical factories.

Here we generate a collection of 168 BDPs in the methylotrophic yeast P. pastoris, using its natural histone promoters as an engineering template. Our library covers a 79-fold range of cumulative expression, has variable expression ratios ranging from parity to a 61-fold difference between sides, and combines different regulatory profiles per side including the possibility for constitutive induction. The utility of these BDPs is demonstrated through the optimization of multi-gene co-expression, and the conserved nature of the framework histone promoters suggests the generalizability of this approach for other eukaryotes.

Results and discussion

Expression capabilities and limitations of natural BDPs. Our study began by searching for nBDPs that might satisfy various engineering needs (Fig. 1a), targeting our search to the yeast P. pastoris. Long favored as a host for heterologous protein production, P. pastoris has recently emerged as a promising chassis for metabolic engineering applications owing to its growth to high cell densities and its excellent protein expression capabilities. In addition, its methanol utilization (MUT) pathway represents one of the largest sets of tightly co-regulated genes in nature, offering transcriptional repression via glucose and inducibility via methanol, making it an ideal target for BDP mining. Bioinformatics approaches (Supplementary Data 1, Supplementary Note 1) identified 1462 putative BDPs in P. pastoris’ genome (Fig. 1b), with a subset of 40 BDPs selected for detailed characterization due to their expected high expression as housekeeping or previous application as MDPs (Fig. 1c, see Supplementary Data 2 for a list of the promoters tested).

All putative MUT pathway and housekeeping gene nBDPs were tested to identify potential regulated and constitutive promoters, respectively. Our promoter screening involved green and red fluorescent protein (FP) reporters (Fig. 1c), normalized with respect to their different relative fluorescence units (rfu), which vary, due to their dependence on the specific quantum yields of the FPs and spectrometer settings, to allow direct comparison of the two promoter sides in our experimental setting (Supplementary Fig. 1, Supplementary Note 2). This normalization factor was applied to all promoter measurements reported in this work. Among MUT promoters, only the DAS1-DAS2 promoter (P_DAS1-DAS2) showed strong expression on both sides, matching the most frequently used monodirectional AOX1 promoter, with concurring with a previous study (ref. 30 and Supplementary Fig. 2a, b, Supplementary Note 3). Other MUT promoters showed only strong monodirectional expression (Fig. 1c). Several putative nBDPs of housekeeping genes showed detectable expression on both sides but weaker than the classical and most frequently applied monodirectional GAP promoter, which was used as a benchmark (Fig. 1c). Though the majority of nBDPs mined provided limited engineering applicability, the histone promoters (P_HHT1, P_HHT2, and P_HHX) showed promise due to their equally strong expression on both sides, matching (Fig. 1c) the F_GAP benchmark during growth on glucose as a carbon source.

Bidirectional histone promoters as useful parts repository. Based on the results from the nBDPs screening (Fig. 1c), we focused subsequent engineering efforts on the three bidirectional histone promoters P_HHT1, P_HHX1, and P_HHX2, where HHT refers to the BDP at the HTA+HTB locus and HHX represents HHT–HHF. These promoters regulate the expression ratios of highly conserved multimeric histone proteins, which are required for packaging DNA into chromatin. They are required to be produced in equimolar amounts in the cell and evolutionary conserved BDPs control these ratios. Note that P. pastoris contains in contrast to S. cerevisiae only a single HTA+HTB locus (HHTX1) and two HHT+HHF loci (HHX1, HHX2).

The function, structure, involvement in gene regulation, and modifications of histones have been extensively investigated in several model organisms, with an emphasis on the cell-cycle-
regulated expression of histone promoters. Histone promoter have even been utilized to drive heterologous gene expression in fungi and plants, but these studies focused solely on monodirectional expression from histone promoters without evaluating their bidirectional potential.

For our studies, because P. pastoris reaches higher specific growth rates and biomass on glycerol compared to glucose, we tested the histone BDPs on both carbons sources. The monodirectional P\(_{GAP}\) benchmark performed better on glucose than glycerol. However, the histone BDPs performed better on glycerol and even outperformed the P\(_{GAP}\) benchmark by up to 1.6-fold (Fig. 2a).

Notably, the bidirectional P. pastoris histone promoters condense the regulatory elements needed for strong bidirectional expression compared to monodirectional benchmark promoters (Fig. 2b). This is exemplified in the length of these promoters (365–550 bp) compared to the monodirectional P\(_{GAP}\) (486 bp) and P\(_{AOX1}\) (940 bp). Nonetheless, both sides of the BDPs reached expression levels comparable to MDPs, reflected by a higher expression strength per promoter length (discussed in greater detailed below).

Noticeably all P. pastoris histone promoters contain clear TATA box motifs (Fig. 2b), meaning they are grouped with a TATA-less promoters that rely on TATA-binding protein to initiate transcription instead of alternative factors. TATA box-containing promoters are typically tightly regulated and involved with cellular stress response genes, including with P. pastoris MUT genes, whereas TATA-less promoters are typically constitutively active. Hence, the TATA boxes in the histone promoters concur with their tight cell cycle-associated expression.

Using the TATA boxes as a hallmark for determining the core promoter length, we observed exceptionally short core promoters in all histone BDPs (55–81 bp, compared to 160 bp in case of the well-studied P\(_{AOX1}\)). Core promoters are the basic region needed for transcription initiation and bound by general transcription factors (TFs) and RNA polymerase II (RNAPII). It is worth nothing that histone core promoter sequences contain the 5’ untranslated region (5’ UTRs) of the natural histone mRNAs, as these cannot easily be functionally separated from the core promoter. Regardless of this complication, the short core promoters/5’ UTRs identified here are desirable tools for promoter engineering as they can be simply provided on PCR primers. Consequently, these short histone core promoters turned out to be an excellent repository of parts for promoter bidirectionalization and the creation of synthetic hybrid promoters.

Creation of BDPs with varied expression strength. Their strong bidirectional expression and short length provided opportunity to use the histone BDPs as a template for mutagenesis strategies to create a library of variants with greater expression flexibility. To
expand the expression capabilities of the natural histone BDPs beyond only a fixed ratio and cumulative expression strength, we utilized truncation and deletion strategies of \( P_{HHX2} \) (Fig. 2c, d) to construct a synthetic BDP (sBDP) library with diversified expression strengths and ratios (Fig. 3c, d). Interestingly, removing the core promoter from one side of a BDP (Fig. 2c, d, Supplementary Fig. 4, Supplementary Note 5) increased monodirectional expression on the other side up to 1.5-fold, hinting a higher flexibility. The 31 variants generated from the native promoters (on glucose) or even exceed (on glycerol) the monodirectional \( P_{GAP} \) promoter. Reporter protein fluorescence of the bidirectional \( P_{HTX1} \) and \( P_{HHX2} \) promoters in comparison to the strong, monodirectional \( P_{GAP} \) promoter in P. pastoris. Cells were grown for 60 h on 1% (w/v) glucose or glycerol in 96-well plates. \( P_{GAP} \) was cloned in forward (fwd) and reverse (rev) orientation and is hence not bidirectional. The reporter protein fluorescence is normalized per biomass (determined by \( \text{OD}_{600} \) measurements) to rule out effects of different biomass yields between the carbon sources. Bi-directional histone promoters are short compared to the commonly used monodirectional \( P_{GAP} \) and feature exceptionally short core promoters (pCore\text{-}HTA1–100). A schematic on the sequence variants is shown (Supplementary Data 2 for exact positions). TATA boxes are denoted by red rectangles. Expression levels after growth for 60 h on glucose are shown. ‘SBFD sequence feature-based deletions (i.e., AT/GC-rich regions and TATA boxes). In a, d, mean values and standard deviations of normalized (using the normalization factor calculated in Supplementary Fig. 1) reporter protein fluorescence measurements of biological quadruplicates grown on the respective carbon sources are shown.

Creation of inducible sBDPs by MDP bidirectionalization. We next sought to introduce inducibility to this library of promoters with varied expression strengths and ratios by incorporating design elements from the inducible MUT pathway. As mentioned, MUT promoters such as \( P_{PDAS2} \) (Supplementary Fig. 2) showed promise because of their expression capacity (Fig. 1c) but are cumbersome to work with due to size (2488 bp). To solve this, we aimed to generate shorter and more flexible inducible BDPs by bidirectionalizing MDPS, fusing a second core promoter in reverse orientation to an MDP (Fig. 3a). As core promoters in eukaryotes typically provide little expression on their own, strong expression generally requires upstream activating sequences, which are also referred to as enhancers or cis-regulatory modules (CRMs)\(^{43}\), with the CRM terminology including repressor-binding sites (Fig. 3a illustration). Here the previously identified short core promoter\(^5\) UTRs of the histone promoters hold utility (Fig. 2b). We hypothesized that adding a short, non-regulated core promoter in reverse orientation upstream of an MDP could duplicate the expression and regulation of the native orientation\(^{24,25}\).

Accordingly, we fused 6 histone core promoters to 12 monodirectional P. pastoris promoters, partly varying the lengths of the core promoters and the MDPS (Fig. 3a). Two thirds of the 30 constructs were successfully bidirectionalized, showing detectable expression from the second core promoter. In the case of three promoters (\( P_{AOX1} \), \( P_{FLD1} \), and \( P_{PDAS2} \)) bidirectionalized expression >50% of the native monodirectional side was reached. The constructs \( \text{PcoreHTA1-81}^{+}P_{PDAS2-699} \) even outperformed strong MDPS. Different core promoter lengths only moderately affected expression, while MDP length had a drastic effect (e.g., \( \text{PcoreHTA1-81}^{+}P_{PDAS2-699} \) vs. \( \text{PcoreHTA1-81}^{+}P_{PDAS2-1006} \) very high vs. no bidirectionalized expression). This was perhaps surprising in light of milestone bidirectionalization studies in higher eukaryotes\(^{24,25}\) where testing only a few promoters in a single length led to suitable BDPs. These dissimilarities may be explained by a different function/distance relationship between CRMs from yeast and higher eukaryotes.

Creation of fusion sBDPs with varied regulation. All bDPs to this point possessed the same regulation on both sides. Having varied regulation can allow for expression cascades, which can be
beneficial when it is necessary to express one gene before another, such as a chaperone before its protein folding target. We generated fusions of constitutive, derepressed, and inducible MDPs[29], creating 30 fusion sBDPs with distinct regulation on each side (Fig. 3b, c; Supplementary Note 4, Supplementary Table 1). These fusions generally maintain each side’s original regulation and individual expression levels, allowing for the creation of variably regulated BDPS with a range of expression ratios between sides (0.16–0.96). A subset of the fusion promoters (Fig. 3c) consisted of combinations of DASI and DAS2 deletion variants (Supplementary Fig. 2, Supplementary Note 3) demonstrating that binding of insulator proteins can decouple regulation of BDPS per side in S. cerevisiae[18], and thus the properties of fusion promoters are difficult to predict. These synergistic effects, though, can be harnessed to design shorter, more efficient promoters and so we expanded this principle to the design of hybrid promoters (Figs. 4, 5c), ultimately finding it successful.

Creation of short hybrid sBDPs. Through the creation of this sBDP library, it became clear that we had little ability to predict function based on promoter length and core promoter properties alone. To help improve our understanding, we assembled short defined CRMs (30–175 bp, Supplementary Fig. 2, Supplementary Fig. 4, Supplementary Note 5) with histone core promoters (Fig. 2b) into compact bidirectional hybrid[48] promoters (Fig. 4). The CRMs were selected from methanol-regulated promoters (Supplementary Fig. 3). These findings contrast previous MDP fusion studies in S. cerevisiae[45,46–47], potentially due to the greater number of promoters and combinations tested here. It is known that binding of insulator proteins can decouple regulation of BDPS per side in S. cerevisiae[18], and thus the properties of fusion promoters are difficult to predict. These synergistic effects, though, can be harnessed to design shorter, more efficient promoters and so we expanded this principle to the design of hybrid promoters (Figs. 4, 5c), ultimately finding it successful.
based on literature data available on PAOX1 \((31, \text{Supplementary Fig. 2})\) and deletion studies on PDAS1 and PDAS2 \((\text{Supplementary Fig. 2, Supplementary Note 3})\). Each CRM was characterized with a single core promoter \((\text{Supplementary Fig. 4b})\), two core promoters, and combinations of CRMs in different positions and orientations \((\text{Fig. 4})\). To create combinations of regulatory profiles, we fused a truncated histone promoter variant \((\text{PHHT2-T3, Fig. 2c, d})\) to a single CRM and one core promoter.

Inducible synthetic hybrid BDPs matched expression from the monodirectional AOX1 reference promoter \((\text{bottom of Fig. 4})\). However, the generated sBDPs were considerably shorter \((179–457 \text{ bp})\) than PAOX1 \((940 \text{ bp})\). To illustrate this length advantage, we characterized their expression strength per bp of promoter length, which we define as normalized fluorescence per bp in this study. As the expression output depends on the reporter protein, these expression strengths per bp of promoter length are dependent upon the fluorescence reporter proteins and even spectrometers used. Hybrid BDPs showed up to 3.3-fold higher expression strengths per bp of promoter length than typically used nMDPs and were 2.1-fold more efficient than the most efficient nBDP \((\text{Fig. 5c})\). In addition, synthetic MDP controls were up to 2.4-fold more efficient than nMDPs \((\text{Supplementary Fig. 4})\). The length of the core promoters and the orientation of the CRMs only marginally affected the expression of the hybrid BDPs. Orientation independency in yeast CRMs has long been known\(^{38}\), and our results demonstrate that this property can also be harnessed to generate strong sBDPs.
BDPs facilitate dual gene co-expression optimization. After developing a cloning strategy to insert the library of BDPs into a cloning junction between genes of interest (Supplementary Fig. 5, Supplementary Note 6), we next aimed to demonstrate the utility of our BDP library for optimizing multi-gene co-expression. First, we optimized dual gene co-expression for production of taxadiene (Fig. 6a), the first committed precursor of the potent anticancer drug Taxol (paclitaxel), which requires expression of geranylgeranyl diphosphate synthase (GGPPS) and taxadiene synthase [30]. So, in the best taxadiene-producing strains, the GGPPS gene was at first repressed, partially activated in the derepressed phase, and then fully activated by methanol [30]. We presume that the high yield of this strain is mostly attributable to the use of \( P_{\text{CAT1}} \) to drive expression of the GGPPS gene, as also the second-best design (\( P_{\text{AOXI-CAT1}} \)) had GGPPS under the control of the same promoter. \( P_{\text{CAT1}} \) is a derepressed promoter, meaning expression starts once the glucose in the media is depleted and is further strongly induced by methanol [30].

Our results showed that constitutive expression worked only for CalB. Constitutive expression of endoplasmic reticulum-localized CYP2D6/CPR may exert too much stress on the cells, leading possibly stress responses and degradation driving its activity below the limit of detection. For taxadiene production, we noticed an approximately 100-fold decrease in transformation rates when the GGPPS gene was under control of a constitutive promoter, with few candidate colonies showing no detectable taxadiene production. For the three gene pairs tested (Fig. 6a–c), there was a 5.2–50-fold difference in activity/yields of the best and worst performing promoter choice. Most strikingly, for taxadiene production, the worst strain produced only 0.1 mg L\(^{-1}\), whereas the best strain (bearing a \( P_{\text{GAP-CAT1}} \) fusion promoter) reached 6.2 mg L\(^{-1}\), in range with engineered \( S. \text{cerevisiae} \) strains (8.7 ± 0.85 mg L\(^{-1}\)) [49].

We evaluated expression in human cytochrome P450s (CYP2D6) and its electron-donating NADPH-dependent reductase partner (CPR) using a subset of strong, differently regulated BDPs from the library (Fig. 6b). Third, we evaluated the effect of the chaperone protein-disulfide-isomerase (PDI) on secretion of the disulfide-bond-rich biocatalyst \( \text{Candida antarctica} \) lipase B (CalB, Fig. 6c).

Fig. 5 The library of 168 BDPs provides different absolute expression strengths, ratios, and regulatory profiles with synthetic BDPs (sBDPs) considerably surpassing the expression strength per bp of promoter length of natural BDPs (nBDPs). a The library of BDPs covers the whole expression space. Normalized upstream and downstream reporter fluorescence is shown (rfu OD\(^{-1}\) as in Fig. 1 to Fig. 4; under optimal growth conditions, by the default orientation in which the BDPs were cloned in the reporter vector). b The library of BDPs offers different ratios between the two sides of the promoters, ranging from equal expression to a 61-fold difference. The ratios were calculated from the normalized reporter protein fluorescence measurements of both sides (under optimal growth conditions) by dividing the lower value by the higher value. Different growth conditions of the strains with differently regulated promoters even extend the ratios achievable. Only promoters clearly exceeding the background signal of the measurements (>500 rfu for eGfp, >100 rfu for dTom) were included in the calculations. c Expression strengths per bp of promoter length of sBDPs exceed nBDPs up to 2.1-fold and nMDPs up to 3.3-fold. “Expression strength per bp of promoter length” is a term introduced in this study to illustrate the relationship between promoter length and promoter strength. The expression strengths per bp of promoter length were calculated by adding up the normalized reporter protein fluorescence measurements of both sides (under optimal growth conditions) and dividing the sum by the length of the promoter (bp). Hence the expression strengths per bp of promoter length are relative terms and will change with different fluorescence reporter proteins used and even with different fluorospectrometers for detection. The monodirectional \( \text{AOXI} \) and GAP promoters are included as references for state-of-the-art nMDPs. Fold differences between the most efficient hybrid promoters and the most efficient nBDPs, hybrid MDPs, and the monodirectional reference promoters are shown.

Cumulative expression, and (3) up to 61-fold expression ratio between sides, meeting the intended design requirements for our library (Fig. 5a, b).
BDPs alongside BDTs simplify multi-gene pathway fine-tuning. Finally, we wanted to assemble a pathway with greater than two components. In doing so, we quickly found that, with increasing numbers of genes, inclusion of bidirectional terminators (BDTs) was necessary. Lack of BDTs in this context results in transcriptional collision as polymerases transcribing opposite DNA strands in convergent orientation stall upon collision\textsuperscript{51–53}. We combined selected MDTs, including heterologous \textit{S. cerevisiae} terminators shown to be active in \textit{P. pastoris}\textsuperscript{30}, into 11 bidirectional fusion terminators by linking them in convergent orientation (Fig. 7). Additionally, natural BDTs (nBDTs) can be used as the \textit{P. pastoris} genome harbors 1461 putative BDTs from genes in tail-to-tail orientation (Fig. 1b). We included two such short nBDTs from both \textit{P. pastoris} and \textit{S. cerevisiae}.

The BDTs were cloned, maintaining the natural transition between stop codon and terminator without any additional restriction sites, into a reporter vector containing two FPs in convergent orientation (Fig. 7). Complete lack of a termination signal in this context, created by leaving only an 8 bp NotI restriction between the reporter genes, resulted in an ~8-fold reduced reporter gene fluorescence, suggesting that transcriptional collision occurs to similar extents in \textit{P. pastoris} as reported in \textit{S. cerevisiae}\textsuperscript{51–53}. Providing either fusion terminators or nBDTs showed clear improvements compared to the no terminator control, restoring 50–90% of reporter protein fluorescence. As in previous work on \textit{P. pastoris} MDTs\textsuperscript{30}, we also noticed that some BDTs functioned as autonomous replicating sequences (ARS) (Supplementary Fig. 7), which may lead to increased background growth and strain instability for episomal replicating sequences. We therefore recommend screening new BDTs for ARS function, as fusion terminators behaved in part differently from the originating MDTs (Supplementary Fig. 7).

With these BDTs available, we tested combinations of BDPs (constitutive, inducible, expression ratios) to optimize expression of the four-gene carotenoid pathway for \(\beta\)-carotene synthesis (Fig. 8a). Monodirectional cassettes using \(P_{\text{AOX1}}\) (inducible) and \(P_{\text{GAP}}\) (constitutive) were included as reference. The bidirectional constructs showed a 12.1-fold range in \(\beta\)-carotene yields, with the highest \(\beta\)-carotene yield coming from the methanol-inducible bidirectional designs (C2/C7, Fig. 8b). This construct surpassed the monodirectional \(P_{\text{AOX1}}\) design two-fold and matched the best MDP-based inducible construct previously reported in \textit{P. pastoris} (5.2 ± 0.26 mg g\textsuperscript{-1} cell dry weight)\textsuperscript{30}. Regarding constitutive/growth-associated expression of the pathway, the bidirectional design based on histone promoters (C11) yielded 14.9-fold higher \(\beta\)-carotene titers than the monodirectional standard \(P_{\text{GAP}}\) design. This improvement may be explained by the regulation of the promoters used. \(P_{\text{GAP}}\) is constitutively expressed and constitutive expression of the \(\beta\)-carotene pathway from this promoter may present too great a metabolic burden. Core histone genes, in contrast, are cell cycle regulated and typically only activated in the late G1 phase to provide sufficient histones for the newly replicated DNA in the S phase\textsuperscript{7}. It appears plausible that cell cycle-associated expression from histone promoters exerted less metabolic burden than entirely constitutive expression from \(P_{\text{GAP}}\), leading to their improved function.

Discussion
Constructing efficiently expressed and well-balanced pathways is paramount for harnessing biology to its full industrial potential. Here, using the natural histone BDPs of \textit{P. pastoris} as template, we combined multiple engineering strategies, including truncation and MDP bidirectionalization, to develop a library of sBDPs with a broad range of expression levels and ratios and with different regulation profiles. We found that this library not only covers diverse expression profiles but also is highly efficient in...
terms of the output expression. Even more, we demonstrated its utility for multi-gene pathway optimization, highlighted by simple optimization experiments for taxadiene and β-carotene production. By screening of our large 168 member library, we identified a subset of highly useful BDPs and compiled a minimal set of 12 BDPs (6 BDPs to be tested in both orientations, Table 1 and Supplementary Data 3 for annotated sequence files). These promoters have regulatory diversity, different strengths, and ratios. In addition, this subset offers extended diversity if cultivated with different carbon sources (glucose/glycerol, methanol). Screening with this initial set provides a foundation for subsequent fine-tuning.

Generating similar BDP libraries in other organisms will require species-specific engineering, especially for obtaining inducible promoters. Methanol-inducible promoters are rather unique to P. pastoris and other methylotrophic yeasts31, whereas other systems will require species-specific promoters such as galactose-regulated promoters in S. cerevisiae32. In higher eukaryotes, where carbon-source-regulated promoters are scarce, inducible BDPs based on synthetic TFSs30 could be generated relying on strategies developed for MDPs54,55.

However, as this library strategy relies on parts from the highly conserved histone BDP architecture, with homologs in S. cerevisiae, Schizosaccharomyces pombe, and even Chinese Hamster Ovary cells, we have reason to believe that the promoter engineering and cloning strategies outlined in this work will be generalizable to other eukaryotes. Hence, the use of similar BDP libraries is likely to expand to many hosts and allow for efficient and rapid pathway optimization, expanding the possibilities of synthetic biology and metabolic engineering.

**Methods**

**Promoter reporter vectors.** The P. pastoris CBS7435 wild-type strain was used for most experiments. The control strain expressing the four genes of the carotenoid pathway under control of four AOX1 promoters was available from Geier et al.56. This strain contains the identically codon optimized genes of the carotenoid pathway used in this study each under control of the AOX1 promoter and terminator. For CalB expression, mutS strains57 were used, as higher productivity on methanol has been reported30.

Details on the promoters and terminators used in this study (including primers for amplification) and the list of primers for generating the reporter vectors and applications (pathway assembly, etc.) are provided in Supplementary Data 2. A subset of annotated sequences of a minimal set of BDPs covering broad regulatory profiles for dual gene expression optimization is provided in Supplementary Data 3 file in GenBank format (and summarized in Table 1 in the main manuscript).

For basic characterizations, a pPyT4,57 based expression vector (Zeocin selection marker) bearing a single eGFP reporter gene reported by Vogl et al.30 was used (pPyT4mutZeomMyl-intARGal-eGFP-BmrStuffer30). This vector contains integration sequences near the ARG4 locus and was linearized with SspI to target integration sequences near the ARG4 locus and was linearized with SspI to target integration sequences near the ARG4 locus and was linearized with SspI to target

**Fig. 7** Bidirectional transcription terminators (BDTs) required for the assembly of bidirectional multi-gene co-expression relieve expression loss associated with transcriptional collision. A reporter construct for testing bidirectional transcription termination was assembled by cloning the genes coding for eGfp and dTom in convergent orientation (small inlet). Two AOX1 promoters were used to drive equal expression of the reporter genes. Monodirectional terminators (MDTs) were combined into bidirectional fusion terminators and two putative natural BDTs (nBDTs) were tested. A negative control lacking termination sequences and bearing solely a Nde restriction site was included. Additional control constructs contain only a single AOX1 promoter, a single FP, and the AOX1* terminator. AOX1TT* denotes the AOX1 terminator sequence used by Vogl et al.30. Some BDTs acted also as autonomously replicating sequences (Supplementary Fig. 7). Mean values and standard deviations of fluorescence measurements after pre-growth on glucose followed by methanol induction of biological quadruplicates are shown.

**Fig. 8** The library of BDPs and BDTs facilitates the assembly and transcriptional fine-tuning of multi-gene pathways demonstrated with the four gene (crtE, crtB, ctrl, crtY) model pathway of β-carotene biosynthesis. a Using BDPs and BDTs for pathway assembly reduces construct length and the number of parts required. Twelve bidirectional constructs were assembled by combining inducible or constitutive BDPs and combinations thereof (Induc.+const.) with a BDT and two MDTs. See Supplementary Fig. 5d for assembly strategy and supporting file Supplementary Data 2 (sheet “Carotenoid pathway constructs”) contains detailed information on the BDPs/BDTs used. For the BDPs, a coloring scheme similar to Fig. 5 was used. T* natural bidirectional terminator between the S. cerevisiae IDP1 and PEX19 genes, T+ natural bidirectional terminator between the P. pastoris TEF1 and GDMT1 genes. The bidirectionalized P366+HHT7-91 was used. b β-Carotene titers obtained with strains based on the bidirectional constructs shown in a span a 12-fold range matching or surpassing conventional P30 and P32-based designs. Mean values and standard deviations of biological triplicate cultivations in shake flasks are shown (HPLC measurements).
Table 1 Minimal set of diverse BDPs covering broad regulatory profiles for co-expression optimization

| BDP          | Regulation                          | Strength                                         | Ratio |
|--------------|-------------------------------------|--------------------------------------------------|-------|
| pYTPxT (HTA1:HTB1) | Constitutive on both sides (cell cycle/growth associated in S. cerevisiae) | Strong on both sides | -1.1  |
| pDAS2-699:µCointTAI-B1 | Methanol inducible (tightly glucose/glycerol repressed) on both sides | Strong on both sides | -1.2  |
| pCAD5-ΔFVDI | Derepressed/methanol inducible on both sides | Weak/moderate under derepression, strong on methanol | -1.1  |
| pAOX1-CAT1 | Methanol inducible (tightly glucose/glycerol repressed)-derepressed, methanol inducible | Strong on both sides | -1.1  |
| pAOX1-GAP | Methanol inducible (tightly glucose/glycerol repressed)-constitutive | Strong on both sides | -1.1  |
| pGAP-CAT1 | Constitutive-derepressed, methanol inducible | pGAP side strong constitutive (moderate on methanol), pCAT side moderate derepressed, strong on methanol | -1.25  |

In dual gene expression applications, each BDP should be tested in forward and reverse orientation. Reporter protein fluorescent of the respective promoters are shown in Figs. 2a and 3a. a. Annotated GenBank files for these promoters are provided as Supplementary Data 3. For multi-gene co-expression furthermore, the three histone promoters HXT1, HXK1, and HXK2 and additional methanol inducible promoters (e.g., SNOB23 [Fig. 4]; FDL1-PMP20, FBA1-TAL2 [Fig. 3b]) are useful.

BBDs reporter vector and cloning of BDTs. The reporter vector for BDTs contained two convergent expression cassettes each consisting of an AOX1 promoter and an eGFP or ΔTOM reporter gene, respectively (see illustration in Fig. 7). The 3’ ends of the reporter genes are separated by a stuffer fragment that can be replaced with a BDT. The reporter vector was assembled by digesting a monodirectional control vector containing an AOX1 promoter upstream of eGFP (pYTP4mutZeomyl-entArg4-eGFP-BmrIstuffer) with NotI and BamHI. Subsequently, the AOX1 promoter fused to the dTomato gene was amplified using primers dTomato-AscIBmrIFWD and AOXTTSbfIAvrIIREV1. To add an additional restriction site, the obtained PCR fragment was used as template for a second PCR. The fragment between them is assembled by olePCR, digested with NotI-digested pPpT4mutZeoMlyI-intArg4-DAS1TT-NotI-AOX1TT backbone and joined by Gibson assembly. The entire inserted fragment was amplified using primers seqEGFP-520..543-fwd and seqTomato-517..540-fwd. The obtained PCR fragment was used as template for a second PCR using primers TomatoAscIBmrIFWD and AOXTTSbfIAvrIIREV1. To add an additional restriction site, the obtained PCR fragment was used as template for a second PCR using primers TomatoAscIBmrIFWD and AOXTTSbfIAvrIIREV1. The newly inserted part was confirmed by Sanger sequencing. This vector was named pYTP4mutZeomyl-entArg4-bidi-dTOM-eGFP-BmrIstuffer. Subsequently, we cloned several natural BDPs and semisynthetic fusion promoters into this vector (primers provided in Supplementary Data 2). The promoters were either inserted in random orientation by TA cloning or directional by Gibson assembly.

Cloning different BBDs for dual gene co-expression. Our screening strategy for the optimal BDP for a certain gene pair (Supplementary Fig. 5a–c) requires an entry vector containing the two genes in the same orientation and the promoter can be easily exchanged. A stuffer fragment in this entry vector is subsequently cut out by BmrI digestion and replaced with BDPs. Note that the genes to be co-expressed must not contain BmrI sites.

The vector for taxadiene co-expression was generated by ordering P. pastoris codon-optimized GGPS and TDS genes. The genes were ordered as synthetic double-stranded fragments (gBlocks by Integrated DNA Technologies) with overhangs for Gibson assembly (gBlock-GGPPS_optTV-AOX1TT-Gib, gBlock-TDS_optTV-Part1 and gBlock-TDS_optTV-Part2-DAS1TT-Gib). A stuffer fragment with complementary overhangs was amplified using primers TDS-BmrI-stuffer-Gib and GGPPS-BmrI-stuffer-Gib. The four fragments were mixed in equimolar ratios with the NotI-digested pYTP4mutZeomyl-entArg4-DAS1TT-NotI-AOX1TT backbone and joined by Gibson assembly. The entire inserted cassette was sequenced. This vector was named pYTP4mutZeomyl-entArg4-DAS1TT-AOX1TT-TDS_optTV-GGPPS_optTV-BmrIstuffer.

After removal of the stuffer fragment by BmrI digestion and gel purification, a set of the respective differently regulated promoters was amplified, cloned into the entry vectors, and verified by sequencing. See Supplementary Data 2 for the exact primers and overhangs used.

In a similar way, entry vectors for CYP2D6/CPR co-expression and CalB/PDI co-expression were generated. The coding sequences were available from previous studies (CYP2D6/CPR (28, 30, PDI (28)). See also Supplementary Data 2 for the exact primers and overhangs used. For CYP2D6/CPR, the monodirectional control strain (Fig. 6b) containing a single copy of a vector with each gene under control of an AOX1 promoter was available from previous work and was generated by cloning each of the two genes into the two vectors FPCR and FZoR via ZeoRI and NotI sites and after the transformation a transformant with a single copy of each plasmid was selected. The monodirectional CalB/PDI control constructs shown in Fig. 6c were generated by

...insertion near the ARG4 locus, as had been established for promoter characterizations in P. pastoris (39, 41). Also the following vectors described below were based on this vector backbone. With the single reporter vector, BDPs had to be cloned twice, once in forward and once in reverse orientation. The P. pastoris nBDPs were initially characterized by these means. To reduce the cloning effort and allow simultaneous detection we designed a bidirectional screening vector. Based on the single reporter vector, we inserted a second reporter gene (a red FP variant termed dTomato (95) between the targeting sequence and the stuffer fragment of pYTP4mutZeomyl-entArg4-eGFP-BmrIstuffer. The vector was assembled by digesting the single reporter vector with Ascl and AvrII. Subsequently, the dTomato fused to a P. pastoris transcription terminator sequence was PCR amplified from a P. pastoris cloning vector using primers TomatoAscIBmrIFWD and AOXTTSbfIAvrIIREV1. To add an additional SfiI restriction site, the obtained PCR fragment was used as template for a second PCR using primers TomatoAscIBmrIFWD and AOXTTSbfIAvrIIREV2. The newly inserted part was confirmed by Sanger sequencing. This vector was named pYTP4mutZeomyl-entArg4-bidi-dTOM-eGFP-BmrIstuffer. Subsequently, we cloned several natural BDPs and semisynthetic fusion promoters into this vector (primers provided in Supplementary Data 2). The promoters were either inserted in random orientation by TA cloning or directional by Gibson assembly.

Cloning different BBDs for dual gene co-expression. Our screening strategy for the optimal BDP for a certain gene pair (Supplementary Fig. 5a–c) requires an entry vector containing the two genes in the same orientation and the promoter can be easily exchanged. A stuffer fragment in this entry vector is subsequently cut out by BmrI digestion and replaced with BDPs. Note that the genes to be co-expressed must not contain BmrI sites.

The vector for taxadiene co-expression was generated by ordering P. pastoris codon-optimized GGPS and TDS genes. The genes were ordered as synthetic double-stranded fragments (gBlocks by Integrated DNA Technologies) with overhangs for Gibson assembly (gBlock-GGPPS_optTV-AOX1TT-Gib, gBlock-TDS_optTV-Part1 and gBlock-TDS_optTV-Part2-DAS1TT-Gib). A stuffer fragment with complementary overhangs was amplified using primers TDS-BmrI-stuffer-Gib and GGPPS-BmrI-stuffer-Gib. The four fragments were mixed in equimolar ratios with the NotI-digested pYTP4mutZeomyl-entArg4-DAS1TT-NotI-AOX1TT backbone and joined by Gibson assembly. The entire inserted cassette was sequenced. This vector was named pYTP4mutZeomyl-entArg4-DAS1TT-AOX1TT-TDS_optTV-GGPPS_optTV-BmrIstuffer.

After removal of the stuffer fragment by BmrI digestion and gel purification, a set of the respective differently regulated promoters was amplified, cloned into the entry vectors, and verified by sequencing. See Supplementary Data 2 for the exact primers and overhangs used.

In a similar way, entry vectors for CYP2D6/CPR co-expression and CalB/PDI co-expression were generated. The coding sequences were available from previous studies (CYP2D6/CPR (28, 30, PDI (28)). See also Supplementary Data 2 for the exact primers and overhangs used. For CYP2D6/CPR, the monodirectional control strain (Fig. 6b) containing a single copy of a vector with each gene under control of an AOX1 promoter was available from previous work and was generated by cloning each of the two genes into the two vectors FPCR and FZoR via ZeoRI and NotI sites and after the transformation a transformant with a single copy of each plasmid was selected. The monodirectional CalB/PDI control constructs shown in Fig. 6c were generated by
cloning the respective promoters into the same pPt4 vector (using the standard AOX1TT).

Assembly of multi-gene cassettes for the carotenoid pathway. Constructs with different BDPs and terminators were designed for the expression of the carotenoid pathway (four genes CrtE, CrtB, CrtI, and CrtY) in P. pastoris and are shown in Fig. 8a. The exact promoters, terminators, and primers for amplification are provided in Supplementary Data 2. The BDPs and terminators were selected based on their layout, length, and sequence characteristics. Combinations of the different strengths and regulations were tested (inducible, constitutive, constitutive + inducible). Also a construct with switched positions of the BDPs was created to evaluate the effect of positioning the promoter between the first two or the last two genes.

The vector backbone pPT4S-DAS1TT-NotI-AOX1TT containing two monodirectional terminators TAOX and TDDAS in opposite orientation with a NotI restriction site in between was used for insertion of the pathway. The genes, BDPs, and terminators were amplified by PCR, using the primers listed in Supplementary Data 2. The primers for the amplification of the promoter and terminator sequences contained overlaps to the carotenoid genes. The fragments were linked by Gibson assembly. In order to increase the efficiency of the Gibson assembly, the number of fragments, which have to be combined, was reduced by a preassembling step via overlap extension PCR. After combining the carotenoid genes with the adjacent promoter or terminator, the preassembled fragments were connected by Gibson assembly and used to transform E. coli. Plasmid DNA was isolated from transformants and the sequences were verified by sequencing.

Cultivation conditions and screening procedures. The P. pastoris cultivations were performed using a high-throughput small-scale 96-deep-well-plate (DWP) cultivation protocol43. Briefly, wells containing 250 µl BMD1 (buffered minimal dextrose medium, as reported44) were inoculated with a single colony from transformation plate and grown for 60 h on glucose. After induction with 0.5% (v/v) methanol concentration of 0.5% (v/v) was used. Cells were induced with 250 µl of buffered media with 1% methanol (BMM2) after 60 h of growth on glucose. After 12 h, 24 h up to 48 h, 50 µL of BMM10 (with 5% methanol) was added for further induction.

P. pastoris cells were transformed with molar equivalents to 1 µg of the empty pPyPT4_S vector Swall linearized plasmids45 (1 µg of the empty pPyPT4_S vector was found to yield predominantly single copy integration5,6,6). Some of the vectors used in this study are, however, considerably larger than the empty pPyPT4_S vector (e.g., the carotenoid pathway constructs), hence in these cases we increased the DNA amount (equal number of vectors. Combinations of the promoter and terminator were amplified by PCR, using the primers listed in Supplementary Data 2. The primers for the amplification of the promoter and terminator sequences contained overlaps to the carotenoid genes. The fragments were linked by Gibson assembly. In order to increase the efficiency of the Gibson assembly, the number of fragments, which have to be combined, was reduced by a preassembling step via overlap extension PCR. After combining the carotenoid genes with the adjacent promoter or terminator, the preassembled fragments were connected by Gibson assembly and used to transform E. coli. Plasmid DNA was isolated from transformants and the sequences were verified by sequencing.

Fluorescence reporter measurements and assays. The fluorescence reporter and OD600 measurements were performed using 96-well microtiter plates (Nunc MicroWell 96-well optical-bottom plates with polymer base, black; Thermo Fisher Scientific) and a Synergy MX plate reader (Biotek, Winooski, VT, USA). Enhanced green fluorescent protein (EGFP) measurements were performed at excitation/emission wavelengths of 488/507 nm40,41. Tomato was measured at excitation/emission wavelengths of 480/507 nm42. dTomato was measured at excitation/emission wavelengths of 488/507 nm30,40.

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Author contributions
T.V. and T.K. contributed equally to this work. T.V. and T.K. selected the nBDPs. T.V. discovered the histone promoters and designed all sBDPs. T.K., L.S., B.A., E.-M.K., P.H., M.B. and T.V. performed the promoter experiments. A.G. recognized the need for an innovative co-expression strategy. T.V. selected the nBDTs and designed the sBDTs. E.-M.K. performed the terminator experiments. The applications of the BDP library for dual gene co-expression were designed by T.V. and performed by J.E.F. and B.W.B. E.-M.K. performed the terminator experiments. The applications of the BDP library for metabolic pathways. The remaining authors declare no competing interests.

Additional information
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Competing interests: T.V., T.K., L.S., and A.G. are inventors on a patent application entitled “Bidirectional promoter” (assignee: Technische Universität Graz/ACIB GmbH; inventors: T.V., T.K., L.S. and A.G.; application number: EP286293; status of application: pending; specific aspect of manuscript covered in patent application: bidirectional promoters). T.V., A.G., and P.K.A. have filed a patent application entitled “Production of terpenes and terpenoids” (assignee: Technische Universität Graz; inventors: T.V., A.G. and P.K.A.; application number: US20180094286; status of application: pending; specific aspect of manuscript covered in patent application: metabolic pathways). The remaining authors declare no competing interests.

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