Synthetic Toolkit for Complex Genetic Circuit Engineering in *Saccharomyces cerevisiae*

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ABSTRACT: Sustainable production of chemicals, materials, and pharmaceuticals is increasingly performed by genetically engineered cell factories. Engineering of complex metabolic routes or cell behavior control systems requires robust and predictable gene expression tools. In this challenging task, orthogonality is a fundamental prerequisite for such tools. In this study, we developed and characterized in depth a comprehensive gene expression toolkit that allows accurate control of gene expression in *Saccharomyces cerevisiae* without marked interference with native cellular regulation. The toolkit comprises a set of transcription factors, designed to function as synthetic activators or repressors, and transcription-factor-dependent promoters, which together provide a broad expression range surpassing, at high end, the strongest native promoters. Modularity of the developed tools is demonstrated by establishing a novel bistable genetic circuit with robust performance to control a heterologous metabolic pathway and enabling on-demand switching between two alternative metabolic branches.

KEYWORDS: synthetic transcription factor, gene regulation, yeast, core promoter, bistable switch

Increased ability to engineer microbial hosts has led to quick expansion of synthetic biology applications ranging from therapeutic cells to biological production of fuels, chemicals, and pharmaceuticals. This ability has been enabled by the availability of novel genome editing tools, such as CRISPR-Cas9, by rapid and affordable DNA sequencing and synthesis services, and by the use of modular and standardized DNA parts that allow quick assembly and optimization of genetic systems. Such tools are especially well established for the commonly used model and production organisms such as *Escherichia coli*. Another widely used model and production host with a comprehensive collection of engineering tools is the yeast *Saccharomyces cerevisiae*. However, development of the tools in *S. cerevisiae* lags behind as compared to *E. coli*. Especially tools for gene expression control need to be developed further.

The orthogonality of gene expression systems is an important feature to limit or, in the best case, completely avoid the cross-talk between the engineered gene circuit and the native cellular regulation. The use of homologous (native) DNA regulatory parts in an expression system can lead to unintended behavior that is caused, for instance, by changing growth conditions during extended bioprocesses. This is due to the often poorly understood, complex native cellular regulation in which several transcription factors and other regulators are involved and can influence the performance of the expression system. Adjustability, expression output stability and predictability, the ability to reach high expression levels, and independence on specific inducer compounds are also desirable features of gene expression tools. To address these challenges, a number of recent studies describe development of orthogonal gene expression tools for *S. cerevisiae*. In most cases, the gene expression tools are based on the use of synthetic transcription factors (sTF) consisting of modular parts, such as DNA binding proteins, receptor domains, and transcription activation domains. Modularity of the different parts is a great advantage as it allows design of sTFs with desired functions and target specificities. In addition, the existing parts typically originate from evolutionarily distant organisms, such as bacterial repressor proteins, zinc finger domains, transcription activator-like effectors (TALEs), or the Cas9 null mutant (dCas9) protein, which have no homologues in yeast and thus potentially provide a high degree of orthogonality.

The expression level of a target gene is, to a large extent, dependent on the (sTF-dependent) promoter strength, which
in turn is defined by the modules used in the expression system. The core promoter\textsuperscript{7,15} as well as the number of sTF binding sites, or their distance from the core promoter, are the most important and commonly modified parameters which determine the synthetic promoter strength.\textsuperscript{3,6,8} In addition, the affinity of the sTF to its binding sites and the capacity of the activation domain to recruit transcription machinery can greatly affect the target expression levels. For example, the use of strong transcription activation domains, such as VP16 or Gal4, can lead to significantly higher expression levels compared to weaker activation domains, such as B42.\textsuperscript{6,7,16} In challenging genetic engineering applications, cellular hosts may be designed to contain complex genetic circuits performing intricate biological tasks. The construction of such systems typically

Figure 1. Characterization of orthogonal activators. (A) Scheme of the synthetic expression system for testing of the engineered transcription activators. Two genome-integrated DNA cassettes provide expression of the synthetic transcription factor (sTF) and the reporter gene (Venus). The selected sTFs are expressed from the \textit{TDH3} core promoter, ensuring a low and constitutive expression level. The sTF-specific binding sites (BS) upstream of the \textit{ENO1} core promoter form the synthetic promoter for the reporter gene (Venus). (B) Orthogonality matrix of the sTFs and their binding sites. The specificities and off-target activities of the sTFs were analyzed in strains carrying all combinations of the sTF and 8 BS reporter cassette pairs. The fold activation represents fluorescence ratio between the strain containing the sTF-BS pair and the corresponding strain lacking the sTF cassette. The values are calculated from three biological replicates. The corresponding fluorescence values are listed in Figure S1A. (C) Tuning of gene expression level by different sTFs. The modified reporter cassettes with sTF-dependent promoters containing varying numbers (0–8) of the sTF-specific BSs were used. The strain "8 BS (wo sTF)" represents a negative control strain lacking the sTF cassette. The values represent the mean of three biological replicates ± standard deviation (SD). (D) Transcription analysis of the selected strains (from C) with 2 or 8 BSs in the sTF-dependent reporter cassette promoters. "P" represents the parental strain without the sTF and reporter genes. The values represent the mean of two biological and two technical replicates ± SD.
Figure 2. Characterization of orthogonal repressors. (A) Scheme of the synthetic repressor (sRep) system for testing of the sReps and the corresponding repressible core promoters. The LexA-sTF functions as a constitutive activator for the reporter gene (Venus). The production of sReps leads to repression through binding within the core promoter and blocking of transcription initiation. The sTF is constitutively expressed from the TDH3cp, whereas the sReps are expressed from the thiamine repressible THI4 promoter. The sRep system is encoded by three DNA cassettes each integrated in separate loci in the genome. (B) Transcription analysis of an example strain containing the sRep system with Bm3R1-based sRep and ENO1cp with two Bm3R1-specific binding sites. Cells were cultivated in varying thiamine concentrations to adjust the level of the sRep expression. The values represent the mean of two biological and two technical replicates ± SD. (C) Reporter (Venus) fluorescence in strains carrying different sRep systems cultivated in various thiamine concentrations. “No rep” represents control strains without the sRep-cassette (cultivated in 400 μg/L thiamine concentration). The values represent the mean of three biological replicates ± SD.
requires simultaneous use of several sTFs each controlling a specific sub-operation in the circuit. For functional establishment of such genetic tools, a spectrum of transcription factors needs to be carefully characterized, especially for the absence of cross reactivity, and also for functionality in relation to different core promoters and binding sites used in the target synthetic promoters. However, many of the systems developed in yeast so far have been based on the use of a single sTF. At the same time, the orthogonality of different sTFs and synthetic promoter modules have not been studied extensively. For example, several synthetic transcription factors have been successfully used in engineered genetic circuits, and a wide variety of promoters and core promoters is also available. However, a comprehensive characterization and comparison of different DNA parts, as well as their detailed analysis once integrated into complex expression systems, is often not available. In addition, the methods used for the characterization vary greatly. This represents a significant challenge for the design and establishment of robust genetic circuits in which precise balancing of expression levels is required.

In this study, we developed and characterized in depth a set of orthogonal transcription factors and synthetic promoter parts for use in complex genetic networks in yeast. The orthogonal transcription factors were engineered to either upregulate (sTF) or downregulate gene expression (synthetic repressor, sRep). In addition, a library of core promoters was tested and characterized. As a result, we established a novel setup for a bistable switch using the developed DNA parts. The bistable switch herein has two distinct expression states and a genetic memory allowing it to retain its programmed status in the absence of inducer compounds over 80 generations (12 days). The functionality of the switch was demonstrated by switching between two branches of the violacine biosynthetic pathway resulting in on-demand formation of different end-products.

Results

Synthetic Activators. To develop a modular toolbox for gene expression in yeast, a set of sTFs were constructed and expressed under the TDH3 core promoter (TDH3cp). We previously showed that TDH3cp provides a low and constitutive expression level. It could thus be well suited even for expression of strong sTFs, which can have detrimental effects on growth if expressed at high level. The test platform for the sTFs was composed of synthetic promoters that control the expression of the reporter gene Venus (yellow fluorescent protein) (Figure 1A). The synthetic promoters contained eight binding sites for the sTF to be tested followed by the ENO1cp. The sTFs are modular and consist of a bacterial DNA binding protein, the SV40 nuclear localization signal, and a C-terminal VP16 activation domain (VP16AD). The DNA-binding protein modules were selected from the previously characterized bacterial repressors with a DNA-binding motif. The tested repressors were LexA, SrpR, PhlF, TetR, Bm3R1, TarA, and IscR. The test system was constructed as an extension of the Venus reporter gene (Figure S2). To achieve adjustable gene expression levels, the sTF-dependent promoters were engineered to contain 0, 1, 2, 4, or 8 sTF binding sites. A clear increase in the reporter gene expression was observed in response to increasing number of the sTF binding sites in strains containing LexA-sTF, SrpR-sTF, PhlF-sTF, or TarA-sTF (Figure 1C). In case of Bm3R1-sTF and TetR-sTF, already one or two binding sites resulted in maximal expression level of these synthetic promoters. As expected, the expression level of the sTFs itself had a strong influence on the range of expression level of the reporter gene (Figure S1B).

To directly assess the functionality of the expression system, transcription level analysis was carried out for strains containing expression systems with two or eight sTF binding sites (Figure 1D). With the exception of the PhlF strains, the relative differences in reporter gene transcription level corresponded to the Venus fluorescence level. In the PhlF strains, the Venus reporter transcription level was similar to those in the TetR and Bm3R1 strains, although the fluorescence signal was similar to those in the LexA and SrpR strains. Comparison of the transcription level to strong native genes (TDH3 and TEF1) revealed that higher expression level was achieved by the strongest versions of the engineered promoter systems as exemplified by the fact that the reporter transcript level significantly exceeded the levels of TDH3 and TEF1.

Synthetic Repressors. The construction of genetic circuits requires tools that enable adjustable and robust gene expression control. The sTFs described above enabled tuning of target gene activation. To achieve tight repression, the DNA binding property of the sTF proteins were considered for use as repressors. To test this, SrpR, PhlF, TetR, Bm3R1, and the previously used LacI were fused to SV40 NLS to facilitate nuclear localization and to generate synthetic repressors (sRep). In addition, the synthetic promoters were modified by the introduction of sRep-specific binding sites in the core promoter downstream of the TATA-box. The concept of the synthetic repression system for testing the repressors is shown in Figure 2A. The test system was constructed as an extension of the LexA-based expression system with four binding sites.
Figure 3. Performance of the bistable switch circuit. (A,B) Schemes of the bistable switch circuit states. The genetic system is encoded by four DNA cassettes, each integrated in separate loci in the genome. The circuit operates in two distinct and mutually exclusive states, TetR and Bm3R1, respectively. Transcriptional activation is indicated by a filled arrow, the vertical line in front of the arrow indicates simultaneous repression of the other direction. An open arrow next to core promoters (CPs) indicates the direction of transcription activation facilitated by the CP. (A) The TetR state is triggered by the absence of thiamine (via the THI4p). Expression of TetR-B42 in the switch cassette activates transcription of the TetR-sTF and, simultaneously, blocks the Bm3R1-sTF expression in the control cassette. By the action of the TetR-sTF, a feed-forward loop is formed to ensure stable maintenance of the TetR state, resulting in expression of Venus in the reporter cassette and KO2 in the diagnostic cassette. At the same time, TetR-sTF acts as a repressor for Bm3R1-sTF, BFP, and mCherry. (B) The Bm3R1 state is activated in the presence of galactose (via GAL1p),
and all the regulatory events in the circuit are reversed (switched) as compared to the TetR state. The Bm3R1-sTF-Cln2-sh activates the expression of the Bm3R1-sTF to form another feed-forward loop triggering a cascade of activations and repressions resulting in a stable state. (C) Time course transcription analysis of the bistable switch during the transition between the states. The cells were switched to the TetR state in the absence of thiamine for 24 h (yellow box) followed by cultivation in non-inducing SCD medium (white box). Subsequent switch to the Bm3R1 state was triggered by a 24 h cultivation in the presence of galactose (gray box), followed by another cultivation in non-inducing SCD medium (white box). In the heat map, the values are relative to the maximum transcript level of each gene (in percent). The value 100% corresponds to the highest measured signal in the time course. The UBC6 transcription was used as a reference value in the analysis. The shown values are the mean values of two biological and two technical replicates. The absolute (numerical) values from this analysis are listed in the Figure S5A. (D) The system was switched several times between the TetR and Bm3R1 states by cultivating cells for 24 h in the inducing conditions, in the absence of thiamine (yellow shading) or in the presence of galactose (gray shading), followed by a 48 h cultivation in non-inducing conditions (SCD). Each cultivation was refreshed every day for the duration of the experiment (16 days). The color of the yeast cells in the scheme represents the main fluorescent protein expressed in each state, Venus (yellow) or BFP (blue). The fluorescence values in the graphs represent the mean of four biological replicates ± SD. (E) Memory test of the bistable switch. Either the TetR or the Bm3R1 state was switched on, followed by cultivation in non-inducing SCD medium for 11 days (culture diluted in fresh medium every day). The fluorescence values represent the mean of four biological replicates ± SD.

(Figure 1A), where the ENO1cp was replaced with a spectrum of modified core promoters that originate from S. cerevisiae (PGK1cp, ENO1cp, THI4cp), Aspergillus niger (533cp, 114cp), and Trichoderma reesei (Tr123cp). A separate sRep expression cassette was introduced which provided the control of repressor abundance via the thiamine repressible THI4 promoter. The activity of THI4p is dependent on thiamine concentration, allowing the dose-dependent regulation of sRep levels (Figure S3A).

A set of yeast strains was constructed with three expression cassettes integrated in single copy in their genomes: (1) the LexA-sTF cassette, expressing sTF under the TDH3cp for constitutive activation of the reporter gene expression; (2) the reporter (Venus) cassette, where the reporter gene was under the control of an sTF/sRep-dependent synthetic promoter; and (3) a corresponding sRep expression cassette. In addition, control strains lacking the sRep cassette were constructed to assess the performance of the fully activated system. The highest activation of the reporter gene expression was observed in the presence of high thiamine concentration (800 μg/L, THI4p repressed) (Figure 2B,C), where the reporter gene expression was at similar level as in strains lacking the sRep (Figure 2C). Lowering the thiamine concentration led to a gradual activation of the sRep expression, resulting in decreased reporter levels and eventually full repression of reporter expression in the absence of thiamine (Figure 2B,C).

As expected, the introduction of the sRep binding site affected the core promoter functionality. In most cases, the modified core promoters showed decreased activity as compared to the wild-type versions (Figure S3B). The results are in line with previous reports showing that the introduction of repressor binding sites affect native core promoter elements which are important for proper transcription initiation.33 It appears that already at low level, some sReps show higher capacity to repress the reporter expression than others. The THI4 promoter, used for the sRep transcription control, is fully repressed at thiamine concentration of 800 μg/L, and it is only mildly activated at 400 μg/L (Figure S3A). The Bm3R1- and TetR-sReps-mediated repression was more sensitive to decreasing thiamine concentration from 800 to 400 μg/L compared to SrpR- and PhilF-sReps (Figure 2C). This could indicate higher affinity of the “stronger” sReps to their binding sites. This is in line with the results obtained for the activation function of the sTFs (Figure 1C), where the Bm3R1- and TetR-based sTFs triggered higher expression levels of the reporter gene than the SrpR- and PhilF-based sTFs.

Establishment of a Bistable Switch. To test the established tools in the control of a complex, multilayered, genetic circuit, a novel type of a bistable switch was designed with a genetically encoded memory function. The structure of the bistable system is shown in the Figure 3A,B (and its alternative version B in the Figure S4A,B). The switch is activated by introduction of a strong temporal signal that causes activation of downstream genes leading to a stable state of the circuit, with the simultaneous repression of the genes required for the opposite state. Upon removal of thiamine, transcription of the TetR-sTF is triggered, while in the presence of galactose transcription of the Bm3R-sTF is switched on. In the system, the signals are mediated by modified versions of Bm3R1- and TetR-sTFs (“Switch cassette”, Figure 3A,B). In the Bm3R1-sTF, a shortened version of the Cln2 degradation tag56,46 was introduced to the C-terminus of the protein to generate Bm3R1-sTF-Cln2-sh. In the TetR-sTF, the VP16 activation domain was replaced by the weaker B42 activation domain (B42AD) to generate TetR-B42. These modifications were made to eliminate toxicity of highly overexpressed sTFs containing VP16AD. Addition of the degradation tag decreases the effective abundance of the Bm3R1-sTF-Cln2-sh protein and also decreases the activity of the VP16AD (our unpublished observation). The overexpression of sTF with B42AD does not have a negative impact on the fitness of yeast cells.7 The expression of these transcription factors was controlled by the galactos-inducible GAL1 promoter (for Bm3R1-sTF-Cln2-sh) and the thiamine-repressible THI4 (for TetR-B42) promoter (Figure 3A,B). The choice of these promoters was motivated by two key features important for the functionality of the bistable switch; they confer very low or no background transcription in the repressing conditions and provide very high expression levels when induced (Figure S3C).

At the core of the system is the “Control cassette” which allows switching between two mutually exclusive expression states: TetR state and Bm3R1 state. The cassette has a novel bidirectional architecture in which two engineered core promoters the ENO1_Bm3R1cp and the S33_TetRcp, selected based on the repression assay (Figure 2C), were fused together in opposite directions. These core promoters control the expression of TetR-sTF and Bm3R1-sTF simultaneously. Once the system is set to the TetR state, the TetR-sTF should activate its own expression, forming a feed-forward loop (Figure 3A). At the same time, the TetR-sTF should act as a repressor for the Bm3R1-sTF through binding at the S33_TetR core promoter. Once the system is in the opposite, Bm3R1 state, the expression of the Bm3R1-sTF forms a feed-forward
loop and simultaneous repression of the TetR-sTF (Figure 3B). An important feature of the “Control cassette” is the moderate strength of both core promoters and the high repression capacity of the sTFs. These features provide mutually exclusive expression of the sTF and the stability of each state.

The output signal of the bistable circuit was monitored via a “Reporter cassette” containing expression cassettes for reporter genes encoding either BFP (blue fluorescent protein) or Venus. In the cassette, upstream of the repressible core promoters, four binding sites for TetR-sTF or Bm3R1-sTF have been implemented to control the expression of Venus (TetR-BSs + ENO1_Bm3R1cp) and BFP (Bm3R1-BSs + S33_TetRcp), respectively (Figure 3A,B). Thus, in the Reporter cassette, TetR-sTF activates Venus expression and simultaneously represses BFP expression, whereas Bm3R1-sTF functions in the opposite manner. In addition, the bistable switch system contains a “Diagnostic cassette” which was used to indirectly monitor the expression pattern and level of the sTFs in response to switch state changes. The Diagnostic cassette contained the same repressible-core promoter architecture as used in the Control cassette, but here the presence of the TetR-sTF activates the expression of KO2 (orange fluorescent protein) gene, whereas the Bm3R1-sTF drives the expression of mCherry (Figure 3A,B).

All four cassettes of the bistable switch system were integrated in a single copy to four separate loci in the S. cerevisiae genome, and the resulting strain was subjected to a series of tests to assess the functionality of the engineered genetic circuit. First, detailed transcription analysis was performed to characterize the dynamics of the transitions between the states (Figure 3C). The initial “synchronization” of the whole cell population into the TetR state was achieved by cultivating the yeast cells in the absence of thiamine. This triggered strong upregulation of the TetR-B42 resulting in activation of the TetR state (i.e., TetR-sTF feed-forward loop and the production of Venus and KO2 and simultaneous repression of the Bm3R1 state) (Figure 3C). When thiamine was provided again (cells transferred into the complete SCD medium), the TetR-B42 transcription was rapidly inactivated. The active TetR-sTF feed-forward loop, however, retained the circuit in the TetR state and provided stable expression of Venus and KO2 while simultaneously repressing the Bm3R1 state. The transition from the TetR into the Bm3R1 state was triggered by transferring the cells into a galactose-containing medium. This led to a rapid induction of the Bm3R1-sTF-Cln2-sh expression from the Switch cassette and inhibition of the TetR-state transcription activity (Figure 3C). There was a several hour delay in the Bm3R1-state activation that is likely due to the presence of residual TetR-sTF protein and its association with the binding sites at the S33_TetRcp that is driving the Bm3R1-sTF expression. Once this sTF was degraded or diluted through cell proliferation, Bm3R1-sTF-
Con2-sh initiated the Bmr3R feed-forward loop and the expression of BFP and mCherry. When galactose was removed (cells transferred into the SCD medium) and the Bmr3R-sTF-Con2-sh transcription was inactivated, the Bmr3R-sTF resumed the control over the circuit.

The functionality and robustness of the bistable switch was tested by extended continuous cultivations and serial transitions between the states. The cells were initially synchronized (switched) to either the TetR or Bmr3R state, and then repeated cycles of switching between the states were performed over 16 days (Figure 3D). After each switch, while the cultivation was continued in non-inducing conditions (SCD), the fluorescent protein production stabilized and remained stable until the next switch, as monitored by the fluorometry measurements on the population level (Figure 3D). The single-cell analysis performed by flow cytometry revealed that virtually all cells in the populations showed the predicted fluorescence pattern in each state (Figure S6A,B). The circuit performed remarkably well, in accordance with the design; importantly, it remained functional over a long time period.

The ability of the bistable switch system to maintain the programmed state for extended time is an essential feature for any application. Therefore, we investigated the stability of the circuit in a memory test. For this, the cells were first synchronized to either the TetR or Bmr3R state and then subjected to continuous growth in non-inducing SCD medium for 12 days (Figure 3E). In the case of TetR state, the system remained highly stable for the duration of the whole experiment. The Bmr3R state showed also a stable pattern, however, at the late stage of the experiment (after 10 days) a small increase of Venus and KO2 fluorescence levels, associated with the TetR state, were detected (Figure 3E). The fluorescence flow cytometry performed after 6 days of cultivations in non-inducing medium showed that virtually all the cells in both populations were in the correct state (Figure S6C,D). These results show that the developed gene expression tools provide a predictable and robust performance.

**Bistable Switch in Control of a Metabolic Pathway.**

The utility of the bistable switch system was tested in a metabolic pathway-engineering example. We employed the system as a transcription regulator of the heterologous violacein pathway in *S. cerevisiae*, allowing switching between two alternative branches of the pathway. The pathway consists of five enzymes—VioA, VioB, VioC, VioD, and VioE—which convert L-tryptophan into violacein via formation of different pigment molecules (Figure 4A). The first part of the pathway, consisting of VioA, VioB, and VioE enzymes, leads to protodeoxyviolaceinic acid (PDVA) formation. This intermediate can be non-enzymatically converted into a green-colored pigment, prodeoxyviolacein (PDV). In the next step, VioD enzyme converts PDVA into protoviolaceinic acid (PVA), followed by the VioC-catalyzed conversion to violaceinic acid, which is spontaneously transformed into the final product, the purple-colored violacin (V). PVA can also be converted non-enzymatically into another green pigment, proviolacin (PV). Expression of the entire pathway results predominantly in the formation of violacein. However, the VioC has also a side activity and can convert PDVA into deoxyviolaceinic acid, which is further spontaneously transformed into a red-colored pigment, deoxyviolacin (DV). Thus, by altering the expression of *vioC* and *vioD* genes in the presence of constitutively produced VioA, VioB, and VioE enzymes, it should be possible to generate a yeast strain capable of producing either red DV or green PV.

To test this, VioA, VioB, and VioE enzymes were constitutively expressed in the *S. cerevisiae* strain forming the VioABE background strain (Figure S7A and Supplementary sequences and strains file). Into this strain, the bistable switch system was implemented to control the *vioC* and *vioD* expression (Figure 4B). For this purpose, we used an alternative version of the bistable switch (Version B, Figure S4), where the core promoters driving the expression of target genes (here *vioC* and *vioD*) did not contain the repression binding sites. This design provides higher expression levels of the target genes. The characteristics of the version B switch are shown in detail in the Figure S4. No Diagnostic cassette was used in the violacein pathway application. The cells were initially switched into either TetR or Bmr3R state, and then transferred and cultivated in the non-inducing SCD medium (Figure 4C). In the TetR state, the expression of *vioD* gene was maintained by the circuit, and the pathway flux was directed toward the formation of PV (Figure 4C and Figure S7B). In the Bmr3R state, the production of red-colored DV was observed through activation of the *vioC* gene, and also a small amount of violacin (V) was detected (Figure 4C and Figure S7B).

Transcription analysis was performed on samples collected during the cultivations (Figure S7C). The analysis confirmed that the bistable circuit maintained the programmed states in the cells. Especially, the TetR state showed stable expression profiles of the genes over the course of the experiment. In the Bmr3R state, however, the transcript level of the *vioC* gene was decreased over time, while there was an increase in the *vioD* gene expression. This corresponds to the appearance of small amounts of violacin (V) in the culture. In conclusion, the results describe a broad toolbox for precise gene expression control in *S. cerevisiae*.

### Discussion

Engineering of increasingly complex synthetic gene circuits requires robust and predictably performing gene expression tools. In this study, we set out to establish such tools and to demonstrate their utility in the implementation of synthetic genetic networks in yeast. The developed toolbox contains a set of orthogonal DNA-binding proteins that, depending on their design, acted either as activators (sTFs) or as repressors (sReps). In addition, we engineered and characterized a library of core promoters, which can be used together with the sTFs and sReps to create functional genetic devices with predictable performance. Finally, we took advantage of the characterized toolbox and developed a novel type of bistable switch as an example for the assembly of a complex genetic circuit using these tools.

The sTFs tested in this study were expressed under the control of the TDH3cp whose basal transcription activity provides sufficient expression of the sTF. The absence of upstream regulatory sequences is a key advantage in using only the core promoters to provide constitutive expression of the sTF. In addition, it is important to maintain the sTF expression level low as the sTF overexpression can lead to toxic side effects. Moreover, some core promoters are functional across a wide spectrum of species. This allows design of broadly applicable expression systems for different hosts. For instance, S33cp, 008cp, and 114cp from *Aspergillus niger*, or Tr123cp from *Trichoderma reesi*, used here for the expression of an sTF (Figure S2) or in the sTF-dependent promoters (Figure 2),
have been successfully employed in an expression system for a broad spectrum of fungal species (Rantasalo et al., manuscript in preparation).

Not all of the tested bacterial repressors were suitable for use as the DNA binding (and dimerization) parts of sTFs. Only 5 out of 11 candidates were able to trigger reporter gene expression without any visible negative impact on the fitness of the modified hosts (Figure S1). Two sTFs were functional in terms of reporter gene activation, but their use resulted in undesirable phenotypes: expression of the TarA-based sTF led to a mild growth retardation (Figure S1B), whereas the GalR-based sTF caused a severe growth defect associated with a high genetic instability of the sTF expression cassette (data not shown). This suggests off-target activation of some endogenous yeast genes by these sTFs with harmful consequences. The BlrR-based expression system was disqualified due to an intrinsic activation of the reporter cassette even in the absence of the sTF, possibly by an endogenous transcription factor (data not shown). The LacI, Orf2, and IscR did not constitute a functional expression system, as no reporter fluorescence was detected in the modified hosts (data not shown). However, among the selected functional sTFs, high, but not equal, expression level of the reporter gene was observed. The Bm3R1- and TetR-sTFs conferred the strongest capacity to activate transcription (Figure 1C), which could indicate a high affinity of these synthetic TFs to their binding sites in the target promoters or higher stability of the sTF proteins in S. cerevisiae.

One of the most important features of the engineered gene expression systems is the possibility to fine-tune expression levels of target genes. An obvious approach, also applied in other synthetic expression systems, is modulating the number of sTF binding sites in the sTF-dependent promoters. We tested a range from one to eight binding sites in the upstream sequence of the sTF-dependent promoters (Figure 1C) and found that expression tuning was possible only for the weaker activators (LexA-, SrpR-, and PhIF-sTFs), while the strong activators (TetR- and Bm3R1-sTFs) had a narrow range of expression level close to the maximum output. This indicates that relatively high basal transcription activity of the TDH3cp, in combination with high affinity of the strong sTFs, could be responsible for full activation of the target gene transcription. We attempted to reduce the expression of the strongest sTF (Bm3R1-sTF) by using weaker core promoters for its expression to decrease its concentration and to enhance regulation based on the binding site-number (Figure S2). This resulted in a broader range of expression outputs likely due to lower occupancy of the binding sites in the promoter and consequently a more pronounced effect of the number of binding sites in transcriptional regulation (especially in case of the very weak 008cp).

Other important parameters of an expression system are the maximal achievable level of transcription and a stable expression pattern in diverse conditions. The level of transcription provided by the expression system developed here exceeds the transcription level of the strongest native genes in S. cerevisiae (e.g., TDH3, Figure 1D). In addition, it seems that the level of reporter expression in the strongest versions of the system, especially the Bm3R1-sTF-based system, conferred a maximal achievable transcription output of the fully activated core promoter used in the sTF-dependent promoter (ENO1cp). It is known that the core promoter, as part of a full promoter, has a strong impact on the overall expression level. Therefore, it is likely that the expression output of the Bm3R1 system could still be increased if a stronger core promoter is used in the sTF-dependent target gene promoter. The strong native promoters (such as TDH3p, TEF1p, PGK1p, etc.) are traditionally used for heterologous gene expression in S. cerevisiae, and they are usually referred to as constitutive promoters with stable expression outputs. In fact, the activity of these promoters is highly dependent on the conditions, conferring the highest activity in the presence of glucose (fermentative growth) and being strongly down-regulated during respiratory and stationary growth phases. This could be a disadvantage in many applications where robust and stable expression is desirable. The expression system developed here is designed to be constitutive, as it does not contain any regulatory sequences (such as native upstream elements in the promoters), which are often subject to conditional control. This makes these novel tools, especially the Bm3R1-based version, an attractive candidate for applications in which high and stable expression levels are needed.

However, the highest possible expression of a target gene is not always the aim in biological engineering tasks. In addition to precise regulation of transcriptional activation, controlled down-regulation of expression can also be an essential feature for the development of genetic circuits. Especially in metabolic engineering, redirecting metabolic fluxes through temporal and/or targeted repression of selected native or heterologous genes could improve availability of crucial substrates or prevent excessive accumulation of intermediates compromising the productivity or fitness of the production organism. Complex genetic circuits typically rely on fast responses and exact expression levels of their parts, which is hard to achieve by only transcriptional activation. We therefore developed a synthetic repression system with a well-characterized set of synthetic repressors (sReps) and corresponding core promoters (Figure 2A). The system was based on engineered core promoters containing binding sites for the sRep in their structure, where the sRep binding aims to block the recruitment of RNA polymerase or general transcription factors to the core promoter. We showed that the level of repression was directly dependent on the sRep concentration (Figure 2). Already very low levels of sRep led to mild repression in the case of Bm3R1-sRep and TetR-sRep. This implies high affinity for these proteins to their binding sites that are present in the engineered core promoters. To achieve complete repression, however, very high levels of the sReps needed to be produced. This could be problematic in applications where tight repression is needed, but an advantage in applications where only partial downregulation of transcription would be beneficial, such as in decreasing flux through an essential metabolic pathway. A tighter repression functionality could be implemented to the current sReps by the addition of a specific repression domain. An important outcome of the repression system is the set of modified core promoters, which confer a broad range of activities (Figure S3B). This information can now be utilized in the construction of sTF-dependent expression systems with a high range of achievable expression levels, and it highlights the importance of the core promoter (together with variable numbers of the sTF-binding sites) for the overall performance of the expression system.

The predictable behavior of expression tools is an essential attribute for efficient strain engineering. However, due to the complex nature of biological systems, even the tools generally considered as “orthogonal” can sometimes behave unexpect-
edly. To demonstrate the predictability of the tools developed here, we established two versions of bistable switch circuits (version A, Figure 3, and version B, Figure S4), where the proper functionality strongly depends on the exact performance of each of its components.

By design, bistable systems have two distinct and stable states, and the transition between the states occurs as a response to temporal input signals.\(^{\text{19}}\) A few artificial bistable switch-like circuits have been previously developed for various hosts, such as for \(E.\ coli\),\(^{\text{12,20}}\) yeast,\(^{\text{11,20}}\) and mammals.\(^{\text{17,51}}\) However, as opposed to previously developed systems in yeast,\(^{\text{49,52}}\) our circuit includes a robust memory function, which was demonstrated to retain its programmed status for at least 12 days of continuous cultivation. The two circuit versions differed only in types of the core promoters used in the Reporter cassette; this however had significant consequences on their functions. While the use of repressible core promoters in version A resulted in faster responses upon switching to the opposite state, in version B the use of unmodified core promoters led to much higher expression levels of the target genes (BFP and Venus). Also other interesting phenomena were associated with the functionality of individual parts. In both versions, it seemed that the transition from the TetR into the Bm3R1 state was slower than the reverse transition. This was particularly visible on the lower level of the Bm3R1 state—specific fluorescence at the beginning of cultivation in the SCD medium after each transfer from galactose, and a longer time needed for stabilization of the Bm3R1-state-specific fluorescence outputs (Figure 3D, Figure S4D). This might be caused by higher affinity and/or longer half-life of the TetR-sTF, which could result in the observed slower transition between the states. In addition, analysis of reporter protein fluorescence confirmed differences between system versions observed at the transcription level. In version B, there was clearly a visible delay in the disappearance of the fluorescent proteins encoded by the Reporter cassette after each switch to the opposite state (Figure S4D). In general, the generated bistable switch systems showed very robust functionality and prompted us to apply this tool in a metabolic engineering task.

The violacein biosynthetic pathway was selected as an example metabolic pathway to demonstrate the utility of the bistable circuit (Figure 4). The bistable switch was used to control switch carbon flux between two branches of the pathway leading to two different pigments. The functional expression of the violacein pathway, namely the upper part consisting of VioA, VioB, and VioC enzymes, in the tested yeast strain resulted in a significant growth defect. This was likely to be associated with a too efficient consumption of L-tryptophan by the pathway, as the addition of excessive amount of L-tryptophan to the culture media partially rescued growth (data not shown). Regardless of this challenge, the bistable switch proved to provide mutually exclusive control of production of the end-product pigments, either proviolacein (PV) or deoxyviolacein (DV), depending on the state of the circuit (Figure 4C and Figure S7B). In the Bm3R1 state, however, also some violacin (V) appeared to be co-produced together with DV. This was most likely caused by the low expression level of the viod gene forming the whole violacin production pathway in the Bm3R1 state (Figure S7C). The architecture of the violacein cassette (Figure 4B), where the viod gene is downstream of the vioC gene, may have caused a weak long-distance activation of the ENO1cp of the viod gene by the Bm3R1-sTF bound in the promoter of the vioC gene. This phenomenon could be eliminated if repressible CPs, such as those in version A of the bistable switch, would be used.

In conclusion, we demonstrated here that these well-characterized and robustly performing DNA parts can be highly useful tools in the development of sophisticated genetic circuits, which can find multiple applications in biotechnology.

### MATERIALS AND METHODS

**Strains.** \(S.\ cerevisiae\) strains which were used as parental strains in this study were CEN.PK102-5B (H3900; ura3-52, his3-delta1, leu2-3,112, TRP1, MAL2-8c, SUC2), CEN.PK111-32D (H3891; URA3, HIS3, leu2-3,112, TRP1, MAL2-8c, SUC2), CEN.PK113-5D (H3895; ura3-52, HIS3, LEU2, TRP1, MAL2-8c, SUC2), CEN.PK113-7A (H3888; URA3, his3-delta1, LEU2, TRP1, MAL2-8c, SUC2), and CEN.PK102-3A (H3899; ura3-52, HIS3, leu2-3,112, TRP1, MAL2-8c, SUC2), which were kindly provided by Dr. P. Köter (Institute of Microbiology, J.W. Goethe Universität). H-number indicates strain number in VTT’s internal collection. List of constructed \(S.\ cerevisiae\) strains are shown in the Supplementary sequences and strains file.

**Media.** Media used in the experiments and the strain construction were yeast extract peptone with \(\delta\)-glucose (YPD, 20 g/L bacto peptone (Becton Dickinson), 10 g/L yeast extract, and 20 g/L \(\delta\)-glucose) and synthetic complete medium (SCD, 6.7 g/L yeast nitrogen base (Becton Dickinson), 20 g/L \(\delta\)-glucose, and 790 mg/L complete supplement mixture (Formedium)) with different dropout variations: SCD without uracil and leucine (SCD-UL), SCD without histidine and leucine (SCD-HL), and SCD without histidine, uracil, and leucine (SCD-HUL). For the CRISPR/Cas9-mediated gene integrations into LYP1 locus, lysine-deficient SCD medium supplemented with 100 mg/L thialysine was used. Medium used in the repression experiments was SCD with varying thiamine concentrations (0, 100, 200, 400, or 800 \(\mu\)g/L). The yeast nitrogen base without thiamine and amino acids (Formedium) was used and supplemented with thiamine (Merck) to the desired concentration. The media used in the bistable switch experiments were SCD supplemented with additional glucose and thiamine (SCD-extra; SCD with 40 g/L \(\delta\)-glucose and 1000 \(\mu\)g/L thiamine), SCD without thiamine (SCD-wo-thi; 20 g/L \(\delta\)-glucose and 0 \(\mu\)g/L thiamine), and SC with galactose (SCgal; SC with 20 g/L \(\delta\)-galactose and 400 \(\mu\)g/L thiamine). Media used in the experiments with bistable switch with violacein pathway were supplemented with an additional 1000 mg/L tryptophan. Media used in the induction analysis of \(THI4p\) and \(GAL1p\) were SCD-extra-wo-thi (SCD with 40 g/L \(\delta\)-glucose and 0 \(\mu\)g/L thiamine) and SCgal-extra (SC with 40 g/L galactose and 1000 \(\mu\)g/L thiamine). Corresponding agar plate media were prepared by supplementing liquid medium with 20 g/L of agar.

**Cloning.** All the plasmids used in this study were cloned according to the manufacturer’s protocol using Gibson assembly (New England Biolabs) or restriction enzyme-based techniques (Thermo Fisher Scientific). Kapa HiFi enzyme (Kapa Biosystems) was used for all PCR reactions. Ligation and Gibson assembly mixes were transformed into \(E.\ coli\) TOP10 by electroporation, and the correctness of plasmids was confirmed by analytical digestion and sequencing. Synthetic DNA fragments and primers were obtained from Integrated DNA Technologies.

**Transformations.** \(S.\ cerevisiae\) transformations were done using the standard lithium acetate protocol.\(^{\text{50}}\) Either the
expression cassettes were released using NotI restriction enzyme from plasmids, or the plasmids were linearized using NotI restriction enzyme prior to the transformation. The cassettes were integrated into yeast genome into the following loci: LEU2 (Venus cassettes, and Reporter or violacein cassettes of bistable circuits), URA3 (sTF and sRep cassettes, and Control cassettes of bistable circuits), GRE3 (Switch cassettes of bistable circuits), HIS3 (LexA-sTF cassette for repressor strains, and Diagnostic cassettes of bistable circuits), or LYP1 (VioABE cassette) (integration flanks used in the expression cassettes are listed in Table 1). Expression cassettes containing violacein pathways genes VioA, VioB, and VioE were PCR amplified from the plasmid using the primers oCC-121 and oCC-122 containing 5′ and 3′ flanking sequences for the integration into LYP1 locus. The genome integration was facilitated using CRISPR/Cas9 by co-transforming the plasmid pCC-036 containing Cas9 under TDH3p and guiding RNA (gRNA) for LYP1 expressed under SNR52p. The gRNA protospeaker sequence was AGACCAGATAGAACATGAGA. The transformed cells were selected on plates containing thiamine.

**Copy Number Analysis.** Single colonies were isolated from transformation plates and regrown on the corresponding selection plates. Phenol—chloroform method was conducted to extract genomic DNA from yeast cells. First, 600 μL of glass beads, 600 μL of 1xTE (pH 7.5), and 600 μL of phenol—chloroform—isoamyl alcohol solution (50% phenol, 48% chloroform, 2% isoamyl alcohol) were mixed, and the extraction was done using Precellys24 homogenizer (Bertin Instruments). The aqueous layer was diluted 100x into water to obtain template for the copy number analysis. Copy number analysis was conducted with quantitative RT-PCR Lightcycler 480II (Roche) using Lightcycler 480 SYBR Green I Master (Roche) according to the manufacturer’s instructions. The signal from target gene was compared to signal from the control. The primers which were used in transcription analysis are shown in the Supplementary sequences, and strains file. Target gene expression levels were normalized using expression levels of ubiquitin-protein ligase encoding gene UBC6, which has been reported to confer exceptional transcription stability in various conditions making it suitable for such use.

**Fluorescence Measurement with Fluorimeter.** Cultivation samples were collected by centrifugation and resuspended with 200 μL of water. A 200 μL portion of the suspension was transferred to Black Clniplate (Thermo Fisher Scientific), and fluorescence was measured with Varioskan (Thermo Electron Corporation). The excitation/emission wavelengths for measurement of BFP (blue fluorescent protein) were 399/456 nm, for Venus (yellow fluorescent protein) 510/530 nm, for mCherry (red fluorescent protein) 587/610 nm, and for KO2 (orange fluorescent protein) 550/570 nm, respectively. A 5 nm bandwidth and 500 ms measurement time were used in each measurement. Cell density (OD_{600}) measurement was done for normalizing fluorescence measurement results. After fluorescence measurement, cells were diluted 100x into water, and then OD_{600} was measured using Varioskan (photometric measurement mode, wavelength = 600 nm, bandwidth = 5 nm, measurement time = 100 ms) using transparent microtitre plate (Nunc 96F, Thermo Fisher Scientific). The arbitrary units (AU) reported in figures were obtained by dividing the fluorescence measurement value by the OD_{600} value.

**Transcription Analysis and RT-PCR.** Cell cultivation samples were pelleted by centrifugation and washed with cold water. Cell pellets were frozen with liquid nitrogen and samples were stored at −80 °C.

Total RNA was extracted using RNeasy Mini Kit (Qiagen; protocol for yeast). DNase treatment (DNase I RNase-free, Thermo Fisher Scientific) was performed to remove residual genomic DNA from the samples. Transcription First Strand cDNA Synthesis Kit (Roche) was used for the cDNA synthesis according to manufacturer’s protocol. Diluted cDNA samples were mixed with primers, and LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer’s protocol. The RT-PCR was conducted using Lightcycler 480II (Advanced Relative Quantification Tool; Roche). The primers which were used in transcription analysis are shown in the Supplementary sequences, and strains file. Target gene expression levels were normalized using expression levels of ubiquitin-protein ligase encoding gene UBC6, which has been reported to confer exceptional transcription stability in various conditions making it suitable for such use.

**Fluorescence Flow Cytometry.** Cells to be analyzed were diluted into 1x PBS prior to the analysis. Venus, and BFP fluorescence was measured from 10 000 S. cerevisiae cells using FACSAria III (BD) flow cytometry. BFP was measured using the 375 nm laser and 450/20 nm filter, whereas Venus was measured with 488 nm laser and 530/30 nm filter. No gating was performed on the data.

**Violacein Pathway Metabolite Analysis.** For the analysis of the intracellular violacein pathway metabolites, yeast cells were collected from 4 mL of a liquid cultivation by centrifugation. The cell pellets were disrupted by adding 500 μL of preheated methanol and incubating at 95 °C for 15 min. Samples were centrifuged and the supernatants were analyzed. Violacein, proviolacein, deoxyviolacein, and prodeoxyviolacein were qualified using the ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Waters) with

| integration locus (protein ID in JGI<sup>49</sup>) | selection marker (selection condition) | S′ integration flank | S′ integration flank |
|---------------------------------------------|-------------------------------------|---------------------|---------------------|
| LEU2 (626)                                  | K. lactis LEU2 (absence of leucine) | 50 bp (−80 to −31 bp upstream of start codon) | 50 bp (+1 to +50 bp downstream of stop codon) |
| URA3 (1899)                                 | K. lactis URA3 (absence of uracil)  | 50 bp (−80 to −1 bp upstream of start codon)  | 50 bp (+1 to +50 bp downstream of stop codon) |
| GRE3 (3067)                                 | KanMX (200 μg/mL G418)              | 48 bp (−109 bp to −62 bp upstream of start codon) | 50 bp (+1 to +50 bp downstream of stop codon) |
| HIS3 (5860)                                 | S. pombe HIS3 (w/o histidine)       | ~1000 bp            | 85 bp               |
| LYP1 (5106)                                 | no selection marker (selection in gRNA plasmid) | 60 bp (−60 bp to −1 bp upstream of start codon) | 60 bp (+1 bp to +60 bp downstream of start codon) |

*Joint Genome Institute’s database, http://genome.jgi.doe.gov/programs/fungi/index.jsf.*
ACQUITY Van Guard pre-column and ACQUITY UPLC HSS T3 2.1 × 100 mm, 1.8 μm analytical column. The columns were maintained at 40 °C; 0.1% formic acid in acetonitrile (eluent A) and in water (eluent B) were used as eluents with the flow rate of 0.5 mL min⁻¹ as follows: 0–1.5 min, 95% A, 5% B; 1.5–7.0 min, decrease 16.9% min⁻¹ A, increase 16.9% min⁻¹ B; 7.0–9.0 min, 2% A, 98% B; 9.0–9.1 min, increase 15.5% s⁻¹ A, decrease 15.5% s⁻¹ B; 9.1–11.5 min, 95% A, 5% B. Injection volume of 2 μm was used for the analytes. Absorbance was measured with a UV/vis detector at 600 nm.

Analysis of Orthogonal Activators. The functionality of each sTF and orthogonality in S. cerevisiae was tested by fluorescence measurements. The experiments were initiated by pre-cultivating S. cerevisiae cells (strains marked with asterisk in the column “Analysis of orthogonal activators” in the Supplementary sequences and strains file) at 30 °C on YPD plates for 24 h. Four ml of SCD-UL medium in 24-well plate was inoculated to initial optical density of 0.2 (OD₆₀₀) by the pre-culture. Three parallel replicates were cultivated for each strain. Cells were cultivated 18 h at 28 °C, 800 rpm. Fluorescence was measured as described in the “Fluorescence Measurement with Fluorimeter” section.

In addition to the fluorescence measurements, transcription analysis was performed for the subset of the strains. Strains containing sTF and Venus expression cassettes either with 2 or 8 sTF binding sites (strains H4603, H4605, H4608, H4610, H4613, H4615, H4618, H4620, H4623, H4625, H4628, H4630, H4878, H4880, H4883, and H4885) were pre-cultivated on SCD-UL plates for 24 h (30 °C). Strain H3888 was used as a control strain (P). Cells were grown in SCD-UL in identical way as described above in the fluorescence analysis, and samples for transcription analysis were collected after 18 h of cultivation. Samples were processed as described in the “Transcription Analysis and RT-PCR” section.

Growth Curves. The growth of strains containing an sTF expression cassette and 8BS version of the Venus expression cassette (strains H4605, H4610, H4615, H4620, H4625, and H4630) were analyzed. H3888 was used as a control strain (WT). Cells were pre-cultivated 24 h on SCD-UL plates at +30 °C. Aliquots of 4 mL of SCD-UL in a 24-well plate were inoculated to optical density OD₆₀₀ of 0.2, and cells were cultivated 24 h (+30 °C, 800 rpm). Optical density was measured after 24 h of cultivation, and this culture was used to inoculate SCD-UL medium to optical density of 0.1. Inoculated SCD-UL medium was transferred to a Bioscreen microtiter plate (250 μL per well), and the growth was followed with Bioscreen C MBR automated turbidometric analyzer (Growth Curves Ltd.) at 30 °C. OD₆₀₀ measurement was taken every 30 min. Five biological replicates were cultivated for each strain.

Analysis of Orthogonal Repressors. The functionality of repressors and engineered core promoters (with repressor binding sites) was tested by fluorescence analysis. The analysis was initiated by pre-cultivating S. cerevisiae cells (strains marked with asterisk in the column “Analysis of orthogonal repressors” in the Supplementary sequences and strains file) on YPD plate at 30 °C for 24 h before starting the experiment. Four ml of SCD medium with 0, 100, 200, 400, and 800 μg/L thiamine was inoculated in 24-well plate to initial optical density of 0.2 (OD₆₀₀). Strains lacking repressor cassette (but containing the integrated cassette with URA3 selection marker) were cultivated only in standard SCD containing 400 μg/L thiamine (standard YNB (Becton Dickinson) used for the SCD preparation). Three parallel replicates were cultivated for each strain at 28 °C (800 rpm) for 18 h, and the fluorescence was measured as described in the section “Fluorescence Measurement with Fluorimeter”. In addition, the activity of THI4p in varying thiamine concentrations was tested using the same experimental setup (strain HS127).

Transcription analysis was performed on the yeast strain containing ENO1_Bm3R1p (strain H4888). Cells were pre-cultivated on YPD plate for 24 h at 30 °C, and 4 mL of SCD containing 0, 100, 200, 400, and 800 μg/L thiamine in 24-well plate was inoculated to initial optical density (OD₆₀₀) of 0.2. Two parallel replicates were cultivated at 28 °C (800 rpm) for 18 h, and the samples for the transcription analysis were prepared as described in the section “Transcription Analysis and RT-PCR”.

Analysis of the THI4 and GAL1 Promoter Induction. Strain containing bidirectional cassette THI4p-Venus and GAL1p-BFP was cultivated overnight in 65 mL of SCD-extra (30 °C, 250 rpm). Cultivation was split into two tubes (32.5 mL per tube) and pelleted by centrifugation. Supernatant was removed, and pellets were suspended into 300 mL of SCD-extra-wo-thi (induction of THI4p) or SCgal-extra (induction of GAL1p) medium. Cells were cultivated at 30 °C (250 rpm), and 50 mL samples (one biological replicate) were collected at 2, 4, 6, 8 and ~18 h of the cultivation. Venus and BFP fluorescence was measured from the samples as described above in the section “Fluorescence Measurement with Fluorimeter”.

Analysis of the Bistable Switch. The continuous-growth tests for the serial switches and the “memory tests” were done by sequential cultivations of the cells in liquid media at 28 °C (800 rpm). The cultivation volume was in each case 4 mL (four biological replicates), and each cultivation was continued for 24 h. The follow-up 4 mL cultivations were inoculated by 30 μL of the previous cell culture, and the length (in days) of each consecutive cultivation is indicated in the figures. The fluorescence measurement was performed after each 24 h of cultivation as described in the section “Fluorescence Measurement with Fluorimeter”.

The serial switch experiment was started by pre-cultivating strains HS206 (switch version A) and HS208 (switch version B) on SCD-UL plates for 24 h at 30 °C. Next, 4 mL of medium, either SCD-wo-thi (initiating the TetR state) or SCgal (initiating the Bm3R1 state), was inoculated to initial optical density (OD₆₀₀) of 0.2. After 24 h of cultivation in these inducing media (28 °C, 800 rpm), the cultivation was continued in SCD-extra for 48 h (culture refreshed after 24 h of cultivation). After cultivation in SCD-extra, cells in the TetR state were transferred into the SCgal medium, and the cells in the Bm3R1 state into the SCD-wo-thi medium for next 24 h. After that, cultivation was again continued for 48 h in SCD-extra (culture refreshed after 24 h of cultivation) prior to next cultivation in either SCD-wo-thi or SCgal. In total, the cultivation was continued for 16 days. Fluorescence flow cytometry was also performed for the samples collected at the end of days 3 and 6, as described in the section “Fluorescence Flow Cytometry”.

The “memory test” was done by switching cells first to either TetR or Bm3R1 state. After that, the cultivation was continued in SCD-extra, and the programmed state of the cells was followed by fluorescence measurements. The experiment was started by cultivating strains HS206 (switch version A) and HS208 (switch version B) in 4 mL of either SCD-wo-thi (TetR state) or SCgal (Bm3R1 state) for 24 h (28 °C, 800 rpm). The
subsequent cell cultivations in SCD-extra were inoculated using 30 μL of cell suspension from each previous cultivation, and the cultivation was continued for the next 24 h (28 °C, 800 rpm). The inoculation of fresh SCD-extra was repeated always after 24 h. The experiment was continued for 12 days in total. Fluorescence measurement was done every day after 24 h of cultivation as described in the section “Fluorescence Measurement with Fluorimeter”. In addition, fluorescence flow cytometry was performed on samples collected at the end of day 6.

In addition to fluorescence measurements, transcription analysis was performed on the bistable-switch strains. Strains H5206 (switch version A) and H5208 (switch version B) were cultivated in 50 mL of SCD-extra-wo-thi for 24 h at 30 °C (250 rpm). This culture was used for inoculation of 150 mL of the SCD-extra medium to initial optical density of 0.2 (OD600). Cells were cultivated at 30 °C (250 rpm), and samples were collected for transcription analysis at time points 0, 2, 6, and 24 h to monitor dynamics of the transcription pattern stabilization after transfer from inducing conditions to non-inducing condition. After 24 h of cultivation in the SCD-extra medium, the cells were transferred to SCGal-extra medium (150 mL, initial OD600 of 0.2). Samples for transcription analysis were collected at 3, 8, and 24 h to monitor dynamics of the transcription responses during switch of the system into the opposite state. Finally, the cells were transferred from the SCGal-extra medium into 150 mL of the SCD-extra medium (initial OD600 of 0.2), and samples were collected at 2 and 6 h to monitor stabilization in the newly gain state. RNA extraction, cDNA synthesis, and RT-PCR were done as described in the section “Transcription Analysis and RT-PCR”.

**Analysis of the Violacein Pathway Control by the Bistable Switch.** The experiment was done in a similar way as the bistable switch “memory test”. Strains HS285 (switch-controlled VioC and VioD) and HS285 (VioABE background strain) were first pre-cultivated on the SCD-extra plates for 24 h at 30 °C. The liquid cultivation was started by inoculating 4 mL of either SCD-extra-wo-thi medium (switch to the TetR state) or SCGal medium (switch to the Bm3R1 state) to OD600 of 0.8 by the pre-cultures. The inducing cultures were carried out for 24 h at 28 °C (800 rpm), after which the cultivations were continued in non-inducing conditions (SCD-extra) for 3 days. Cells were transferred into fresh medium every 24 h using 100 μL of previous culture. All the media (liquid and plates) used in this experiment were supplemented with additional 1000 mg/L tryptophan.

Samples for transcription analysis (two biological replicates) and for liquid chromatography were collected after every 24 h. Samples were pelleted by centrifugation and washed with cold water. Cell pellets were frozen in liquid nitrogen and stored at −80 °C. Transcription analysis and liquid chromatography were conducted as described in the sections “Transcription Analysis and RT-PCR” and “Violacein Pathway Metabolite Analysis”, respectively.

**Statistics.** The number of used replicates in each experiment is shown in figure legends as well as in the methods discussion. The results represent averages of individual data values. Error bars given in graphs represent ± one standard deviation.

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