MINIREVIEW

The synthetic biology toolbox for tuning gene expression in yeast

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ABSTRACT

Saccharomyces cerevisiae can serve as a key production platform for biofuels, nutraceuticals, industrial compounds, and therapeutic proteins. Over the recent years, synthetic biology tools and libraries have expanded in yeast to provide newfound control over regulation and synthetic circuits. This review provides an update on the status of the synthetic biology toolbox in yeast for use as a cell factory. Specifically, we discuss the impact of plasmid selection and composition, promoter, terminator, transcription factor, and aptamer selection. In doing so, we highlight documented interactions between these components, current states of development, and applications that demonstrate the utility of these parts with a particular focus on synthetic gene expression control.

Key words: synthetic biology; yeast; terminators; promoters; aptamers

INTRODUCTION

Saccharomyces cerevisiae can serve as a key production platform for biofuels, nutraceuticals, industrial compounds, and therapeutic proteins (Nevoigt, 2008; Curran and Alper, 2012). This organism naturally has several favorable production traits, including a large cell size, which allows for ease of separation, higher tolerance to byproducts and acids, relatively low temperature requirements (when compared to Escherichia coli), and a lack of susceptibility from phages (Liu et al., 2013). More recently, a broad and expanding array of information and genetic tools have become available that strengthen our capacity to study and engineer yeast. Many of these tools are a direct result of the advances in the field of synthetic biology.

As a relatively new field, synthetic biology sits at the intersection of engineering principles and biological design. While the exact definition of synthetic biology is continuously expanding (Andrianantoandro et al., 2006; Purnick and Weiss, 2009), the overarching goal has remained the same: gaining a better understanding of how to coordinate and regulate pathways and gene expression in organisms. In particular, the engineering of synthetic systems involves practical and rationale design of disparate synthetic parts that all must work together to obtain the desired function. Expression systems have distinct elements (such the promoter, transcription factor binding sites (TFBS), terminators, and vector plasmids), which can be synthetically combined to achieve the desired regulation. Each of these components (among others) contributes to the overall synthetic biology toolbox in yeast. However, this toolbox of parts is much smaller in comparison with that of Escherichia coli, and its regulation tends to be more complex (Blount et al., 2012). Nevertheless, great strides have been made in the past few years to expand synthetic biology in yeast.

To this end, this review will highlight the synthetic parts and approaches available for regulating gene expression systems in S. cerevisiae and will describe the areas that need to be further explored. We will first consider the plasmids and basic multigene expression systems available for introducing synthetic functions. Next, we will discuss the expansive synthetic part libraries for controlling and optimizing gene expression, including native promoters, synthetic promoters, and terminators. Finally, this
Figure 1. Engineering tools used in metabolic engineering of S. cerevisiae. Plasmid copy number (1) determines the overall copies of plasmid present in the cell. Synthetic transcription factors, including those guided by RNA (2) and others activated by exogenous small molecules (3), can be employed to activate a promoter (4) by binding to transcription factor binding sites (TFBS) in the upstream activating sequence (4a), thereby assisting transcription initiation at the TATA box in the core (4b). Restriction sites (RS) (5) and sequences upstream of ATG but downstream of the transcription start site (TSS) determine the 5′ UTR (6), which can affect translation efficiency by the ribosome. Riboswitches (7), specifically designed 5′ UTR, can be used to stop translation by binding to a small molecule. The half-life of an mRNA is determined by the sequence of the terminator (8) due to its contribution to the 3′ UTR and, thus, overall stability (9).

PLASMID SELECTION

Plasmid selection has three attributes that can affect the transcriptional and translational efficiency as well as global impact on growth. These attributes include (1) the origin of replication, (2) the selection marker, and (3) the multicloning sites. We briefly describe the importance of these elements in synthetic design below.

The origin of replication is the most commonly altered component and often dictates the plasmid copy numbers present in the cell (Fig. 1.1). In general, low-copy plasmids are more stable and robust to different fermentation processes than high-copy plasmids and can often handle larger heterologous expression cassettes (Li et al., 2013). However, high-copy plasmids allow for overexpression of the gene of interest. Plasmids can be categorized by their plasmid copy number into two families typically used in metabolic engineering: YCp, a low-copy plasmid, and YEp, a high-copy plasmid. YCp plasmids have a yeast origin of
replication (ARS) and a centromere sequence (CEN). These have been shown to maintain only 1–2 copies per cell (Clarke and Carbon, 1980). YEp plasmids, on the other hand, are based on the native 2 μ (2 micron) episomal plasmid found in S. cerevisiae. These plasmids can maintain over 10 copies per cell.

Aside from the origin of replication, selection markers present in the plasmid should be considered. Because yeasts do not respond significantly to antibiotics, auxotrophic yeasts are used for screening. Therefore, the use of auxotrophic markers, such as TRP1, HIS3, and LEU2, has allowed the positive selection of yeast cells which have correctly incorporated the respective plasmids. Additionally, other markers, such as URA3, convert harmless substances into compounds toxic to the cell, allowing for the negative selection of cells. In general, the wide selection of these auxotrophic markers allows for flexibility. However, studies have shown that the choice of auxotrophic markers can affect plasmid copy number and growth of their host cell. For example, a gene expression cassette could significantly affect the expression of a nearby selection marker; divergent arrangement of the two can lead to suppression of the marker and, as a result, hinder growth (Lee et al., 2014). It is also well known that selection pressures can alter plasmid copy number (Erhart and Hollenberg, 1983; Wittrup et al., 1994; Chen et al., 2012). Additionally, Karim et al. showed that the use of certain auxotrophic markers, such as LEU2, gave a decrease in cell growth compared to other auxotrophic markers tested. The choice of promoter in the expression cassette, as well as the inclusion of an antibiotic resistance marker such as KanMX, was also seen to affect plasmid copy number, indicating that the origin of replication is not the sole determinant of copy number (Karim et al., 2013). A review covering markers in yeast has been previously published (Da Silva and Srikrishnan, 2012).

Lastly, the multicloning sites (MCS) present in the plasmid (often for convenience of restriction-enzyme based cloning) can significantly affect the expression of a gene of interest (Fig. 1.5). As these MCS regions are interstitial between the promoter and gene of interest, they will impose distance between these two elements and also contribute to the 5′ untranslated region (UTR) of the resulting transcribed mRNA. Crook et al. (2011) demonstrated that this contribution can affect overall expression of the gene and that thermodynamic models can be used to minimize secondary structures in the 5′ UTR and expedite translation of the mRNA. It should be emphasized that other assembly methods are available that do not necessarily rely on constructs such as MCSs (these include techniques such as Gibson assembly and Golden Gate cloning). For more information on available assembly methods, see a recent review by Chao et al. (2014). However, regardless of which cloning assembly method is chosen, it is important to realize that there will always be a 5′ UTR, and such a sequence should be considered as it can impact expression levels. Thus, the exact structure and composition of all components in expression vectors (from origin to selection marker to placement of synthetic elements) should be considered holistically. Conveniently, a review with an extensive list of plasmids available has been written (Da Silva and Srikrishnan, 2012).

**Multigene expression cassettes**

In addition to a single-gene plasmid arrangement, characterized multigene plasmids are also available to consolidate expression of multigene pathways onto a single vector (Ishii et al., 2014). When incorporating a long biosynthetic pathway into yeast, a multigene plasmid is preferred over numerous single-gene plasmids as only one selection marker will be required. As with before, origins of replication, selection markers, and multicloning sites/5′ UTR regions will need to be considered for multigene plasmids. Beyond this, the specific DNA arrangement of the multiple genes needs to be considered. For example, promoter-terminator interactions have been observed in synthetic systems when two expression cassettes are placed in tandem without sufficient spacing, leading to transcriptional deficiencies (Greger and Proudfoot, 1998). This interaction indicates that synthetic parts do indeed interact and thus are influenced by genetic context and cannot be assessed only in isolation. Furthermore, optimal multigene pathway engineering requires the balancing of two or more enzymes to obtain maximum titer and avoid accumulated intermediate metabolites, especially toxic ones. To accomplish this task, typically distinct promoters are chosen both to optimize expression and to avoid the instability caused by homologous recombination of repeated elements. Interestingly, it has been demonstrated that high-copy plasmids can magnify the differences between promoter levels, leading to an imbalance of enzyme levels (Du et al., 2012; Li et al., 2013). Thus, low-copy plasmids may be more desirable than high-copy plasmids in some pathway engineering applications. As an example, by only using a low-copy plasmid to avoid these imbalances, xylitol titers in yeast were increased 1.7-fold (Li et al., 2013). Similarly, the combination of plasmid factors (including copy number and promoters) has been systematically optimized in E. coli to reduce an intermediate toxin and increase overall production of a taxol precursor (Ajikumar et al., 2010). It is likely that a similar approach can be used to optimize synthetic pathways and circuits in yeast.

**PROMOTERS**

**Overview**

Once the expression cassette and mode of delivery is chosen, the choice of promoter is usually the most sought after synthetic tool (Fig. 1.4). In S. cerevisiae, a wide variety of promoters are available for use in metabolic engineering and synthetic biology. In this review, we will discuss libraries of promoters that are either constitutive or inducible in nature. Constitutive promoters offer a constant level of expression with no need for induction or repression. In contrast, inducible promoters allow for considerable control as to when a gene is expressed. Such control is desirable when the gene poses a burden on the cell when expressed. However, before choosing to use an inducible promoter, several factors should be considered, such as the financial cost of the metabolite inducing expression, sensitivity of the promoter to the metabolite, time for induction, and background expression in the absence of the metabolite.

Initially, both constitutive and inducible promoters were isolated from endogenous sequences in the genome. Within the last decade or so, entirely synthetic promoters have been developed either by screening large mutagenic libraries or through rational design. We briefly cover both native and synthetic promoter libraries for yeast synthetic biology applications.

**Native promoters**

The most widely used promoters are those isolated from the genome. The vast majority of native, constitutive promoters have been found in the glycolytic pathway, yet their expression still depends on the presence of glucose (Da Silva and Srikrishnan, 2012). These promoters have a long history in synthetic biology and metabolic engineering and are still used
in many contemporary engineering efforts. For example, \( P_{TEF1} \) was used in the first ever production of glycolic acid in yeast (Koivistoinen et al., 2013), \( P_{PGK1} \) and \( P_{TUB3} \) were used in a novel co-fermentation to co-produce ethanol and xyitol (Zha et al., 2013), and a newly identified xylose isomerase was successfully expressed in yeast using \( P_{XIL1} \) (Hector et al., 2013). The strengths of these promoters, as well as \( P_{TEF1} \), \( P_{PYK1} \), and \( P_{GAP1} \), were characterized by Partow et al. (2010) and Sun et al. (2012). Most studies agree that the TEF1 promoter is among the strongest, TPI1 and PYK1 are a medium strength, and ADH1 is among the weakest (Partow et al., 2010; Sun et al., 2012). It should be emphasized that the overall native promoter library in yeast is vastly under the limitations described here and efforts have been made to characterize more native promoters. For example, recently, 859 endogenous promoters from a wide variety of cellular functions, processes, and compartments were characterized under ten growth conditions using a fluorescence reporter (Keren et al., 2013; Lubliner et al., 2013). Characterizations of common promoters have been tabulated in a recent review (Da Silva and Sririkrishnan, 2012).

Several inducible promoters have been used in yeast, with GAL1, GAL7, and GAL10 being the most common and tightly regulated native elements (Hawthorne, 1964; Bassel and Mortimer, 1971). In the presence of glucose, GAL promoters are completely off, and in the presence of galactose, a 1000-fold increase in expression can be achieved in just 4 h (Adams, 1972). Beyond primary carbon sources, the native copper-inducible promoters, \( P_{CUP1} \), can induce 20-fold expression in the presence of Cu\(^{2+} \) (Etcheverry, 1990). Aside from inducible promoters, repressible promoters have also been isolated from the genome. As examples, \( P_{ADE2} \) displays a 100-fold reduction in expression in the presence of glucose (Price et al., 1990) and \( P_{PHO5} \) has a 200-fold repression in the presence of inorganic phosphate. Collectively, there is a growing, but limited, library of native yeast promoters. However, as always with any synthetic, genetic context is critical. For example, it is possible to convert an inducible promoter into a constitutive promoter. In the context of a gal80 deletion, even the strong GAL promoters can be converted into constitutive promoters (although with increased plasmid instability (Özaydin et al., 2013)).

**Synthetic promoters**

Native promoters provide a starting point for synthetic biology in yeast. To increase the yeast promoter toolbox (both with respect to sequence and functional diversity), a collection of yeast synthetic promoters has been developed. Two strategies are currently being utilized to create synthetic promoters: screening and rational design. The latter allows for a more pronounced manipulation of sequence space, resulting in synthetic promoters with low sequence homology to native promoters, a desirable trait for synthetic biology. For additional information on synthetic promoters not presented below, an in-depth review on promoter engineering approaches has been published (Blazeck and Alper, 2013).

One method to create synthetic promoter libraries is to perform error-prone PCR mutagenesis on a native promoter to create sequence and hence functional diversity (Jensen and Hammer, 1998; Alper et al., 2005; Hammer et al., 2006; Nevoigt et al., 2006; Du et al., 2012). For yeast, this technique was performed on the constitutive native promoter \( P_{TEF1} \) to yield 11 mutant promoters with strengths ranging from 8% to 120% of the native \( P_{TEF1} \) (Alper et al., 2005; Nevoigt et al., 2006). It was also performed on the oxygen-regulated promoter \( P_{GAL1} \), where a mutant promoter was isolated with maximal expression level equal to that of fully induced \( P_{GAL1} \), the most widely used inducible yeast promoter. The isolated versions also had a quicker induction time compared to the wild-type starting sequence (Nevoigt et al., 2007). To specially design synthetic promoters to work in a specific system, customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER) was developed. In this method, a library of mutant promoters driving the expression of a gene in a multigene plasmid was screened for a desired phenotype. In just one single round of COMPACTER, a xylose-utilizing industrial strain was engineered with 69% of the xylose consumption rate of the fastest reported xylose-utilizing strain in *S. cerevisiae*. In another single round of COMPACTER, they were also able to construct an industrial strain with the highest consumption of cellobiose ever reported (Du et al., 2012). COMPACTER was further improved by utilizing HPLC as a screening method for small molecules that do not induce phenotypic changes (Lee et al., 2013).

While these examples (and several others) demonstrate the capacity to make synthetic promoter libraries via error-prone PCR, the resulting sequences have high homology to the native scaffold. An alternative approach to create synthetic promoters involves stitching transcription factor binding sites (TFBS) with spacer regions based on the architecture of a native promoter of interest (Fig. 1.4). In such libraries, random or partially defined oligonucleotides can be placed in the interspacing regions between the TFBS (Jeppsson et al., 2003; Ligr et al., 2006; Blount et al., 2012b). As an example, by stitching RPG and CT boxes using the ENO1 promoter as a guide, 37 promoters were identified that covered three orders of magnitude in expression (Jeppsson et al., 2003). Similarly, Blount et al. isolated 36 promoters with a 10-fold range in expression by carefully selecting a model constitutive promoter with limited natural regulation (Blount et al., 2012). Furthermore, in another promoter context, increasing TFBS from 1 to 8 yielded a fourfold expression range (Khalil et al., 2012). Synthetic promoters and libraries can also be created by rational design. As native promoters are generally nucleosme free (Fig. 1.4), utilizing homopolymeric stretches of deoxyadenosine nucleotides to disfavor nucleosome formation can alter promoter strength (Iyer and Struhl, 1995; Ravesh-Sadka et al., 2012; Sharon et al., 2012). It was discovered that longer tracts and those placed closer to Ccn4p TFBS in the HIS3 promoter could induce higher expression levels (Ravesh-Sadka et al., 2012).

One additional method for designing synthetic promoters and libraries is through a hybrid promoter engineering approach. This approach harnesses the modularity of promoter design by coupling disparate upstream activating sequences (UASs) and core promoters (Blazeck et al., 2012). Fundamentally, a UAS is characterized by nucleosome depletion and a high number of TFBS (Hahn and Young, 2011) (Fig. 1.4a), yet this element lacks the necessary sequences for transcription to occur. In contrast, a core promoter contains the necessary and sufficient components of transcription initiation, including a TATA box and a transcription start site (Fig. 1.4b). In *S. cerevisiae*, it is possible to use a hybrid promoter engineering approach to both amplify the expression of a core promoter and create libraries. For example, multiple UAS elements can be used in tandem to increase transcriptional strength. With this method, Blazeck et al. (2012) was able to obtain a constitutive promoter based on the \( P_{TUB1} \) that had the same expression level as induced \( P_{GAL1} \), and Liang et al. (2013) was able to create synthetic promoters of an eightfold range, which were employed to express an...
inducible five-gene zeaxanthin biosynthetic pathway into yeast. Furthermore, simple TFBS for Gal4 can be coupled with core promoters to yield a wide variety of galactose-inducible promoters ranging 50-fold in expression levels (Blazeck et al., 2012). A similar hybrid promoter engineering strategy can work in other yeast systems to generate strong promoters and libraries of expression (Blazeck et al., 2011, 2013). Finally, a recent study has demonstrated the potential to both redesign native promoters and design synthetic de novo promoters in S. cerevisiae through the tuning of nucleosome architecture (Curran et al., 2014). In this study, purely synthetic, de novo promoters were designed that achieved high expression levels. This work represents a new approach for DNA-level specification of synthetic parts.

As described previously, these promoters (and any MCSs) all contribute to the 5′UTR (Fig. 1.6) of the resulting mRNA and therefore have the potential to impact both mRNA half-life and translational efficiency. By utilizing recent global mapping of 5′UTR in S. cerevisiae (Nagalakshmi et al., 2008) and their respective mRNA decay rates (Munchel et al., 2011), the GAL1 5′UTR was swapped with one from GAL80 and SOL1, both previously shown to produce more stable transcripts (Munchel et al., 2011). Unfortunately, these substitutions resulted in deleterious protein production (McIsaac et al., 2014). Instead of simple swaps, modeling the 5′UTR effects on gene expression has proven to be a valuable tool in successful 5′UTR engineering. Crook et al. (2011) and Dvir et al. (2013) have both used models to manipulate protein levels (Crook et al., 2011; Dvir et al., 2013). Beyond models, Lamping et al. (2013) have placed 17 small GC-rich mRNA stem loops in the 5′UTR to downregulate gene expression, which resulted in a reduction range of 99% to 50%. Thus, it is possible that these regions will be further studied in creating functional promoter libraries for yeast.

**TERMINATORS**

**Overview**

The terminator region is also a critical component of all expression cassettes and is important for both mRNA half-life and stopping transcription (Fig. 1.8). However, in almost all yeast synthetic biology and metabolic engineering applications, only a handful of common terminators are used in an expression cassette. Unlike promoters, very few studies have examined the influence of terminators on net protein expression. However, terminators have since been shown to affect the stability and abundance of their respective mRNA (Abe and Aiba, 1996; Yamanishi et al., 2013), suggesting that these components should be equally considered as part of the synthetic biology toolbox.

**Characterizing terminators**

Most yeast synthetic biology and metabolic engineering applications utilize a handful of native terminators with well-studied 3′end sequences efficient at stopping transcription. As a result, nearly all expression and shuttle vectors utilize terminators from only a couple of genes, such as the phosphoglycerate kinase (PGK1) (Mellor et al., 1983; Vreken and Raué, 1992), alcohol dehydrogenase (ADH1) (Vernet et al., 1987), and the cytochrome c (CYC1) (Zaret and Sherman, 1982; Mumberg et al., 1995) genes. With respect to synthetic biology applications, the choice of these terminators in the toolbox was relatively arbitrary as little was known about the mechanism of termination. However, since then, the polyadenylation and cleavage of mRNA transcripts from RNA Polymerase (RNAP) have begun to be understood (Mischo and Proudfoot, 2013), and major sequence elements have been identified. Moreover, a minimal terminator sequence has been synthesized (Guo and Sherman, 1996a,b), which can form the basis for new synthetic terminators. However, the rationale for including synthetic terminator libraries is still being studied.

One recent study performed a genomewide analysis of all the native terminator regions in a S. cerevisiae strain (Yamanishi et al., 2013). In this report, Yamanishi et al. used a fluorescent reporter protein to study over 5300 terminator strains for their relative expression levels and ranked the top and bottom 30 terminators. The top 5 most active terminating regions (RPL41B, RPL15A, DIT1, RPL3, and IDP1) were shown to enable protein expression more than twice that of an expression cassette containing a native PGK1 terminator (Yamanishi et al., 2013). These terminators were tested in vivo with a yeast secretion system for endoglucanase II, whereby they were able to boost cellulase production 2.2 times that of a similar system by swapping the PGK1 terminator with a DIT1 terminator (Ito et al., 2013). Therefore, increased protein expression was achieved simply by changing the terminating region in the expression cassette.

Another study by Curran et al. characterized 34 different terminators relative to the common CYC1 terminator used in the Mumberg expression vectors (Mumberg et al., 1995; Curran et al., 2013). The most active of these terminators, taken from the CPS1 gene, was shown to increase transcript half-life by a 2.5-fold increase, which led to an overall 6.5-fold increase in protein production. These terminators were used in combination with promoters to demonstrate that the high expression of a gene is achievable by coupling lower-strength promoters with more highly active terminators. Moreover, combining various promoters with a collection of terminators was more effective at improving a xylose isomerase pathway than promoter optimization alone (Lee et al., 2012; Curran et al., 2013). An analogous study by Shalem et al. tested 85 different 3′UTR regions taken from ribosomal protein and galactose metabolism genes in the yeast genome (Shalem et al., 2013). Similarly, they correlated terminator regions with mRNA abundance, attributing terminating regions to different protein production dynamics. However, despite these recent studies, the synthetic biology toolbox for terminators in yeast is still rather small. Further studies are needed to engineer synthetic terminators with an increase or decrease in strength relative to native terminator sequences.

**SYNTHETIC TRANSCRIPTION FACTORS**

The yeast synthetic biology toolbox is also expanding through the creation of synthetic transcription factors (sTF). These elements provide a unique advantage over endogenous transcription factors (TF) by providing another layer of control in systems in which production can be easily scaled up, and avoiding the cross talk between natural and synthetic systems by remaining completely orthogonal (Blount et al., 2012). Additionally, sTF can be easily modified to bind to millions of different promoter sequences and in effect, providing an ever-expanding toolkit for S. cerevisiae. The most common strategy to engineer sTF is to fuse a DNA binding domain with a transcription activating or repressing domain. In this regard, the modular construction allows for both specificity and function to be prescribed by two distinct parts. While several DNA binding domains are possible, we will focus most of this section on zinc finger sTF, whose DNA
binding domain consists of a zinc finger, as these are the most common versions found in literature.

Zinc finger sTF are comprised of a zinc finger DNA binding domain fused to an activator or repressor domain. Zinc finger DNA binding domains are attractive due to their small size (about 30 amino acids), functional independence, modular construction, and ability to be combined with activating and repressing domains while conserving binding abilities (Elrod-Erickson et al., 1996; Beerli and Barbas, 2002). Each native zinc finger recognizes a 3–4 base pair unique DNA sequence and can be engineered to bind to sites other than their native recognition sequences (Jamieson et al., 1996; Beerli et al., 1998; Dreier et al., 2000). Zinc finger DNA binding domains have been combined with activator and repressor domains to yield hundreds of rationally designed zinc finger sTF (Sera, 2009). Park et al. successfully screened a library containing 100,000 possible zinc finger sTF fused to either a repressing or activating domain for various phenotypes, such as drug resistance, thermotolerance, or osmotolerance in yeast (Park et al., 2003). In another study, the same group utilized a zinc finger sTF library strategy to improve yields of human growth hormone in S. cerevisiae 2.5-fold, reaching titers of 250 mg L⁻¹ (Park et al., 2005). Most recently, McIsaac et al. (2013) created estradiol-inducible systems by fusing a human estrogen receptor domain to a polydactyl zinc finger binding domain and showed that a 50-fold expression induction could be attained in yeast after just 15 min of induction (Fig. 1.3). This system was further improved using a modified GAL1 promoter containing six binding sites for the sTF (McIsaac et al., 2014). Additionally, gene networks constructed using zinc finger sTF can be used to confer memory of a stimulus in yeast where 90% of cells retained activation after the stimulus was removed (Ajo-Franklin et al., 2007). These examples provide a brief overview of the power of this sTF in synthetic biology applications, and more extensive applications have been reviewed by others (Sera, 2009).

For activation and induction, estradiol is attractive due to its limited number of unintended gene activations. A 200-fold induction by estradiol was obtained by fusing the hormone binding domain of the human estrogen receptor to the DNA binding domain of GAL4 and to the activation domain of VP16 (Fig. 1.2) (Louvion et al., 1993). McIsaac et al. (2011) improved this system further by creating a gradient response to estradiol concentration and fully characterizing it in a number of contexts. And most recently, the employment of the P65-activation domain in an estradiol-activated stTF improved the production level of zeaxanthin in yeast 50-fold from a previously reported strain (Liang et al., 2013).

On the DNA-binding side, clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems and transcription activator-like effectors (TALEs) can afford unique opportunities to augment and bypass the need for zinc finger proteins and other protein-based DNA binding elements discussed thus far. TALEs are bacterial proteins that infect plants by binding to the host’s promoters and activating expression of plant genes that aid in infection. While TALEs have been primarily exploited for genome editing and gene activation in plant and mammalian cells (Zhang et al., 2014), a recent study was able to manipulate them to repress a yeast synthetic promoter. Termined TAL orthogonal repressors (TALOR), these sTF were able to repress transcription 80% when expressed (Blount et al., 2012). Additionally, CRISPR/Cas systems hold the promise of more facile engineering than previously discussed sTF by avoiding screenings of large libraries, a strategy previously discussed to engineer zinc-finger-based sTF. By removing endonuclease activity of the RNA-guided Cas9, dCas9 can be used for gene silencing (Gilbert et al., 2013). More importantly, dCas9 can be fused to an activation domain to act as a sTF in which binding specificity is dictated by CRISPR. By fusing a SV40 nuclear localization sequence and four tandem VPS16 sequences to a dCas9, a 70-fold activation of a synthetic promoter containing 12 binding sites for this RNA-guided sTF was achieved when it was expressed (Fig. 1.2) (Farzadfard et al., 2013). Therefore, while the synthetic biology toolbox of synthetic transcription factors is small, it is rapidly expanding and can enable both library-based screening and target-specific binding function in yeast.

**APTAMERS AND RIBOSWITCHES**

**Overview**

A final area of rapid growth in yeast synthetic biology is in the area of aptamer and riboswitch design for regulating gene expression (Fig. 1.7). Certain molecules and signals have been shown to have binding interactions with a number of proteins that are responsible for nuclear localization (Chook and Suel, 2011; Marfori et al., 2011), extracellular secretion (Macauley-Patrick et al., 2005; Idris et al., 2010), and enzyme scaffolding (Smith and Scott, 2013). These structured molecules (especially RNAs and peptides) have been shown to have weak but very selective binding to metabolites and proteins (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Gold et al., 1995). Therefore, a combination of mutation and selection has been used to identify folding structures with improved specificity for a desired ligand. More recently, computational design of these elements (especially RNA aptamers) has proven to be possible. These aptamers have started to make their way into yeast as a synthetic tool.

**Use of aptamers in yeast synthetic biology**

Aptamers can serve as potent biosensors and bio-actuators due to the conformational changes in the RNA or peptide molecules that occur upon ligand binding (Cho et al., 2009). For example, synthetic aptamer molecules have been designed with fluorescent and quenching subunit tags that enable a shift in fluorescence when the ligand is present (Hammaguchi et al., 2001). Other aptamers have been developed in external electrochemical biosensors (Hansen et al., 2006; Zhou et al., 2007; Abe et al., 2014), which have the potential to detect cancer and leukemia cells (Bagalkot et al., 2007; Sefah et al., 2009), while other aptamers have been used to localize and high-throughput screen small molecules and proteins in Saccharomyces cerevisiae cells with a higher specificity than other methods (Zipor et al., 2009; Haim-Vilmovsky et al., 2011; Michener and Smolke, 2012; Kraut-Cohen et al., 2013). In most cases, these biosensors involve the use of fluorescent tagging in their function.

The use of aptamers and riboswitches, when properly placed in an expression cassette, can be used in gene regulation for knocking down or turning up expression. Previous work has shown the utility of gene knockdowns in yeast systems (Wang et al., 2010b; Crook et al., 2013), and aptamers designed for gene knockdowns in yeast have had successes. As an example, it had been shown that when an aptamer sequence in the 5’ UTR of a transcript bound to its ligand, translation of the transcript can be inhibited (Westruck and Green, 1998). By placing a tetracycline aptamer sequence in front of a gene coding for the green fluorescent protein (GFP) (Hanson et al., 2003; Suess et al., 2003), gene expression was able to be inhibited...
50-fold in vitro and sixfold in vivo in the presence of tetracycline (Hanson et al., 2005). Another study had found that an aptamer-controlled RNase III could modulate gene expression over a 44% dynamic range based on the ligand concentration, allowing for tunable expression (Babiskin and Smolke, 2011). Other aptamers have also been shown to knockdown gene expression by competing against native gene activation factors (Wang et al., 2010a). Others have used the high selectivity of aptamers to increase homologous recombination efficiency, increasing genetic targeting 32-fold in S. cerevisiae (Ruff et al., 2014). Finally, aptamers have been used to control gene regulation by focusing the RNase III enzyme Rntp1 toward the end of the mRNA in an effort to increase or decrease the rate at which an RNase cleaves mRNA (Babiskin and Smolke, 2011b). As is evident, an expanding toolkit of aptamers and riboswitches are being used in yeast synthetic biology.

CONCLUSIONS

Synthetic biology enables a newfound control over cellular processes. However, multiple components interact to enable this control, especially in eukaryotic organisms such as yeast. Thus, this review looked at progress in controlling many of these aspects with an emphasis on the varied level of control and applications. In total, these different parts can be used to control the net expression of genes and synthetic circuits. However, there are still limitations in metabolic engineering, particularly those for yeast systems such as S. cerevisiae, which have yet to be overcome. As of now, libraries of these synthetic parts (promoters, terminators, etc.) are still small and lack both sequence and orthogonality. Nevertheless, these synthetic systems lack a significant amount of the orthogonality that characterize E. coli synthetic biology efforts and thus may impact net expression in unexpected ways (Andrianantoandro et al., 2006; Lu et al., 2009). Nevertheless, the field of synthetic biology in yeast is certainly expanding, and the toolbox of parts and libraries provides a great promise for the future.

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AUTHORS’ CONTRIBUTION

H.R. and N.M. contributed equally to this work.

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