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Short Synthetic Terminators for Improved Heterologous Gene Expression in Yeast

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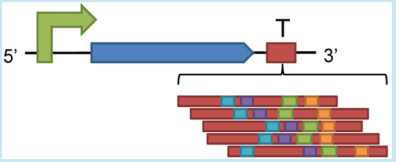
ABSTRACT: Terminators play an important role both in completing the transcription process and impacting mRNA half-life. As such, terminators are an important synthetic component considered in applications such as heterologous gene expression and metabolic engineering. Here, we describe a panel of short (35−70 bp) synthetic terminators that can be used for modulating gene expression in yeast. The best of these synthetic terminator resulted in 3.7-fold more fluorescent protein output and 4.4-fold increase in transcript level compared to that with the commonly used *CYC1* terminator. These synthetic terminators offer several advantages over native sequences, including an easily synthesized short length, minimal sequence homology to native sequences, and similar or better performance characteristics than those of commonly used longer terminators. Furthermore, the synthetic terminators are highly functional in both *Saccharomyces cerevisiae* and an alternative yeast, *Yarrowia lipolytica*, demonstrating that these synthetic designs are transferrable between diverse yeast species.



KEYWORDS: *terminator, 3′ UTR, yeast, Saccharomyces cerevisiae, heterologous gene expression*

Terminators are essential components in expression cassettes and can influence net protein output by controlling mRNA half-life. Mechanistically, eukaryotic terminators signal and recruit the machinery responsible for stopping transcription, cleaving the nascent mRNA, and polyadenylating the mRNA. In addition, a terminator is the genetically encoded element that defines the 3′ untranslated region (UTR) sequence and structure and thus contributes to mRNA stability and corresponding mRNA half-life.[1,2] As a result of this importance in mRNA processing, genes lacking terminators produce extended transcripts that are often too unstable for translation to occur.[3] Moreover, some terminators have been implicated in higher-order interactions across the genome, such as the case with gene looping in the yeast *Saccharomyces cerevisiae*.[4]

Despite their importance, terminators have not received as much attention in the yeast synthetic biology community when compared with that for other synthetic parts such as promoters. Nevertheless, recent work in *S. cerevisiae* has aptly demonstrated that varying the choice of native terminators can influence mRNA half-life of heterologously expressed genes and result in increased or decreased protein levels.[5−7] The underlying force responsible for these variations in gene expression is disparate stabilizing elements found in different native terminators.[2,8] However, the design of *de novo* synthetic terminators would provide additional benefits over these native terminators in the same way that synthetic promoters can outperform native promoters.[9−11] First, synthetic terminators would not rely on a native sequence scaffold and possess little to no homology to the native genome, thus minimizing any risk of undesirable homologous recombination. Second, purely synthetic terminators could be designed with only minimal elements, thus enabling a much shorter sequence with the same net effect as that of native terminators. The resulting DNA savings would provide a tangible benefit when constructing large genetic circuits or pathways. Moreover, shorter minimal sequences are more likely to be codified into predictive models for future design. Third, an understanding of synthetic terminators could provide the opportunity to create a library with tunable mRNA half-life or other characteristics. Finally, synthetic terminators that contain the essential elements for termination and mRNA half-life stability may be more portable for other organisms (such as other yeast, in this case), as these elements are less likely to recruit strain-specific factors, which is the case with promoters.

To create a series of synthetic terminators, we first turned to the molecular understanding of native terminator function in *S. cerevisiae*.[1] One type of terminator class is known to be composed of several important consensus elements, including an efficiency element, positioning element, the polyadenylation (poly(A)) site, and T-rich regions surrounding the poly(A) site (Figure 1a). To demonstrate that these elements comprised a minimal, essential set necessary for termination in yeast, Guo et al. stitched these elements together and demonstrated functional termination and mature mRNA formation (Figure 1b).[12] Yet, this minimal construct was never tested for protein expression levels in comparison to those with native

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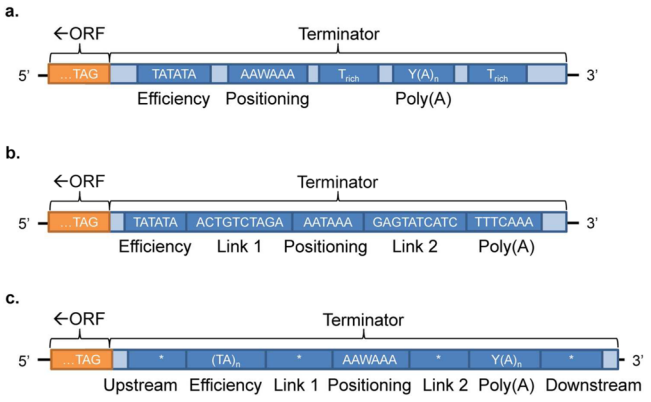

Figure 1. Terminator diagrams. (a) General diagram of a yeast terminator. (b) Diagram of synthetic terminator from Guo et al.[12] (c) Diagram of synthetic terminators tested in this work. For Upstream, Downstream, Link 1, and Link 2 elements, see Table 1 for the list and description of tested sequences.

terminators. In this work, synthetic terminators were designed *de novo* using the same set of essential elements required for transcriptional termination in yeast. Sequence variations both in the actual elements and in spacer regions between and flanking these elements were explored in the context of protein expression level and termination efficiency (Figure 1c). On average, these synthetic terminators were 49 bp long, compared to typical lengths of several hundred base pairs for standard native terminator elements. However, most of these synthetic terminators perform similarly to or better than the best native sequences previously reported.[5] Specifically, the best of these synthetic terminators resulted in 3.7-fold more fluorescent protein production and 4.4-fold more transcript than that with the commonly used *CYC1* terminator and had similar or enhanced performance characteristics as those of native terminators in the context of a metabolic pathway. Moreover, several of these designs were tested and confirmed to be functional in the alternative yeast *Yarrowia lipolytica*. To this end, several of these terminators resulted in nearly 60% more fluorescent protein production compared to that with a commonly used *CYC1* terminator in *Y. lipolytica*. These results demonstrate that these synthetic terminator designs are transferrable across diverse yeast species and are highly functional as short elements.

RESULTS AND DISCUSSION

Developing a Panel of Synthetic Terminators Composed of Minimal Sequence Elements. As mentioned previously, the first attempt to define a minimal set of elements required for yeast termination was described by Guo et al.[12] in 1996 (for the purpose of this work, this terminator scaffold has been named T_{Guo1}; see Figure 1b). In their effort, it was determined that a minimal set composed of the consensus sequences for an efficiency element (TATATA), positioning element (AATAAA), and poly(A) site (TTTCAAA), each spaced 10 bp apart, was sufficient to make mature, polyadenylated transcript. However, this construct was not compared to native terminators on the basis of transcript abundances or corresponding protein levels. Moreover, no subsequent work has been done with this element.

In this work, we created a panel of short synthetic terminators ranging in size from 38 to 75 bp in length. To accomplish this, we cloned prospective terminators into a heterologous expression cassette consisting of the *TEFmut3* promoter[13,14] and the *yECitrine* gene for yellow fluorescent protein expression. This cassette was expressed in a centromeric p413 plasmid,[15] based on the pRS plasmid series. As a point of reference, the T_{Guo1} construct, as well as the commonly used endogenous *CYC1* terminator, was also cloned into this construct. Variants of the T_{Guo1} construct were inspired by elements identified in native terminators that correlate with relatively long mRNA half-lives and increased relative protein abundance.[5] For example, favorable native terminator structures include extended efficiency elements (as seen in *SPG5* and *HIS5* terminators) or poly(T) tracts before or after the poly(A) site (as seen in *CPS1* and *IDP1* terminators). Variations were also created to allow for alternate consensus sites for the efficiency element ($(TA)_n$ instead of TATATA), positioning element (AAWAAA instead of AATAAA), and poly(A) element ($Y(A)_n$ instead of $C(A)_3$). Moreover, we explored alterations in spacer region length and percent GC content as well as the addition of upstream and downstream sequences. Final sequences of all 30 terminator variants tested as well as a description of the modifications are provided in Table 1.

To confirm that these sequences were significantly distinct from those of native *S. cerevisiae* terminators, the sequences were aligned against the *S. cerevisiae* S288C genome (blastn, word size of 7 bp). The highest degree of similarity was found to be the repetitive efficiency elements $(TA)_{6n}$ of more than 6 bp in T_{synth2}–T_{synth3} and $T_{synth25}$–$T_{synth30}$. This is not unexpected, however, as the motif is quite common in both promoters (TATA boxes) and terminators in the yeast genome. Outside of the alignment of these long efficiency elements, the highest degree of similarity was found to be a 41 bp section in only one of the terminators, $T_{synth18}$, to a region within the *NPR2* ORF on Chr.V (*E*-score of 0.006). We therefore conclude that that the synthetic terminators are significantly different from native terminators in the *S. cerevisiae* genome.

Evaluating Heterologous Protein Expression from a Panel of Short Synthetic Terminators. Once cloned, the panel of short synthetic terminators described above was evaluated on the basis of *yECitrine* fluorescence via flow cytometry (Figure 2). This test revealed several important features of the terminator panel.

First, even without any alteration, T_{Guo1} enables a nearly 2.3-fold higher expression in comparison to that with the commonly chosen *CYC1* terminator. As such, this terminator is not only sufficient for termination of a transcript as described previously but also would likely be preferred over many current standard terminators on the basis of improved protein output. Additionally, this terminator is only 39 bp long (compared to the *CYC1* terminator that is 240 bp long), enabling an ease of cloning using standard length, inexpensive oligos.

Second, several of the synthetic terminators in the panel resulted in expression that varied significantly (both higher and lower) from that of the T_{Guo1} base construct. The first set of variants constructed (T_{synth1}–T_{synth5}) was designed exclusively by altering the length of the efficiency element, $(TA)_n$. There is clear, positive correlation between the increased fluorescent protein expression and length of the efficiency element (Figure 3). Furthermore, it is clear that a minimum efficiency element length of 6 bp (TATATA) is required for function, as terminators with elements shorter than this length (T_{synth4} and T_{synth5}) resulted in severely decreased function on the basis of fluorescence (Figures 2 and 3). In exploring the impact of

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Table 1. Synthetic Terminators Sequence and Description[a]

terminator	sequence	description
T_{Guo1}	**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	Terminator from Guo et al.[12]
T_{synth1}	**TATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	Efficiency element of 12 bp
T_{synth2}	**TATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	Efficiency element of 24 bp
T_{synth3}	**TATATATATATATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	Efficiency element of 36 bp
T_{synth4}	ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	Efficiency element of 0 bp
T_{synth5}	**TATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	Efficiency element of 4 bp
T_{synth6}	**TATATA**TTTAATAAAGAGTATCATC*TTTCAAA*	Link 1 changed to 3 bp, GC content = 0%
T_{synth7}	**TATATA**ACTGTCTAGAAATAAATTT*TTTCAAA*	Link 2 changed to 3 bp, GC content = 0%
T_{synth8}	**TATATA**AACTCATTTACTTATGTAGGAATAAAGAGTATCATC*TTTCAAA*	Link 1 changed to 20 bp, GC content = 30%
T_{synth9}	**TATATA**ACTGTCTAGAAATAAAACTCATTTACTTATGTAGG*TTTCAAA*	Link 2 changed to 20 bp, GC content = 30%
$T_{synth10}$	**TATATA**CACCCGTCGAGCCTGTCCGAAATAAAGAGTATCATC*TTTCAAA*	Link 1 changed to 20 bp, GC content = 70%
$T_{synth11}$	**TATATA**ACTGTCTAGAAATAAACACCCGTCGAGCCTGTCCGA*TTTCAAA*	Link 2 changed to 20 bp, GC content = 70%
$T_{synth12}$	**TATATA**GGTGCAGGCAAATAAAGAGTATCATC*TTTCAAA*	Link 1 changed to 10 bp, GC content = 70%
$T_{synth13}$	**TATATA**ACTGTCTAGAAATAAAGGTGCAGGCA*TTTCAAA*	Link 2 changed to 10 bp, GC content = 70%
$T_{synth14}$	**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TAAAAA*	Poly(A) site changed to TAAAAA
$T_{synth15}$	**TATATA**ACTGTCTAGAAATAAAGAGTATCATCC*AAA*	Poly(A) site changed to CAAA
$T_{synth16}$	**TATATAA**CTGTCTAGAAATAAAGAGTATCATCC*AAAAA*	Poly(A) site changed to CAAAAA
$T_{synth17}$	**TATATA**ACTGTCTAGAAAAAAAGAGTATCATC*TTTCAAA*	Positioning site changed to AAAAAA
$T_{synth18}$	**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*TTTTTTTTTT	3′ sequence of 10 T residues added
$T_{synth19}$	TTTTTTTTT**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence of 10 T residues added
$T_{synth20}$	CGCATTGGCCGGCCAATGCGTA**TATAA**CTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ stem-loop added
$T_{synth21}$	AATCAAATTC**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added AATCAAATTC
$T_{synth22}$	TGGGTGGTA**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added TGGGTGGTA
$T_{synth23}$	GTGGTATGT**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added GTGGTATGT
$T_{synth24}$	TGGGTGGTATGT**TATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added TGGGTGGTATGT
$T_{synth25}$	TTTTTTTTT**TATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence of 10 T residues added and Efficiency element of 24 bp
$T_{synth26}$	AATCAAATTC**TATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added AATCAAATTC and Efficiency element of 24 bp
$T_{synth27}$	TGGGTGG**TATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added TGGGTGGTA and Efficiency element of 24 bp
$T_{synth28}$	GTGGTATGT**TATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added GTGGTATGT and Efficiency element of 24 bp
$T_{synth29}$	TGGGTGGTATGT**TATATATATATATATATATATATA**ACTGTCTAGAAATAAAGAGTATCATC*TTTCAAA*	5′ sequence added TGGGTGGTATGT AND Efficiency element of 24 bp
$T_{synth30}$	TTTTTTTTT**TATATATATATATATATATATATA**AACTCATTTACTTATGTAGGAATAAATTT*TTTCAAA*	5′ sequence of 10 T residues added, Efficiency element of 24 bp, Link 1 changed to 20 bp, GC content = 70%, and Link 2 changed to 3 bp, GC content = 0%

[a]In each terminator, the efficiency element is in bold, the positioning element is underlined, and the poly(A) site is in italic.

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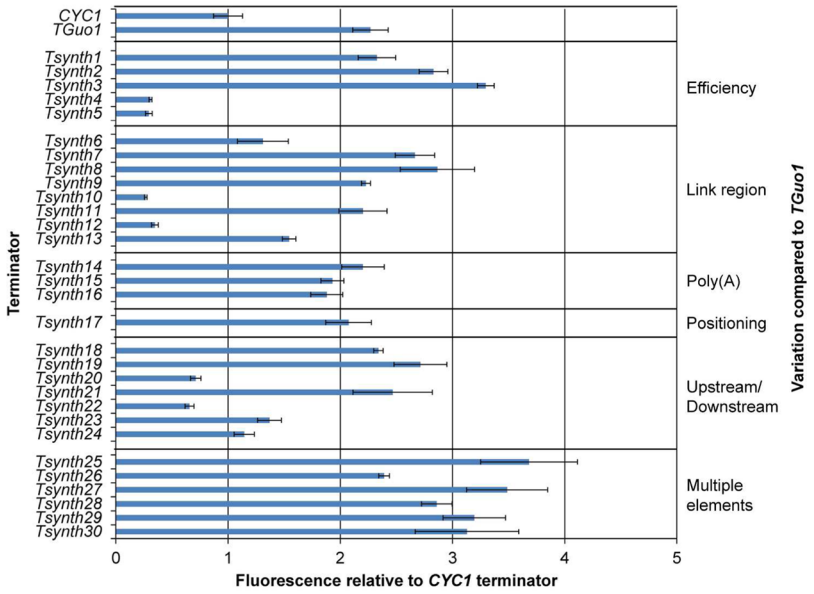

Figure 2. Fluorescence of yellow fluorescent protein produced from heterologous expression cassettes containing synthetic terminators. Values are relative to the *CYC1* terminator, the standard terminator available in the plasmids described in Mumberg et al.[15] Terminators are divided into categories based on the terminator element that was varied in comparison to the T_{Guo1} termiantor. Error bars represent standard deviation of three biological replicates. See Table 1 for terminator sequences.

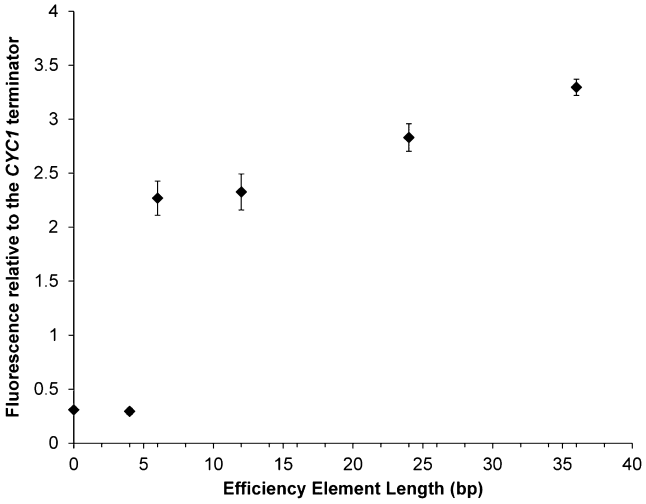

Figure 3. Correlation of efficiency element length and protein expression level. Efficiency element length correlates with relative protein expression, and there is a clear requirement for elements to be at least 6 bp long. Error bars represent standard deviation of three biological replicates.

efficiency element length, a very strong terminator (T_{synth3}) emerges with a motif of $(TA)_{18}$ and a protein output that is nearly 3.3-fold higher than that with *CYC1* terminator.

Third, the length and GC content of spacer regions between consensus elements have an impact on terminator function. In general, link regions with high GC content ($T_{synth10}$–$T_{synth13}$) were detrimental to protein expression, and, in some cases, links of low GC content increased protein expression (such as in T_{synth7} and T_{synth8}). Additionally, a short, 3 bp all-T link (T_{synth6}) was detrimental to protein expression, whereas a 20 bp sequence of low GC content (30% for T_{synth8}) increased protein expression relative to that with T_{Guo1}. These results demonstrate that the spacing and GC content of the link regions between terminator elements are critical, especially for the region between the efficiency and positioning element (Link 1).

Fourth, several variants in the motif sequence for the positioning element and poly(A) site were tested ($T_{synth14}$–$T_{synth17}$). In general, these variations tested did not have a drastic effect on fluorescent protein expression and thus can be used interchangeably with the canonical motif sequence. Only a slight (but statistically significant, $p < 0.04$) decrease in fluorescence output was observed for some of the poly(A) sites tested ($T_{synth15}$ and $T_{synth16}$).

Finally, the impact of additional sequences upstream (5′) and downstream (3′) of the terminators elements was evaluated. Initially, poly(T) tracts were added in both upstream and downstream regions. The addition of poly(T) tracts had no impact for the 3′ location ($T_{synth18}$) and proved to be slightly beneficial for the 5′ location ($T_{synth19}$). This result aligns with recent evidence that poly(T) tracks in the 3′ UTR can form stabilizing stem-loop structures with the poly(A) tail and lead to more stable transcripts.[2] On this basis, a separate stem loop was designed such that the resulting 3′ UTR would be more difficult to degrade and therefore result in a higher half-life ($T_{synth20}$); however, this element proved to be detrimental to expression in this case. In contrast, GC-rich stem loops have been shown to increase mRNA half-life when inserted into native terminators.[2] We hypothesize that sufficient spacing of the secondary structure in the 3′ UTR may be critical to allow for efficient translation. Next, several additional elements identified in a global bioinformatics study[8] to occur in the first 20 bp of terminators associated with high mRNA half-life were tested in the 5′ region. Interestingly, several of these elements resulted in lower expression overall when placed in the base construct ($T_{synth22}$–$T_{synth24}$) but higher expression overall when placed into a terminator with an extended efficiency element ($T_{synth27}$–$T_{synth29}$). These results again illustrate the need to appropriately space terminator sequence elements. A final combination of the best individual elements from each category ($T_{synth30}$) did not increase protein

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expression further, indicating either that the elements are not necessarily additive or that the variants have reached a local maximum in the explored sequence space. Furthermore, an analysis of the 3′ UTR secondary structure of the terminator sequences using mfold[16] revealed some localized trends among sequences and function, but these trends were not fully predictive of terminator function (Figure 4). This is not

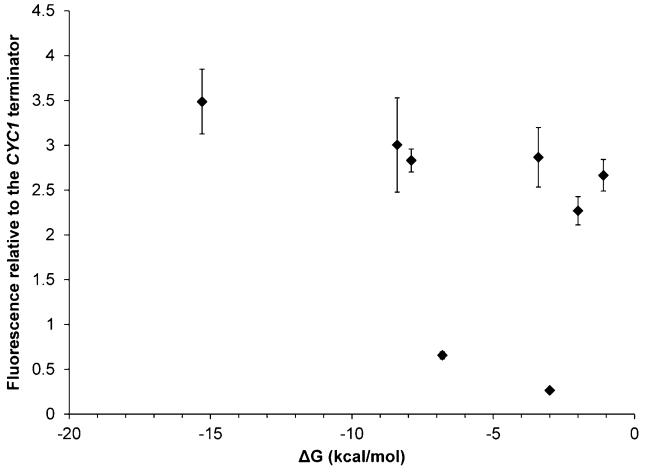

Figure 4. Loop free-energy decomposition of selected terminators versus fluorescence relative to the CYC1 terminator. Error bars represent standard deviation of three biological replicates.

unexpected, as it is likely that the synthetic terminators are affecting multiple mRNA characteristics simultaneously (i.e., polyadenylation level and 3′ UTR structure).

To test whether the observed increase in expression was due to an increase in transcription as expected, quantitative PCR was performed on a subset of the terminator constructs and compared to the fluorescence previously measured for each (Figure 5). The strong correlation between transcript and fluorescence suggests these synthetic terminators are affecting protein expression at the transcript level.

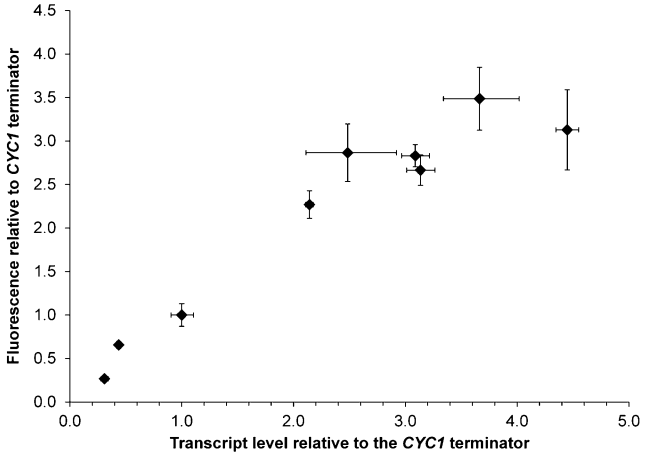

Figure 5. Correlation of yECitrine fluorescence and transcript level. Transcript level, measured by qPCR, correlates well with the relative protein level, measured by fluorescence of yellow fluorecent protein. Vertical error bars represent standard deviation of three biological replicates, and horizontal error bars represent standard deviation of three technical replicates.

Transcription Termination Efficiency of Synthetic Terminators. While terminators must be efficient at increasing protein expression through mRNA stability, they also must serve their primary function of full transcription termination and disengagement of RNA polymerase II. This latter function becomes especially important when multiple heterologous genes are cloned in close proximity in a construct that requires differential expression of each gene. Moreover, the use of short terminators places less sequence space between adjacent genes, which may cause higher amounts of transcriptional read through or interference. To test this impact, several of the synthetic terminators designed in this work were tested on the basis of termination efficiency. To do so, an additional gene (*mStrawberry*, encoding a red fluorescent protein) was cloned immediately 3′ of the terminator using the same constructs as described above. The main promoter used upstream of the *yECitrine* gene was changed to the inducible *GAL1* promoter to also enable a test for latent promoter activity by these synthetic terminators when cells were grown on glucose (Figure 6a). To test termination efficiency, the transcript levels of both *yECitrine* and *mStrawberry* were measured in both *GAL1* induced and repressed states (with galactose and with glucose, respectively). In the repressed state, *yECitrine* was off, and any observed *mStrawberry* transcription is likely due to cryptic promoter activity in the terminator and surrounding sequence. In the induced state, *yECitrine* transcription was very high, and any *mStrawberry* transcription is likely due to read through or incomplete transcription termination (Figure 6b,c). With a few exceptions, the synthetic terminators compare favorably (low read through and low latent promoter activity) to the native terminators. In general, these short synthetic terminators gave *mStrawberry* transcript levels comparable to the *CYC1* terminator in both the induced and repressed states, indicating these sequences are sufficient to terminate RNA polymerase II transcription. Two synthetic terminators in particular (T_{synth8} and $T_{synth27}$) had extremely favorable profiles with respect to read through and latent promoter activity. In addition, several terminators (T_{synth2} and $T_{synth30}$) saw over a 3-fold increase in transcript level relative to that of the *CYC1* promoter in the induced state, even when using this very strong *GAL1* promoter. This demonstrates that these short synthetic terminators have the potential to improve protein expression levels in contexts where very high protein expression is needed.

Short Synthetic Terminators Also Function in an Alternative Yeast Host. Unlike promoters that heavily rely on specific transcription factor binding, terminators are potentially a more universal synthetic part. Consequently, we sought to test several members of our terminator panel in the yeast *Y. lipolytica* to determine whether these short synthetic constructs could be portable and used across yeast families. *Y. lipolytica* is a phylogenetically distinct[17] oleaginous yeast in the family *Dipodascaceae*. This yeast is of particular industrial interest due to a well-established genetic toolbox and ability to accumulate high levels of lipids.[18]

Seven of the synthetic terminators (T_{synth2}, T_{synth7}, T_{synth8}, $T_{synth10}$, $T_{synth22}$, $T_{synth27}$, and $T_{synth30}$) along with T_{Guo1}, *CYC1*, and *TEF1* terminators were cloned into a heterologous expression cassette expressing the green fluorescent protein gene *hrGFP* along with a strong hybrid promoter developed previously.[19] The *CYC1* terminator from *S. cerevisiae* and a 250 bp region assumed to be *TEF1* and *CYC1* terminators from *Y. lipolytica* were also included for comparison. With the exception of $T_{synth30}$, the seven tested synthetic terminators exhibited a

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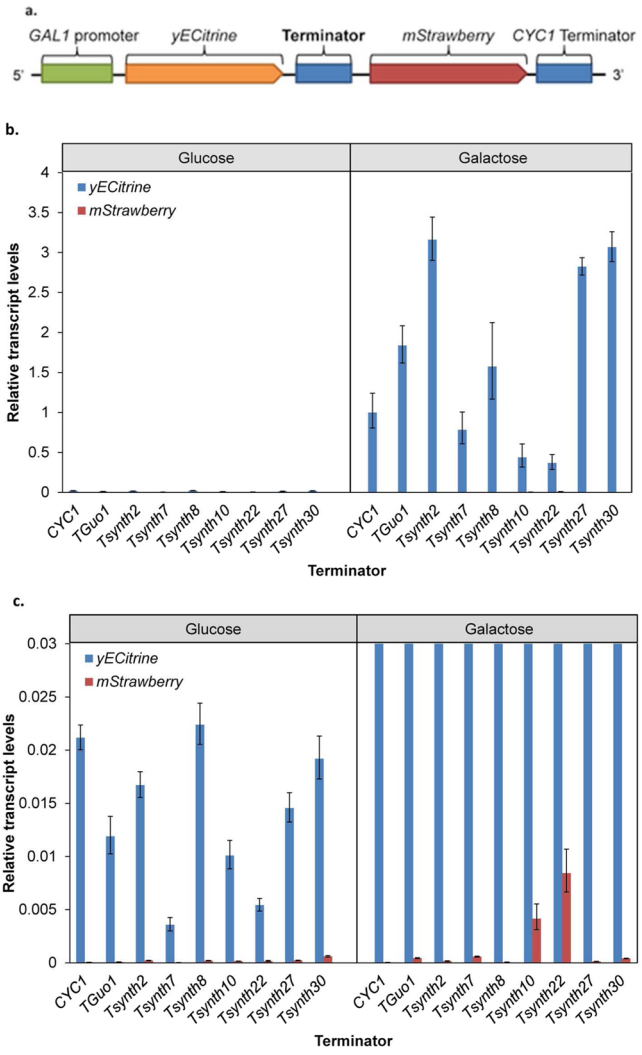

Figure 6. Termination efficiency of selected native and synthetic terminators. (A) Diagram of construct to test termination efficiency. Transcript levels of *yECitrine* and *mStrawberry* placed on either side of the terminator of interest were measured. In glucose media, the *GAL1* promoter is repressed, and higher mStrawberry transcript levels likely indicate cryptic promoter activity in the terminator. In galactose media, the *GAL1* promoter is induced, and higher mStrawberry transcript levels indicate insufficient transcription termination and read through. (B, C) Relative transcript levels of *yECitrine* and *mStrawberry*. All values are relative to the transcript level of the construct containing the *CYC1* terminator in galactose. In panel C, the *y* axis is adjusted to show the smallest values. Error bars are propagated standard deviations from three technical replicates of each qPCR.

similar performance in *Y. lipolytica* as that seen in *S. cerevisiae*, indicating the portability of these synthetic parts across yeast strains (Figure 7). Included among this set is one synthetic terminator, $T_{synth10}$, which performed poorly in both hosts (due to high GC content in Link 1), demonstrating that *Y. lipolytica* terminators are likewise sensitive to the same constraints of sequence space seen in *S. cerevisiae*. The majority of synthetic terminators performed as well as or better than the native terminators, including a nearly 70% improvement over the currently used *CYC1* terminator. These results demonstrate that short synthetic terminators can be highly functional across species and can potentially simplify the design and testing of constructs across multiple yeast hosts.

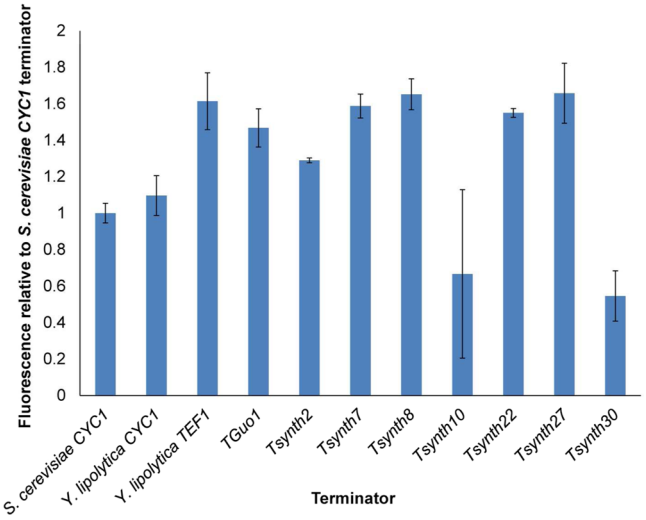

Figure 7. Fluorescence of hrGFP in *Y. lipolytica* using synthetic terminators. Values are relative to the construct containing the *S. cerevisiae CYC1* terminator expressed in *Y. lipolytica*. Error bars represent standard deviation of three biological replicates.

Altering the Expression of a Metabolic Pathway Using Synthetic Terminators. As a proof of concept for using these synthetic terminators to control metabolic flux, we utilized these components to express a heterologous enzyme in *S. cerevisiae* to enable the production of itaconic acid. Previously, we showed that *S. cerevisiae BY4741* strains expressing a codon-optimized *cis*-aconitic acid decarboxylase (*CAD1*) gene from *Aspergillus terreus* were able to produce low amounts of itaconic acid.[20] Specifically, optimized culturing of these trains and improved expression led to 59 mg/L of itaconic acid. This improvement was mainly accomplished by the strong over-expression of *CAD1* driven by a high-strength, enhanced GPD promoter.[20] Here, *TEFmut3*, a significantly weaker promoter, was used to drive the expression of the *CAD1* gene in an effort to determine if synthetic terminator modification could help to tune metabolic pathways. An analysis of culture supernatants using HPLC revealed that all of the terminator constructs led to itaconic acid production (Figure 8). While the use of this significantly weaker promoter led to low titers, each of the synthetic terminators showed at least comparable itaconic acid production when compared with that when using the *CYC1* terminator. Slight improvements in titer were seen with some of the stronger terminators (like $T_{synth27}$). Nevertheless, this effort demonstrates that short synthetic terminator sequences are able to effectively express heterologous proteins for metabolic pathway purposes just as well as that of longer native terminator elements. Combining the benefits of higher strength promoters with synthetic terminators will likely have a combinatorial impact on metabolic pathway engineering.

CONCLUSIONS

This study systematically and rationally created short synthetic terminators for use in yeast. These terminators offer many advantages over endogenous terminators, including being short and therefore easy to clone or synthesize, having decreased homology to the native yeast genome, and being highly functional in several yeast species of industrial interest. Performance of these parts was upward of 3.7-fold better than that of the most common endogenous parts. The analysis of these synthetic terminators offered several initial design

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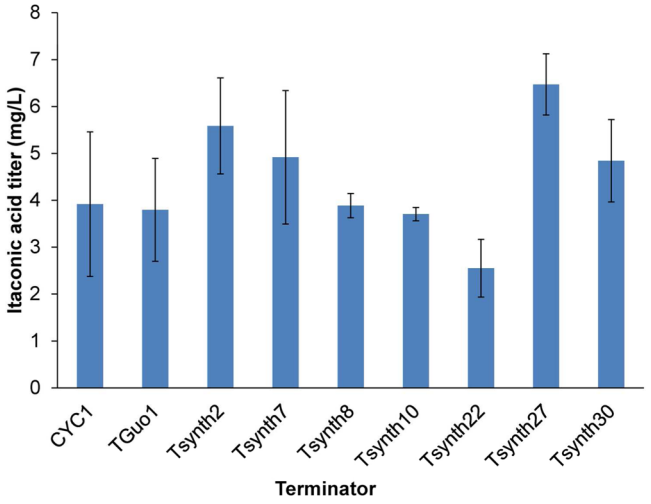

Figure 8. Itaconic acid production from strains expressing CAD1 with variable terminators. Itaconic acid levels were measured for cells expressing the *CAD1* enzyme with various terminators in this study. Error bars represent standard deviation from technical triplicates.

rules, including the best consensus elements to use and their spacing and GC content. Terminator efficiency was also tested in these short elements and resulted in levels that were equivalent to or better than that of native terminators previously studied and used. These results demonstrate a new panel of synthetic terminators that have distinct advantages over longer endogenous elements.

METHODS

Strains. *Saccharomyces cerevisiae* strain BY4741 (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) (EUROSCARF) and *Yarrowia lipolytica* strain PO 1f (*MatA, leu2−270, ura3−302, xpr2−322, axp-2*) (ATCC no. MYA-2613)[21] were used as the host strains in this work. *S. cerevisiae* and *Y. lipolytica* strains were routinely propagated at 30 °C in yeast extract peptone dextrose (YPD) medium or yeast synthetic complete (YSC) medium. YPD medium is composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. YSC-His medium is composed of 6.7 g/L yeast nitrogen base, 20 g/L glucose or galactose, and CSM-His supplement (MP Biomedicals, Solon, OH). *Y. lipolytica* strains were propagated in YSC-Leu medium at 30 °C. This YSC medium contains CSM-Leu supplement (MP Biomedicals, Solon, OH). *Escherichia coli* strain DH10B was used for all cloning and plasmid propagation. DH10B was grown at 37 °C in Luria−Bertani (LB) broth supplemented with 50 μg/mL of ampicillin. *E. coli* and *S. cerevisiae* strains were cultivated with 225 rpm orbital shaking. *Y. lipolytica* strains were cultivated in a rotary drum (CT-7, New Brunswick Scientific) at speed seven to facilitate oxygenation of the cultures. Yeast and bacterial strains were stored at −80 °C in 15% glycerol.

Plasmid Construction. All plasmids used in this study were based on the vectors in Mumberg et al.[15] The *TEFmut3* promoter, the *yECitrine* gene, and the *S. cerevisiae* codon-optimized *cis*-aconitic acid decarboxylase (CAD1) gene from *A. terreus* (NCBI GenBank database accession no. KJ653453) were cloned via PCR from plasmids described previously.[13,14,20,22] The *CYC1* terminator is the terminator from the parent plasmid,[15] which was originally cloned from the D311-3A strain.[23] Synthetic terminators (see Table 1 for sequences) were created by annealing oligos ordered from Integrated DNA Technologies. Specifically, complementary oligos were mixed in HF Phusion buffer to a final concentration of 2.5 mM, heated at 98 °C for 2 min, and then cooled to 45 °C at 0.1 °C/s. For synthetic terminators greater than 60 bp in length, primers were ordered such that the forward and reverse primers overlapped by 20 bp. The annealing protocol as stated above was then followed with the addition of dNTPs and HF Phusion polymerase to the reaction mix per the manufacturer's instructions, and a 30 min extension step at 72 °C was added after the annealing step. Synthetic terminators were then purified using the MER-maid spin kit (MP Biomedicals). All cloned terminators were inserted into the plasmid using the SalI and EagI restriction sites. The SalI site, in addition to the SpeI site at the beginning of the multicloning site, was also used

Table 2. Primers Used in This Study

primer	target	sequence
1	*TEFmut3* promoter	TGACTGAGCTCATAGCCTCAAAATGTTTCTACTC
2	*TEFmut3* promoter	GGCGCTACTAGTTCTAGAAAACTTAGATTAGATTGCTATGCTTTC
3	*GAL1* promoter	CAAAGAGCTCCTAGTACGGATTAGAAGCCG
4	*GAL1* promoter	GGCGCTACTAGTTCTAGAATCCGGGGTTTT
5	*yECitrine*	GGCGCTACTAGTATGTCTAAAGGTGAAGAATTATTCACTGG
6	*yECitrine*	ACGCGTCGACTTATTTGTACAATTCATCCATACCATG
7	*CAD1*	gactgacactagtATGACTAAACAATCAGCTGATTCA
8	*CAD1*	cgactgctgtcgacTTAGACCAATGGCGACTTTAC
9	*mStrawberry-*T_{CYC1}	catcggccgATGGTGAGCAAGGGCGA
10	*mStrawberry-*T_{CYC1}	ttagccggcCAAATTAAAGCCTTCGAGCGTCC
11	*yECitrine* qPCR	TTCTGTCTCCGGTGAAGGTGAA
12	*yECitrine* qPCR	TAAGGTTGGCCATGGAACTGGCAA
13	*mStrawberry* qPCR	CATGGCCATCATCAAGGAGTT
14	*mStrawberry* qPCR	ACCCTTGGTCACCTTCAGCTT
15	*ALG9* qPCR	ATCGTGAAATTGCAGGCAGCTTGG
16	*ALG9* qPCR	CATGGCAACGGCAGAAGGCAATAA
17	*Y. lipolytica* T_{TEF1}	ccTTAATTAAGCTGCTTGTACCTAGTGCAACCCCAGTTTGTTAAAAATTAGTAGTCAAAA
18	*Y. lipolytica* T_{TEF1}	gcgccGTTTAAACACTGAGTGACAGAGCCCTCTCATGTTTGGAGAGAAGACTAAGTACAA
19	*Y. lipolytica* T_{CYC1}	ccTTAATTAAGCGTCTACAACTGGACCCTTAGCCTGTATATATCAATTGATTATTTAAAG
20	*Y. lipolytica* T_{CYC1}	gcgccGTTTAAACGACGCAAGAGAAGCCGTCGCCCCACGGAGTATC

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to insert the gene *yECitrine* for flow cytometry analysis and the *CAD1* gene for itaconic acid quantification so that there was only a single restriction site between the end of the gene and the terminator. To determine termination efficiency, select terminators were cloned into a plasmid with the *GAL1* promoter driving *yECitrine* expression. Then, the *mStrawberry* gene and a *CYC1* terminator were cloned after each terminator using the EagI and NaeI sites available. See Table 2 for primers. *Y. lipolytica* plasmids were modified centromeric, replicative plasmids as described in earlier work.[19] The vector pMCS-UAS1B$_8$-hrGFP was used to create all plasmids. Synthetic terminators were prepared with annealing and extending in HF Phusion buffer as described above. The native terminator regions were obtained via PCR from genomic DNA purified with the Wizard genomic DNA purification kit (Promega). PCR reactions were run with recommended conditions using HF Phusion polymerase (New England BioLabs, Inc.). Cloned terminators were inserted into the plasmid vector via PacI and PmeI restriction sites using T4 DNA ligase (Thermo Scientific).

All plasmids were transformed using the Zymo EZ freeze yeast transformation kit II (Zymo Research, Irvine, CA) according to manufacturer's instructions.

Flow Cytometry. Fluorescence from *S. cerevisiae* strains expressing the *yECitrine* gene was measured with a FACS Fortessa (BD Biosciences) using a YFP fluorochrome in biological triplicates. Cells were grown to mid log phase overnight from a starting OD_{600} = 0.005, and 10 000 events were collected using the Fortessa with a fluoresce voltage of 413 V. *Y. lipolytica* strains were initially propagated from individual colonies on YSC-Leu plates into 2 mL of fresh YSC-Leu media. After 48 h of incubation in a rotary drum, cultures were normalized to an OD_{600} of 0.03 in 2 mL of fresh YSC-Leu media. Cultures were grown 48 h before being harvested. To harvest, cultures were spun at 1000*g* for 5 min, washed with 5 mL of ice-cold water, and then 100 μL of this wash was added to 1 mL of ice-cold water. Fluorescence from *Y. lipolytica* expressing the *hrGFP* gene was measured using the GFP fluorochrome, a voltage of 319, and 10 000 events. Day-to-day voltage variability was mitigated by measuring all comparable strains on the same day. FlowJo (Tree Star Inc., Ashland, OR) was used to analyze data and to compute mean fluorescence values.

Quantitative PCR. The relative abundance of heterologous mRNA was determined using quantitative RT-PCR. RNA was extracted from mid log phase cells via cell wall digest with Zymolyase per the manufacturer's instructions coupled with the Zymo quick RNA miniprep kit. For termination efficiency experiments, cells were grown in both YSC-glucose and YSC-galactose media prior to RNA extraction. cDNA was prepared using the Applied Biosystems high capacity reverse transcription kit (Life Technologies, Carlsbad, CA). Primers were obtained from Integrated DNA Technologies (see Table 2 for primers). Quantitative PCR was performed on a ViiA7 real time PCR system (Life Technologies) using fast start SYBR green master mix (Roche, Penzberg, Germany), following the manufacturer's instructions with an annealing temperature of 58 °C. *ALG9* was used as the reference housekeeping gene.

Itaconic Acid Quantification. Itaconic acid production in *S. cerevisiae* strains was measured as mentioned previously.[20] Briefly, *S. cerevisiae* strains were cultivated in 2 mL of YSC-H media for 3 days. After growth, a 1.0 mL sample was pelleted for 3 min at 10 000*g*, and the supernatant was filtered using a 0.2 μm syringe filter (Corning Incorporated). Filtered supernatant was analyzed with a HPLC Ultimate 3000 (Dionex) and a Zorbax SB-Aq column (Agilent Technologies). A 2.0 μL injected volume was used in a mobile phase composed of a 99.5:0.5 ratio of 25 mM potassium phosphate buffer (pH 2.0) to acetonitrile with a flow rate of 1.25 mL/min. The column temperature was maintained at 30 °C, and UV−vis absorption was measured at 210 nm. An itaconic acid standard (Sigma-Aldrich) was used to detect and quantify itaconic acid production.

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Notes

The authors declare the following competing financial interest(s): K.A.C. and H.S.A. have filed a provisional patent application in 2014 related to the synthetic terminators developed in this study.

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