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REVIEW

Recent developments in terminator technology in *Saccharomyces cerevisiae*

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Metabolically engineered microorganisms that produce useful organic compounds will be helpful for realizing a sustainable society. The budding yeast *Saccharomyces cerevisiae* has high utility as a metabolic engineering platform because of its high fermentation ability, non-pathogenicity, and ease of handling. When producing yeast strains that produce exogenous compounds, it is a prerequisite to control the expression of exogenous enzyme-encoding genes. Terminator region in a gene expression cassette, as well as promoter region, could be used to improve metabolically engineered yeasts by increasing or decreasing the expression of the target enzyme-encoding genes. The findings on terminators have grown rapidly in the last decade, so an overview of these findings should provide a foothold for new developments.

[**Key words:** Terminator; Metabolic engineering; Genetic switch; Gene expression; Synthetic biology; *Saccharomyces cerevisiae*]

In order to realize a sustainable society, many studies have been conducted to develop microorganisms that efficiently produce useful organic compounds using metabolic engineering (1). *Saccharomyces cerevisiae* is considered to have high utility as a platform for these genetically-modified microorganisms for reasons such as high fermentation ability, high safety and easy handling (2). In metabolic engineering, when multistage enzymatic reaction is required for biosynthesis of target organic compounds which yeast does not originally produce, it is necessary to control the expression level of a battery of enzyme-encoding genes involved in the biosynthesis, respectively (3,4). In the expression cassette of the enzyme-encoding genes, terminator is placed downstream of the coding region to control mainly the termination of transcription while promoter is placed upstream of the coding region to regulate mRNA transcriptional activity. In eukaryotes, we assume that protein production level of exogenous genes is determined mainly by the synergistic action of the transcription promoter and terminator. That is, in metabolic engineering, the terminator-exchange strategy is considered to be the greatest advantage in that it produces synergy effects without competing with other expression level modulation techniques such as codon optimization as well as promoter-exchange strategy. In 2010, when my group started its research into metabolic engineering, strong promoters had been developed but there was little information on, or development of, terminators. However, since then, many advances have been made, and here I review the most important developments in terminator technology in *S. cerevisiae*. This review contains the following 7 sections: (i) basic functions of the terminator, (ii) terminator activity and the modulation of protein expression, (iii) application of terminators with strong activity in metabolic engineering, (iv) terminator selection for optimization of gene expression, (v) use of terminators as genetic switches, (vi) mechanism of action of highly active terminators and (vii) challenges in artificial terminator development.

BASIC FUNCTIONS OF THE TERMINATOR

In the eukaryotic genome, transcription terminators are located downstream of protein-encoding regions. The primary functions of terminators are termination of the transcription process, processing of immature mRNA, and addition of poly(A) to the 3′-terminal end of mRNA (polyadenylation) (5).

Initially, in *S. cerevisiae*, three functional sequences essential for transcription termination were identified in the terminator regions of several genes (6–11). These functional sequences, arranged in order from the 5′ end, are the efficiency element, TATATA (6–9); the positioning element, AATAAA (10); and the polyadenylation signal, $Py(A)_n$ (Py represents a pyrimidine) (11). However, after the whole genome of *S. cerevisiae* was sequenced, it was found that the terminator regions of many other genes also contain these functional sequences (12–14). In addition, it has been demonstrated that a 39-bp synthetic sequence containing only these three functional sequences is able to function as a transcription terminator in *S. cerevisiae* (15).

Eukaryotic terminators contain a sequence known as the 3′-untranslated region (3′-UTR), which, among other functions, modulates mRNA decay rate, transcriptional and translational efficiencies, nuclear export of mRNA, and cellular localization of mRNA (16). The average length of 3′-UTRs in *S. cerevisiae* was examined by several groups using different approaches and was found to be about 300 bp (17–20). This suggested that terminators in *S. cerevisiae* contain *cis*-regulatory elements with biological functions, and for example it is reported that *cis*-regulatory elements within the 3′-UTR and their cognate RNA-binding proteins

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may interact during post-transcriptional regulation to affect mRNA degradation and localization. In eukaryotes, the Pumilio-*fem-3*-binding factor (PUF) family comprises a set of RNA-binding proteins that regulate mRNA degradation and inhibit mRNA translation (21). In *S. cerevisiae*, the PUF binding motif is located in the 3′-UTR of the mRNA derived from many genes, and it has been shown that several PUF proteins are involved in the promotion of mRNA degradation (22–26). Another RNA-binding protein, She2p, binds to the mRNA of the transcriptional regulatory Ash1 protein (*ASH1*) at four sites, one of which is located in the 3′-UTR in *S. cerevisiae* (27–29). The *ASH1* mRNA–She2p complex recruits another adapter protein, She3p; interacts with the motor protein Myo4p; and then the *ASH1* mRNA localizes to germinating daughter cells (28). An estimated 567 RNA-binding protein genes exist in the genome of *S. cerevisiae*; however, the functions of most of these genes remain unknown (30).

TERMINATOR ACTIVITY AND THE MODULATION OF PROTEIN EXPRESSION

Around 30 years ago, simulation using a simple dynamic model revealed that the longer the half-life of mRNA, the higher the expression of the encoded protein (31). More recently, DNA microarray analysis has shown that the half-life of *S. cerevisiae* mRNA ranges from 3 to 90 min (average, 23 min; median, 20 min) (32). In *S. cerevisiae*, it has been shown that the 3′-UTR of the *MFA2* gene encoding the mating pheromone is a determinant of the half-life of its mRNA and it confers a half-life of only 3.5 min (33–35). When this 3′-UTR was fused downstream of the *PGK1* gene, the half-life of *PGK1* mRNA was reduced by 50% compared with that of the mRNA of the wild-type gene (34). This suggests that whereas some terminators reduce mRNA half-life, others may increase it and would be useful for constructing yeast strains with increased protein production.

In a comprehensive analysis of the half-lives of mRNAs derived from 4987 *S. cerevisiae* genes, the mRNAs derived from four genes involved in trehalose metabolism (*TPS1*, *TPS2*, *TPS3*, *TSL1*) were found to have remarkably long half-lives (105 min) (32), and it was hypothesized that the terminators of these four genes may enhance protein production by other genes. To verify this possibility, a series of recombinant yeasts harboring constructs each containing one of these terminators downstream from the coding regions of the green fluorescent protein reporter gene and *TDH3* promoter were constructed. Fluorescence intensity was used as an index of terminator activity, with a strain harboring the *PGK1* terminator (the *PGK1t* strain) used for comparison (36). Although the *TPS2t*, *TPS3t* and *TSL1t* strains had comparable terminator activities with the *PGK1t* strain, and the *TPS1t* strain had 20% greater terminator activity than the *PGK1t* strain, indicating that mRNA half-life and terminator activity are not always correlated.

In a separate study, the activities of 5302 terminators derived from approximately 90% of the genes of *S. cerevisiae* were measured, again by using green fluorescence as an index of terminator activity but this time a *CEN*-based single-copy plasmid was used instead of genome insertion (37). The top-5 terminators ranked by activity (Table 1) showed around 2.5 times the activity of *PGK1t*, whereas the weakest *GIC1t* showed only 0.04 times the activity. In addition, the dynamic range of terminator activity was as narrow as about 70 times, whereas that of the promoter was several-thousand times.

Independent of the two previous studies (36,37), terminator activities in 30 and 100 species of *S. cerevisiae* were evaluated by using the similar method but the somewhat different experimental materials (e.g., parent strain, medium and reporter genes) (38,39). In these studies, the relative terminator activities measured were comparable with the results of the previous study (37). In addition, terminator activity was evaluated by using different promoters (inducible promoter, constitutive strong promoter, and weak promoter), and the results showed that terminator activity is not promoter-dependent. The effects of various promoters, reporters, and carbon sources on the activities of the top-5-ranked terminators (*RPL41Bt*, *RPL15At*, *DIT1t*, *RPL3t*, and *IDP1t*) have been investigated, and *DIT1t* was found to have the highest activity under almost all of the conditions examined (40). The *DIT1* gene is involved in the synthesis of a spore cell-wall component (41,42) and its induction of gene expression is strictly regulated only at specific stages of sporulation (43). In addition to the inducible *DIT1* promoter, another mechanism may also exist to tightly control the production of Dit1 protein. Thus, we hypothesized that the time-specific yet transient production of Dit1 protein is presumably attributed to the strong activity and the special features of the *DIT1* terminator (described later).

APPLICATION OF TERMINATORS WITH STRONG ACTIVITY IN METABOLIC ENGINEERING

The strong terminators identified in the studies discussed in the previous section have been used to produce metabolically-engineered yeast strains (Table 2) (38,40,44–48).

The first use of terminator selection for metabolic engineering in *S. cerevisiae* was the construction of a xylose-assimilating strain (38). *S. cerevisiae* harboring a gene expression cassette containing either *CPS1t* or *PRM9t*, both of which have strong activity, downstream of the xylose isomerase gene (*xylA*) from *Piromyces* sp. were cultivated in a medium containing xylose as the carbon source, and both strains grew faster than a strain containing well-used *CYC1t*.

Next a strain of *S. cerevisiae* able to degrade cellulose was produced. *S. cerevisiae* cannot directly assimilate cellulose derived from plant biomass, so expensive pretreatment with cellulase is needed to saccharify cellulose when fermenting ethanol from biomass. Therefore, ethanol production costs could be decreased with the construction of a cellulase-secreting strain of *S. cerevisiae*. Strains of *S. cerevisiae* harboring the gene from *Trichoderma reesei* that encodes cellulose endoglucanase 2 (*TrEG2*) were prepared with the top-5-ranked terminators and cultured until the stationary phase (40). The cellulase activities of the culture media of the strains harboring these terminators were more than 20% higher than those of control strains harboring *ADH1t* or *CYC1t*. The cellulase activity of the strain harboring *DIT1t* was particularly high, about twice that of the *CYC1t* strain. In other studies, strains of genetically modified yeast secreting cellobiohydrolases (CBH1 from *Talaromyces emersonii* and CBH2 from *Phanerochaete chrysosporium*), which are enzymes that perform an exoglucanase reaction alone or in combination with EG2, were prepared (Table 1) (44,45). Strains harboring the high-activity terminators *RPL41Bt* and *DIT1t* secreted

TABLE 1. Representative terminators classified by activity.

Strong	Moderate	Weak	References
RPL41Bt, RPL15At, DIT1t, RPL3t, IDP1t	*ADH1t, CYC1t, PGK1t*	*GIC1t, ICY2t, MFA2t*	Yamanishi et al. (37) and Ito et al. (40)
PRM9t, CPS1t, HIS5t, SPG5t	*SPO1t, VPS13t, CYC1t*	–	Curran et al. (38)
HOG1t	*SDH1t, RAD53t, SOR1t*	*RAD14t, TDH3t*	Wei et al. (39)

TABLE 2. Gene and terminator combinations in metabolically engineered yeast strains.

Metabolic pathway	Enzyme genes	Terminator	References
Xylose isomerization	*xylA*	*CPS1t*, *PRM9t*	Curran et al. (38)
Cellulose degradation	*TrEG2*	*RPL41Bt*, *RPL15At*, *DIT1t*, *RPL3t*, *IDP1t*	Ito et al. (40)
Cellulose degradation	*CBH1*, *CBH2*, *TrEG2*	*RPL41Bt*, *DIT1t*	Ito et al. (44,45)
Acyclic sesquiterpene (nerolidol) production	*ERG10*	*NAT1t*	Peng et al. (46)
	ERG13	*EFM1t*	
	tHMG1	*EBS1t*	
	ERG12	*NAT5t*	
	ERG8	*IDP1t*	
	MVD1	*PRM9t*	
	IDI1	*RPL15At*	
	ERG20	*RPL3t*	
	AcNES1	*RPL41Bt*	
	EfmvaS	*EFM1t*	
	EfmvaE	*EBS1t*	
Fatty acid biosynthesis	*3hpcs*, *prpE*, *pccE*	*ACT1t*	Krink-Koutsoubelis et al. (47)
	aibC, *accA1*, *ter*	*ADH1t*	
	mcr, *pccB1*	*CYC1t*	
	paaH1	*ENO1t*	
	aibB, *acr*, *ccr*, *pccB1*, *birA*	*HXT7t*	
	aibA, *crt*	*PGK1t*	
	bktB	*SSA1t*	
	liuC, *3hpcd*, *accA1*, *prpE*	*TDH3t*	
Nitrogenase	*nifH*, *nifS*, *nifM*, *nifD*, *nifK*, *nifE*, *nifN*, *nifB*, *nifU*	*ADH1t*, *BUD6t*, *CYC1t*, *DPP1t*, *ECM10t*, *EFM1t*, *NAT1t*, *PRM9t*, *RPL15At*, *RPL41Bt*, *RPS14At*, *TIP1t*, *VMA16t*, *VMA2t*, *YHI9t*, *YOL036Wt*, *ADH2t*	Burén et al. (48)

more cellulase than strains harboring well-used terminators, and their ability to assimilate cellulose was also higher.

The terpenoids are a class of biological substances that contain isoprene, a 5-carbon alkene, as a constitutional unit (49). More than 40,000 terpenoids have been identified, and many terpenoids, such as the antimalarial drug artemisinin, have useful functions (50). The mevalonate pathway, the synthetic pathway of many common terpenoid precursors, consists of a multi-step enzymatic reaction. Therefore, improving metabolic flux within the mevalonate pathway would help improve the production of many terpenoids, whereas in the most important example of metabolic engineering modification of *S. cerevisiae*, i.e., artemisinin-producing yeast, only *CYC1t* and *TDH1t* were used (51). Recently, to produce a yeast strain capable of producing the acyclic sesquiterpene nerolidol (46), 11 enzyme genes, including 8 mevalonate pathway enzyme genes, under the control of 11 strong terminators were introduced into *S. cerevisiae* (Table 1). A high production of nerolidol was observed, indicating that these strong terminators may be useful for developing yeast strains producing various other terpenoids.

The development of yeasts that produce 10–20-carbon alkanes, which are the main components of light oil, is an attractive research subject in metabolic engineering. The surface of plant leaves is covered with a wax that suppresses evaporation, and alkanes are a main component of this wax. Therefore, it was hypothesized that plants must contain enzymes that produce alkanes or free fatty acids directly from acyl-coenzyme A, and, indeed, an alkane synthase that uses acyl-coenzyme A as its substrate was identified in the model plant *Arabidopsis thaliana* by using a budding yeast expression system (52). Building on this finding, an alkane-producing yeast was developed that harbored 19 fatty acid synthesis pathway genes under the control of 8 different terminators to improve metabolic flux within the fatty acid synthesis pathway (47). Most of the terminators used in designing this yeast were derived from genes involved in glycolysis.

TERMINATOR SELECTION FOR OPTIMIZATION OF GENE EXPRESSION

To maximize the amount of target product obtained from a metabolically engineered yeast, transgene expression must be optimized. There are two ways to do this: the first is through rational design using metabolic flux analysis and simulation techniques (53), and the second is through combinatorial screening of combinations of promoters and terminators (54); both approaches require a library of gene-expression cassettes with a wide dynamic range.

To demonstrate the feasibility of the combinatorial screening approach, a library of 32 gene-expression cassettes with a dynamic range of 30,000 times was constructed by combining 11 promoters and 5 terminators (*RPL41Bt*, *CYC1t*, *MFA2t*, *STD1t*, and *GIC1t*) of different strengths (44). Four of these cassettes, which contained 4 different artificial promoters and 2 different terminators (*CYC1t* and *RPL41Bt*), were then used to produce a series of recombinant yeast strains harboring three different cellulase genes (*CBH1*, *CBH2*, and *TrEG2*) (45). After three separate transformations of each gene, 368 strains of genetically modified yeast with high to low activities were obtained.

To ensure that heterologous multi-unit enzyme complexes function correctly inside cells, the amount of each enzyme subunit must be controlled stoichiometrically. However, such optimization is difficult. One such complex is a nitrogenase enzyme found in several microorganisms that fixes nitrogen in the air as ammonia, which can then be used as fertilizer (55). Currently, most ammonia is produced via the Haber–Bosch process, which requires a large amount of energy. Therefore, establishing an ammonia production process using metabolically engineered yeast strains would be useful for reducing energy consumption. In *S. cerevisiae*, the heterologous expression of 9 genes (*nifHDKUSMBEN*) encoding the subunits of a nitrogenase complex isolated from prokaryote *Azotobacter vinelandii* was examined (48). Ninety-four strains were produced via a combinatorial approach using 29 promoters and 18 terminators, and protein production by these strains was optimized for each of the 9 different nitrogenase genes. Developing the method used for the above research (48), the same group devised a method of gene-expression cassette selection in which part of the expression of a target transgene is determined and a model is used to statistically estimate the rest (56). By using this method, a library of gene-expression cassettes was constructed with a dynamic range of 174 times depending on the promoter–terminator pair. Then, by using this library, 6 heterologous genes involved in the biosynthetic pathway of itaconic acid, which is a raw material used in the production of resins and plasticizers, were introduced into *S. cerevisiae*, and combinatorial screening was performed to obtain a strain that produced a high yield of itaconic acid (1.3 g/L) (56).

USE OF TERMINATORS FOR GENETIC SWITCHES

The higher the time-resolution for controlling the expression of a transgene, the more precise the observation of the targeted phenomenon can be. For this reason, various synthetic–biological genetic switches have been developed (57,58), one of which uses a site-specific DNA recombinase (Cre) and its recognition sequence (*loxP*) (59,60). In these Cre–*loxP* genetic switches, when expression of the Cre recombinase is transiently induced, *loxP* sequence-specific recombination occurs and the promoter and target gene cDNA coding region, which are separated by a spacer sequence, are brought into proximity, resulting in expression of the target gene (59).

Using such a genetic switch, our group has been able to control the expressions of two different transgenes (61). A combination of a strong inducible *GAL* promoter and a weak *MFA2* terminator was used to transiently express the Cre gene. To accelerate expression of the target gene, *TPS1t*, which has relatively strong activity, was placed downstream of the target gene (Table 3). Initially, the yeast produced ethanol via pyruvate decarboxylase using pyruvate as the substrate; however, after the switch was activated the yeast produced lactic acid via lactate dehydrogenase using the same substrate.

To accelerate the expression of a target gene after recombination, two different types of genetic switches in which a strong terminator (*DIT1t*) was used have been reported: one is a protease-type genetic switch inside the cell (62), and the other is a membrane receptor–type genetic switch that is activated via signal transduction from a membrane receptor (63).

Protease-type genetic switches function by digesting the gene product to be controlled, which genetically fuses a protease-recognition peptide to the target protein. Therefore, a prerequisite for this type of switch is the strict control of the induction of the cognate protease gene. In one study examining this type of switch, the function of a group of genes hypothesized to be involved in meiosis was investigated (62). Many of these genes were known to be essential for vegetative growth, so in many cases it was impossible to produce disruptants. Even if a temperature-sensitive mutant was obtained, it would have been difficult for the gene to lose its function specifically in meiosis. Therefore, to elucidate the functions of these genes in meiosis, a genetic switch capable of rapidly degrading these gene products specifically at the sporulation stage was used. Genetically engineered yeasts were produced in which these genes were replaced with a copy of the same gene fused with the degradation tag (TDegF) sequence recognized by tobacco etch virus (TEV) protease (Table 3). To obtain transient yet powerful induction of TEV protease gene expression, a gene-expression cassette containing the sporulation-specific promoter *IME2* and the strong terminator *DIT1* was used. The protease-inducing effect of the terminator was then compared with that of *CYC1t*; TEV protease activity in the strain harboring *DIT1t* was about 3 times that in the strain harboring *CYC1t*. This finding is consistent with the results using *DIT1t* for the expression of fluorescent proteins and cellulases (62).

TABLE 3. Terminators used to improve gene switch performance.

Type of genetic switch	Activated genes	Terminator	Reference
Cre-loxP	*Cre*	*MFA2t*	Yamanishi et al. (61)
	mKO2, LDH	*TPS1t*	
Protease-dependent degron	*TEV*	*DIT1t*	Renicke et al. (62)
GPCR signaling	*ymUkG1*	*DIT1t*	Fukuda et al. (63)

G-protein-coupled receptors (GPCRs) are seven-transmembrane receptors that bind to ligands such as extracellular neurotransmitters and hormones to transduce signals across the cell membrane (64). More than 800 GPCRs have been identified in the human genome and they form the largest known gene family. Around 50% of GPCRs are odor receptors; however, the ligands of many GPCRs are unknown. Currently, 30–40% of marketed drugs target a GPCR, and therefore GPCRs are very important targets in drug development (65). Because it is very expensive to screen drug candidates for GPCR antagonist activity by using human cells, many researchers are exploring the use of inexpensive screening methods using yeasts (66). It has been reported that incorporation of the *DIT1* terminator into a membrane receptor–type of genetic switch improved the performance of a yeast-based system for detecting human GPCR antagonists (63). In this genetic switch, expression of the fluorescent reporter gene *ymUkG1* is suppressed because guanosine triphosphate is bound to the α-subunit of GPCR in the presence of the agonist; conversion of guanosine triphosphate to guanosine diphosphate in the presence of a G-protein antagonist produces a heterotrimer that releases the suppression of the expression of the *ymUkG1* gene. Then, by using fluorescence intensity as an index of antagonist response, it can be determined whether the target chemical is an antagonist of GPCR. In the improved yeast-based system, a yeast strain harboring *DIT1t* downstream of *ymUkG1* gene showed approximately twice the fluorescence intensity than a strain harboring *ADH1t*.

MECHANISM OF ACTION OF HIGHLY ACTIVE TERMINATORS

The main way in which terminators increase or decrease protein production of the upstream gene is generally believed to be via their effect on the 3′-UTR–dependent half-life of mRNA. It is thought that decomposition by 3′–5′ exonuclease is affected by steric hindrance resulting from the tertiary structure formed by the 3′-UTR of the mRNA and RNA binding proteins (67). Gene looping, in which the promoter and terminator regions are physically associated, allowing for efficient use of the same transcription complex, has also been proposed as an alternative mechanism (68).

Through a post-transcriptional mechanism other than the one described above, in *S. cerevisiae* the strong *DIT1* terminator was shown to increase the protein expression of a gene encoded upstream (69). In a previous study (40), *DIT1t* activity was shown to increase from the logarithmic growth phase to the stationary phase, suggesting the existence of a *DIT1t*-activating factor. Indeed, two *DIT1t*-activating genes were subsequently identified by using a reverse genetics approach (69) and both were found to be RNA-binding proteins: Nab6 (nucleic acid-binding protein 6) and Pap1 (poly(A) RNA polymerase). Together with previous biochemical analysis results (70), it was concluded that Nab6 protein specifically binds to the *cis* sequence in the *DIT1* 3′-UTR to recruit Pap1 protein, which increases expression of the upstream gene. However, in a *DIT1t* strain in which the *NAB6* and *PAP1* genes were simultaneously overexpressed, only a 10% increase in mRNA expression of the upstream gene was observed, even though the protein production was increased by more than doubled. This suggests that mRNA degradation and gene looping do not trigger *DIT1t* activation. Currently, the factors involved in the activation of *DIT1t* via the *NAB6* and *PAP1* genes are hypothesized to be post-transcriptional but remain unknown.

Another proposed mechanism underlying high terminator activity is improvement of transcription termination efficiency as a result of reduction of nucleosome formation in the terminator region (71–73). As discussed in the Basic functions of the terminator section, the terminator region contains three functional elements that are essential for transcription termination (efficiency element, the positioning element, and polyadenylation signal), and these elements are adenosine and thymine rich. In the eukaryotic genome, nucleosome formation decreases in the terminator region at locations where there are many adenosine and thymine bases, and this decrease is associated with high transcriptional activity. This knowledge has been used to develop highly active artificial terminators (74).

CHALLENGES IN ARTIFICIAL TERMINATOR DEVELOPMENT

As Alper and colleagues assert, it may be possible to construct an artificial terminator that is better than natural terminators by considering the following 5 points (38,74). (i) Artificial terminators should contain nucleotide sequences with no homology with natural terminators to avoid unintended homologous recombination. (ii) Artificial terminators should be shortened to 50 bp or less so that the energy required for mRNA synthesis and degradation is reduced, resulting in a reduction in fermentation production cost. (iii) Artificial terminator libraries containing terminators of various strengths should be constructed for rational design and combinatorial screening. (iv) Artificial terminators will likely be effective not only in *S. cerevisiae* but also in other eukaryotes, including non-conventional yeasts, because transcription termination is a well-conserved fundamental process. (v) Artificial terminators will likely not severely disturb host cell function because they will have fewer interactions with endogenous proteins and nucleotides than natural terminators.

In a previous study (15), the minimum length for an artificial terminator is 39 bp, and 30 artificial terminators with lengths from 35 to 70 bp for use in *S. cerevisiae* have been fabricated (74). These artificial terminators have activities of various strengths, with the most active showing 3.7 times the activity of *CYC1t*; it was demonstrated that some of these artificial terminators function normally in the non-traditional yeast *Yarrowia lipolytica*.

To prepare a recombinant yeast that produces a target substance via a multi-step enzymatic reaction, it is necessary to introduce into the genome a construct linking several gene-expression cassettes. Gap-repair cloning by homologous recombination has been used for a long time in *S. cerevisiae* (75) and it is an efficient way of linking DNA fragments. Promoters and terminator sequences located close to homologous recombination sites at the ends of DNA fragments are required to have low homology with other DNA fragments, and therefore an artificial terminator capable of designing its sequence in a less-restrictive manner was proposed to satisfy this requirement. This idea was examined using 10 artificial terminators, and its effectiveness was demonstrated (76).

DIT1 terminators with point-mutated or deleted regions that have higher activity than wild-type *DIT1t* have been reported (69). The effect of *DIT1t* activation by overexpression of the *NAB6* and *PAP1* genes is 5 times that of the standard expression obtained with *PGK1t*. Since the growth of this overexpressing strain is conspicuously delayed, attention is considered necessary for high production of the target protein by overexpressing these genes. In a strain of *S. cerevisiae* without *NAB6* and *PAP1* overexpression but harboring *DIT1t* containing two deletion mutations, it was found that protein production was increased up to 1.5 times that in a strain harboring wild-type *DIT1t*, without affecting growth (69). This *DIT1t*–d22 terminator has the strongest activity reported to date in *S. cerevisiae.*

FUTURE PERSPECTIVE

Terminator technology is a promising means of developing improved yeasts for the production of many useful organic compounds. The usefulness of metabolic engineering has been demonstrated for the production of terpenoids, and this approach is beginning to spread to the development of metabolically engineered yeasts for the production of alkaloids and polyketides, which are often useful medicinal substances, and flavonoids, which are useful as pigments, via multistage enzymatic reactions (77,78). Thus, development of artificial terminators is expected to increase rapidly in the near future, and it is hoped that an artificial terminator with an activity that greatly exceeds that of existing terminators will be reported soon.

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