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Recent Progress on Systems and Synthetic Biology Approaches to Engineer Fungi As Microbial Cell Factories

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Abstract: Filamentous fungi are remarkable organisms naturally specialized in deconstructing plant biomass and this feature has a tremendous potential for biofuel production from renewable sources. The past decades have been marked by a remarkable progress in the genetic engineering of fungi to generate industry-compatible strains needed for some biotech applications. In this sense, progress in this field has been marked by the utilization of high-throughput techniques to gain deep understanding of the molecular machinery controlling the physiology of these organisms, starting thus the Systems Biology era of fungi. Additionally, genetic engineering has been extensively applied to modify well-characterized promoters in order to construct new expression systems with enhanced performance under the conditions of interest. In this review, we discuss some aspects related to significant progress in the understating and engineering of fungi for biotechnological applications, with special focus on the construction of synthetic promoters and circuits in organisms relevant for industry. Different engineering approaches are shown, and their potential and limitations for the construction of complex synthetic circuits in these organisms are examined. Finally, we discuss the impact of engineered promoter architecture in the single-cell behavior of the system, an often-neglected relationship with a tremendous impact in the final performance of the process of interest. We expect to provide here some new directions to drive future research directed to the construction of high-performance, engineered fungal strains working as microbial cell factories.

Keywords: Systems biology, Synthetic biology, Fungal engineering, Regulatory networks, Synthetic promoters.

1. INTRODUCTION

The field of Systems Biology targets the collection and assembly of information contained in the natural biological systems at many scales of complexity, from small molecules and biochemical reactions, toward pathways, cells and tissues [1, 2]. In the case of fungi, which have natural systems to efficiently degrade and utilize lignocellulosic biomass, the study of these systems perfected by nature for millions of years gives the possibility to integrate the mechanisms for biomass utilization into bioprocess for biofuel production. For this purpose, the high-throughput approaches, collectively known as 'omics', enable a comprehensive view of the cell and make possible the discovery and understanding of its functionalities. Several genomic approaches, together with transcriptomic and proteomic analyses, have enabled the investigation of the intricate regulatory network coordinating the expression of cellulases when organisms are exposed to complex biomass material [3]. As cells can alter the pattern of gene expression in response to changing environmental conditions, investigating their behavior using omics tools allows understanding how their networks operate. In this sense, this new data can provide a crucial knowledge to perform genetic modifications in organisms with enhance performance for biotechnological applications [4, 5].

2. SYSTEMS BIOLOGY APPROACHES TO UNDERSTAND CELLULASE EXPRESSION IN FUNGI

In filamentous fungi, plant biomass exposure triggers the expression of many proteins (hydrolases, accessory enzymes and accessory proteins) that deconstructs this complex material into simpler sugars [4, 6]. Cellulose, hemicellulose, pectin and lignin are the major components of the plant cell wall. These distinct portions of the cell wall are formed by diverse constituents linked one to another in different orientations, consisting in polymers formed by sugar backbones (glucose, xylose, manose, galactose, rhamnose, etc) or polygalacturonic acid backbone. These structures can link other sugars (such as arabinofuranose and fucose) or methyl/acetyl groups, or even be impregnated with aromatic compounds such as ferulic acid [7]. Cellulose (chain made by the linking of β-D-glucose) comprises the core of the plant cell wall and is embedded with hemicellulose. Pectin overlays the hemicellulose and fills the space between celullose and lignin, the last layer. To degrade lignocellulosic material, many fungi are endowed with cellulases, pectinases, esterases, lyases and accessories proteins with carbohydrate binding domains [4]. Additionally, there are swollenins and auxiliary redox enzymes, which are also required for efficient cellulose degradation. Swollenin is a new class of protein that

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works as expansin-like polypeptides with cellulose disrupting abilities [8]. In turn, auxiliary redox enzymes comprise accessory proteins that acts on cellulose or lignin in an oxidative way, grouping together polysaccharide monooxygenases and redox enzymes involved with lignin breakdown, integrating a new group of CAZy enzymes named Auxiliary Activities [9].

The difference in composition of the plant cell wall contributes to the induction of several groups of genes related to biomass deconstruction. In fact, genomic and transcriptomic studies in *Trichoderma reesei* allowed the identification of a group of genes especially induced when this organisms is exposed to cellulosic material (such as Avicel, pretreated spruce or complex plant material containing cellulose) [10]. Many of these genes are specifically induced in the presence of sophorose, a glucose disaccharide formed by a transglycosylation reaction from cellobiose during cellulose hydrolyses [10-12]. Additionally, many other genes are mainly expressed in presence of xylans [10]. Yet, in the same way that gene induction depends on biomass material composition, different organisms have specific responses to the plant cell wall components, as in the case of *Neurospora crassa* where xylan induces hemicellulose but not cellulase-related genes [13]. In this sense, while many of the regulatory players are present in different fungal species, the regulatory network of each organism has its own particularities that should fulfill the specific requirements of the host [4, 6].

From a more biochemical point of view, the degradation of cellulose can be described in three main steps: (i) endoglucanases attacks the polymer backbone and releases sugar oligomers; (ii) exoglucanases or cellobiohydrolases generates the disaccharide cellobiose and; (iii) β-glucosidases cleaves cellobiose into two glucose molecules [4]. Regarding the production of biomass-degradation enzymes, the filamentous fungi *T. reesei* is considered the major cellulase-producer organisms [10]. For instance, a quantitative secretome study revealed that about 50% of the proteins secreted by this organism when exposed to lignocellulose material were cellulose hydrolyzing and hemicellulose degrading enzymes [14]. Additionally, when *T. reesei* is exposed to sophorose, cellulases are expressed in a higher level than when exposed to glucose, cellulose or glycerol [11, 12, 15]. However, sophorose is not a good inducer in *Neurospora* or other filamentous fungi [4, 16]. By the same token, Castro and co-workers have identified four sophorose-induced genes encoding proteins that were specific to species of *Trichoderma*, suggesting that this organism have a specialized sophorose metabolization system [11]. In another quantitative proteomic approach, the analysis of the proteins secreted by *N. crassa* grown in cellulose revealed that only 13 proteins comprises 91% of the total *N. crassa* secretome [17]. Between identified proteins, four of them were of particular interest, since they represent 65% of extracellular proteins: CBH-1 (cellobiohydrolase, CAZy family GH7), GH6-2 (cellobiohydrolase, CAZy family GH6), GH5-1 (endoglucanase, CAZy family GH5) and GH3-4 (β-glucosidase, CAZy family GH3). The fifth more abundant protein found in *N. crassa* secretome was a new player GH61 protein, which is a copper dependent polysaccharide monooxygenase that cleaves cellulose in an oxidative manner [18]. The accumulation of data regarding gene regulation and protein production in fungi unequivocally demonstrate that the expression of genes related to biomass degradation in these organisms is tightly controlled at the transcriptional level, where a highly hierarchical regulatory network is responsible for the integration of multiple external and internal signals to control the systems response [4, 6]. Furthermore, another important observation from these studies is that genes related to lignocellulose depolymerization respond to plant biomass in a coupled manner, forming the so-called "regulons". One example of this process is the "Avicel regulon" found in *N. crassa* [16], where 212 genes have increased expression in response to Avicel. More importantly, more than 50% of the proteins encoded by these 212 genes are predicted to enter to the secretory pathway [16].

In addition to the efforts to quantify the global response of fungi to complex biomass material, many works have identified regulatory proteins able to coordinate these transcriptional responses. In this sense, XYR1 is the major transcriptional activator of genes encoding hydrolases in *T. reesei*. In fact, deletion of the *xyr1* gene abolished the induction of cellulases by cellulose and sophorose and decreased the induction of hemicellulases genes involved in the degradation of xylan and arabinan [19]. In the same way, Häkkinen and co-workers (2014) described the gene *ace3* (activator of cellulase expression 3), whose deletion abolish completely cellulase activity against methylumbelliferyl-β-D-lactoside [20]. In *N. crassa*, major activators of cellulase production are CRL-1 and CRL-2 transcription factors [21, 22]. Deletion of these regulators showed loss of inducible cellulase gene expression and no activity against carboxymethyl cellulose, but hemicellulase activity on xylan was not affected [21]. Furthermore, Coradetti *et al.* (2012) suggested that CLR-1 is activated by cellobiose or its products, which in turn promotes the expression of CLR-2 that induces cellulases and some hemicellulases for the depolymerization of plant material. In the case of *Aspergillus nidulans*, in which *clrA* and *clrB* are homologs of *clr-1* and *clr-2* of *N. crassa*, induction of cellulase genes required *clrB* but not *clrA* [21]. While these regulators have been proved as important for cellulase expression in *N. crassa* and *A. nidulans*, no evidence has been provided for their role in the regulatory network of *T. reesei*. In addition to specific transcriptional factors, expression of cellulase genes requires basal chromatin remodeling machinery in order to expose the regulated promoters to the transcriptional apparatus. In this sense, the HAP2/3/5 complex operates in the remodeling of DNA in *T. reesei* by binding to the CCAAT *cis*-element present in cellulase promoters, which is required for efficient gene expression [23, 24]. Alternatively, Lae1 is a methyltransferase that acts as regulator of cellulases in *T. reesei* [25-27], as well as the master cellulase regulator XYR1 [27].

In order to coordinate cellulase expression in response to environmental signals, filamentous fungi are endowed with a carbon catabolite repression (CCR) system represented by the CreA/CRE1 homologues [28-30]. During CCR, genes involved in degradation of complex carbon sources are repressed in the presence of high concentrations of glucose [29, 31]. CreA/CRE1 also affects genes related to nutrient transport and oxidative metabolism, as well as genes encoding other transcription factors [32-34], and its mechanism of action appears to relay on chromatin remodeling [35]. Ana-

lyzing the hyper-producer *Penincilium decumbens* JU-A10-T and the wild-type 114-2, Liu and co-wokers (2013) described a frame-shift mutation at the C-terminus of the *creA* gene that changed the sequence of the last 16 aminoacid residues of the CreA regulator. As in *T. reesei*, modification of de carbon repressor gene alters the perception of glucose in CCR of cellulolytic enzyme production [36]. Perhaps in connection with the control mechanisms related with CCR, it has been demonstrated for *A. niger* that starvation response can also lead to the production of hydrolytic enzymes such as chitinases and glucanases [34]. In this sense, these enzymes could perform scouting functions that could generate the specific inducers to trigger high expression level of hydrolytic enzymes when this organism is presented to a biomass material. Another environmental signal that plays a role in cellulase expression is represented by pH variations. In filamentous fungi, pH sensing is mainly performed by the Rim101p homologue PacC [37-41]. In *T. reesei*, pH variation has been demonstrated to influence cellulase expression through the PacC homologue TrPac1, since in neutral pH, the expression levels of *cbh1* (cellobiohydrolase), *bgl1* (β-glucosidase) and *egl1* (endoglucanase) increased in the deletion mutant Δ*Trpac1* [42]. Additionally, Castro and coworkers (2014) founded that this PacC homologue (protein ID 120698) was regulated in *T. reesei* in a carbon source dependent manner, showing a higher expression level in the presence of cellulose, reinforcing the potential role of this transcriptional factor in cellulase expression.

In addition to the above-mentioned transcriptional factors, several regulators have been implicated in the control of cellulase expression in filamentous fungi. For instance, ACE1 is a transcription factor that represses the expression of biomass deconstruction enzymes. Deletion of the *ace1* gene in *T. reesei* resulted in an increased expression of cellulases and hemicellulases genes in presence of cellulose and hemicellulose [43]. Conversely, deletion of *ace2* gene in *T. reesei* decreased the level of transcripts of cellulases and cellulase activity obtained on medium containing Solka floc cellulose from 30 to 70% [44], revealing its role as activator of cellulase expression. Also in *T. reesei,* BglR regulator induces genes encoding β-glucosidases that cleaves the cellobiose liberated by the depolymerization of cellulose, generating free glucose molecules [45]. Finally, the putative xylanase 2 repressor Xpp1 has been identified based on its capability to bind to the *xyn2* promoter of *T. reesei* [46], adding a novel player in the coordination of hydrolases expression in this fungus.

In general, 'omics' approaches (genomics, transcriptomics, proteomics, secretomics), along with classical genetic and biochemical techniques, have allowed important progress in the elucidation of the intricate network that conducts cell behavior during biomass degradation. Further development of these Systems Biology approaches to study the deconstruction and depolymerization capabilities of fungi will certainly make possible to generate a comprehensive global view of these networks. Moreover, the understanding of such networks related to cellulase expression in fungi will provide a solid ground for further engineering strategies aiming the construction of recombinant strains with superior performances in industrial processes.

3. ENGINEERING MICROBIAL CELL FACTORIES

The conversion of lignocellulosic biomass into fuels through biological processes has an enormous potential for energy production. In particular, the Consolidated BioProcessing (CBP), which combines cellulase production, cellulose hydrolysis and fermentation in a single step, has the potential to reduce considerably the cost of biomass processing due to the elimination of operating and capital costs associated with enzyme production and efficient biomass solubilization [47]. Even though no natural microorganism possesses all properties of lignocellulose utilization and ethanol production preferred for CBP, some bacteria, yeast and fungi display some of the needed features [48].

Currently, there are two main approaches to produce CBP microorganisms: the native strategy (also called category I CBP) which aims at engineering a cellulase producer strain to make it ethanologenic, and the recombinant strategy (category II CBP), which intends to engineer an ethanologen into a cellulolytic organism (Fig. **1**). It is important to highlight that, although there are numerous studies reported in literature about the two strategies, efforts have so far been focused mainly on CBP category II, especially in the bacteria *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca*, as well as in the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* [5, 49-52]. While several studies have reported successful production and secretion of different cellulolytic enzymes, as well as the growth of the microorganisms on lignocellulose as sole carbon source and proper sugar fermentation, there are still significant restrictions that need to be overcome. An example of a CBP category II microorganism extensively studied is *S. cerevisiae*, which have been reported to be able to grow on cellulose, hemicellulose, cellobiose, xylose and arabinose after its genetic modification [53-56]. In order to generate those engineered organisms, genes coding for cellulases, hemicellulases, β-D-glucosidases, as well as xylose-utilizing and arabinose-utilizing enzymes from various species have been introduced in *S. cerevisiae* [53-56]. However, expression of *T. reesei* cellobiohydrolases I and II (CBH I and CBH II) in this yeast is generally poor, which is the main limitation of these approaches since these enzymes play an essential role in cellulose degradation [57]. Yet, each of the principal constrains regarding the utilization of CBP category II microorganisms (as well as CBP category I) might be solved by using approaches based in Synthetic Biology (Fig. **1**). This relatively new research field provides a wide range of choices to manage the expression of multiple heterologous genes and modulate the performance of biosynthetic pathways to generate metabolically efficient organisms [58, 59].

The main goal of Synthetic Biology is to use well-standardized biological party (such as genes, promoters, regulators, enzymes, etc.) to construct new-to-nature circuits for some specific applications. For this, the biological parts are interconnected using specific assembly strategies [60-62] and usually mathematical modelling is used to generate the layout of interest in order to allow the circuit to work in a predictive way [63]. In this sense, biological circuit engineering tries to mimic the strategies used to construct electronic devices in order to reprogram the cell or the organism [62]. Currently, there are several examples of new biological

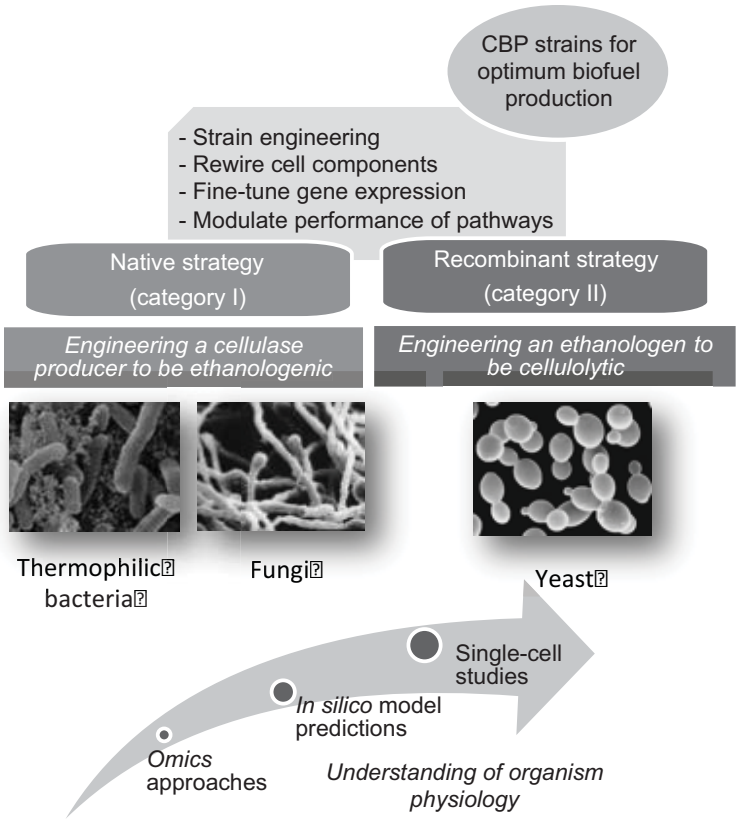

Fig. (1). Organism engineering strategies (native and recombinant) and related approaches to reach a successful CBP microorganism. Examples of the most promising candidates for starting each strategy are given, such as fungi and thermophilic bacteria for the native approach and yeasts for the recombinant one. While Systems Biology approaches supply the basis for understating metabolic microorganism functioning, Synthetic Biology provides new tools to implement the preferred features for effective production of biofuels.

circuits that have been successfully designed and implemented in microorganisms for biofuel production [58, 59, 64-67]. For instance, *S. cerevisiae* was engineered with the genes encoding xylose reductase and xylitol dehydrogenase from *P. stipitis* to enable it to utilize xylose for ethanol production [68]. However, experimental overexpression of both genes leaded to an imbalance of the cofactors NADH and NADPH, which was corrected by the use of predictions from metabolic models, leading to an increase in ethanol production and reduction in by-product synthesis [69]. In another elegant work, Steen (2010) and co-workers used synthetic operons in *E. coli* to produce structurally tailored fatty esters (biodiesel) directly from simple sugars. Furthermore, they showed the engineering of the biodiesel-producing cells to express hemicellulases. The further production of this biodiesel from hemicellulose, a major component of plant-derived biomass, demonstrates a necessary and promising achievement towards realizing a consolidated bioprocess [64].

In a different scenario, the key requirements and challenges to construct a CBP organism through the engineering of a natural cellulase producer include tasks such increasing the ethanol yield, the elimination of by-products and the improvement of ethanol tolerance, among others [48] (Fig. **1**). For instance, the cellulolytic thermophilic bacteria *Clostridium thermocellum*, *Geobacillus thermoglucosidasius*, *Thermoanerobacterium saccharolyticum* and *Thermoanerobacter mathranii* have been described as potential candidates for category I CBP [70]. The most successful example in this field is the engineering of *T. saccharolyticum*, which can normally ferment xylan and biomass-derived sugars [71]. The modified *T. saccharolyticum* strain can produce ethanol at high yield, and several organic acids are no longer detectable after the knockout of genes involved in their formation [71]. At this point, it is important to highlight that thermophilic organisms are endowed with enzymes that display a huge threshold of robustness and versatility. Moreover, there are a number of advantages regarding the utilization of a thermophilic over a mesophilic organism in an ethanologenic process. For example, the remarkable tolerance of thermophiles to stand fluctuations in pH and temperature, the easier downstream ethanol recovery at high temperatures, or the possibility to reduce energy costs, which is required to cool mesophilic fermentations [72]. However, while thermophilic bacteria have been proven as useful candidates, only fungi naturally produce the needed titers of cellulases required for the complete saccharification of pre-treated lignocellulose, as in the case of the mesophilic *T. reesei* [73]. This filamentous fungus is the favourite alternative as a CBP organism due to its high level of production and secretion of enzymes related to lignocellulose degradation. The extensive knowledge on its physiology and native cellulolytic machinery, as well as on the mechanisms involved in regulation of cellulase production [74-77] and the availability of tools for its genetic

manipulation, enhance its preference as a category I CBP organisms [78]. In addition, another remarkable feature is that *T. reesei* has all the metabolic pathways necessary to utilize all the lignocellulose sugars for the production of ethanol [79]. Yet, *T. reesei* also presents some challenges that must be addressed before it can become an efficient CBP organism. The main limitations are related to the low ethanol yield and rate of production, low ethanol tolerance, and difficulties during fermentation associated to its filamentous cell morphology (for more details, see [48]).

In addition to *T. reesei*, there are alternative fungi presenting a significant potential to become a CBP organism. For instance, filamentous fungi belonging to the genera *Neurospora*, *Aspergillus*, *Rhizopus*, *Paecilomyces* and *Fusarium* have been reported to hold the ability to directly ferment cellulose to ethanol [79-81]. As an example, several studies have shown the potential of *Aspergillus* spp. in (hemi) cellulases production at industrial scale, since *Aspergillus* species are the major agents of hemicellulose decomposition and thus possess the capability to produce a broad range of (hemi)cellulolytic enzymes [82]. Yet, the main limitations of these strains consist in very low ethanol yields and the formation of the by-product lactate. On the other hand, strains of the genera *Fusarium* (e.g. *F. oxysporum*) show a robust cellulose degradation capability and present the particularity of producing cellulases able to work in a broad range of temperature and pH, which add a difference from the usually used sources of cellulases from the *Aspergillus* and *Trichiderma* spp [83]. In addition, as *F. oxysporum* produce both cellulases and xylanases, it is not necessary to perform a separate enzymatic hydrolysis of the lignocellulosic raw material for bioethanol production [84-88]. Still, main limitations of using *F. oxysporum* as a CBP organism are the slow conversion of cellulose to ethanol and formation of significant amounts of acetic acid as a by-product. Nevertheless, several studies showed promising results when homologous or heterologous overexpression of different enzymes were performed in *F. oxysporum* to increase ethanol production [89-91].

Finally, it is clear that significant advances have been made in organism development for CBP, while at the same time essential problems have still to be solved. For both strategies, it is necessary a better understanding of microorganisms physiology under a variety of conditions (i.e., ethanol exposure, growth on different lygnocellulosic materials as carbon source, under oxygen limitation, etc) in order to perform subsequent strain improvements. However, such a task cannot be solved by using a single approach and requires the synergic implementation of multiple methodologies (Fig. **1**). In this sense, Synthetic Biology can provide new tools to rewire the cell components (promoters, regulators, terminators, enzymes, operons, transporters, etc) in order to reach the desired features for the production of economically viable biofuels. Yet, continuous advances in Systems Biology approaches (e.g., *omics* studies, single-cell approaches, *in silico* model prediction of new pathways) properly combined with approaches based in Synthetic Biology will help to better understand and optimize CBP candidate organisms for optimum production of biofuels (Fig. **1**).

4. SYNTHETIC PROMOTER ENGINEERING FOR FUNGAL BIOTECHNOLOGY

When considering the engineering of microorganisms for a particular application, perhaps the first strategy that comes to the mind of any researcher is the development of a more efficient expression system. In this sense, heterologous gene expression in prokaryotes [92] and eukaryotes [93] allowed the production of native and recombinant proteins that now are broadly used in medical, biopharmaceutical, biotechnological, engineering, food and chemical industries [94, 95]. Several characteristics should be considered when aiming the improvement of gene expression to produce an economically viable bioprocess [96, 97]. Firstly, the appropriate host strain must be selected based on features such as the ability to sustain fast growth on a cheap media, the ability to be genetically modified and, for some applications, the capability to achieve post-transcriptional modification of enzymes. In the second place, selection between chromosomal or plasmid-based expression systems is also relevant [98]. Additionally, the recombinant protein should be easily extractable in a minimum number of steps. Finally, the strain must be considered as safe for its application to produce a recombinant protein [94, 95]. Historically, engineering of microorganisms has focused on the modification of single genes in a very try and error approach [99], but more recently, Synthetic Biology has developed an overriding role in genetic engineering of living organisms [100, 101]. Below, we present some useful concepts on the engineering of biological systems and their application in Fungal Synthetic Biology.

4.1. Synthetic Biology to Design Genetic Circuits

As discussed above, the field of Synthetic Biology is mainly characterized by the integration between several engineering disciplines, such as chemical and electrical engineering, and biological sciences including biophysics, biochemistry and molecular cell biology [99, 100]. Its inherent diversity allows the design, manipulation and construction of new biological parts, devices, systems, as well as the understanding and re-design of existing natural biological systems in order to construct new regulatory networks in the cell [101-106]. Some of the first synthetic genetic devices successfully constructed and characterized were implemented in *E. coli*, such as the "repressilator" and the "toggle switch" [107-110]. After those initial attempts, others synthetic parts were engineered and characterized in a number of eukaryotic hosts, such as *Dictyostelium, Saccharomyces spp., Pichia spp., Yarrowia* and *Mus musculus* [111-115]. In order to construct more robust and higher-order genetic circuits with predictable activity, it is need first to understand how the biological parts of a network operate in isolation [102, 104, 105]. In this sense, transcription, the dynamics of protein translation and its interaction with acid nucleic (DNA or RNA), are the main processes that need to be considered in a biological circuit [100]. In eukaryotes, transcription has been considered the main mechanism to regulate gene expression, usually considered as a "switch" system [116]. This "ON-OFF" regulation mechanism is modulated by the interaction between *cis*-acting elements located upstream of the transcription start site (SST) with *trans*-acting factors [116-118]. In this regard, one of the focus of Synthetic Biology in eukaryotes is the development of strong and controllable pro-

moter elements that enable the fine-tuning of gene expression in the organism of interest [95, 119]. In this sense, several approaches have been implemented to modify/engineer eukaryotic promoters, and some of particular interests are discussed below.

4.2. Generating Tunable Promoters to Synthetic Biology Uses

4.2.1. Classic Approaches

The most classical way to obtain suitable promoters for metabolic engineering has been the identification of promoter elements displaying strong or controllable activities under the experimental conditions of interest (e.g., during the fermentation of a carbon source or after exposure to a compound of interest). In this sense, several native promoter sequences have been isolated from eukaryotic species and used to construct expression system for these organisms. This is true for example for *P. pastoris*, a methylotrophic yeast specie widely used for heterologous protein production. By using classic methodologies, several strong promoters have been identified and characterized, such as the alcohol oxidase I (AOX1) promoter [120], the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter [121], the translation elongation factor 1 (TEF1) promoter [122], the glutathione-dependent formaldehyde dehydrogenase (FLD1) promoter [123], the phosphate-responsive (PHO89) promoter [124] and the glycolyticenzyme3-phosphoglycerate kinase (PGK1) promoter [125]. For the filamentous fungus *T. reesei* and for the thermophilic fungus *Talaromyces emersonii*, some relevant promoters, such as those from the cellulase-coding genes *cbh1* and *cbh2*, were also mapped using these classical approaches and are widely used to engineer these organisms [126, 127].

4.2.2. Homology Approach

Once some good expression systems are known and characterized for standard model organisms, omics data could be used to "mine" homologous elements in other organisms of interest. For example, genome sequencing and microarray experiments could be used to identify potential promoter sequences for further validation. In this sense, Stadlmayr and coworkers established two strategies to find new promoters. In the first strategy, hybridization of *S. cerevisiae* with *P. pastoris* in microarray provided 15 genes with higher expression, indicating strong promoter sequences as good candidates for circuit engineering. In the second one, mining of genome sequences allowed the identification of strong promoter candidates, such as in the case of *P. pastoris*, in which 9 promoters were identified and characterized [128]. This homology approach is particularly useful to identify functional promoter elements in native hosts were the final circuit should be implemented.

4.2.3. Mutational Approach

After the identification and mapping of functional promoter elements, it soon became clear that native expression systems would not be the perfect solution for the engineering of complex regulatory circuits. This is because natural promoters are endowed with their original regulatory interactions that could lead to non-functional circuits, due for example to the cross-talk with other endogenous pathways of the host [6]. For these reasons, and in order to engineer fine-tuned promoters with enhanced performance, mutation-based engineering has been used recently. First efforts were made to construct promoter libraries based on a constitutive promoter that would allow steady-state gene expression and ensure transcriptional homogeneity. For this, approaches based on the introduction of mutations in the wild-type promoter have been widely used, such as error-prone PCR and site-directed mutagenesis. Using these techniques, 200 variants to TEF1 promoter [59], 33 variants to GAP [129] and ~5,000 variants to AOX1 promoters [130] have been generated, demonstrating the potential of this approach to expand the repertoire of genetic elements for circuit engineering.

4.2.4. Tunable Approach

Once the functional elements existing in a particular eukaryotic promoter are known, its activity can be *fine-tuned* though the directed modification of these elements in order to change its regulatory behavior. Such type of rational engineering process is focused on the removal or introduction of some particular regulatory element at the wild-type promoter sequence. In this sense, earlier relevant works have been focused on the design of short synthetic promoter variants to use in metabolic engineering. Juven-Gershon and coworkers, using the parts that composes a core promoter *in vivo*, designed and characterized an optimized *de novo* core promoter that achieve high levels (>25 fold) of transcription than strong promoters of cytomegalovirus (CMV) and adenovirus major late (AdML), when assayed *in vitro* and *in vivo* in HeLaS3 eukaryotic cells [131]. Similarly, Hartner and colleagues, using known putative transcription factor binding sites (*cis*-elements) within the AOX1 promoter, performed rational deletions and mutations of these sites to modulate gene expression [132]. This work achieved three major goals. In the first place, it demonstrated that those *cis*-elements are able to modulate gene expression by its association with transcription factors. In the second place, it generated a library with regulatory properties different from wild-type AOX1 promoter with an activity range between 6% and >160% of the original sequence. And finally, it showed that even the strong AOX1 promoter has still considerable potential to be improved by engineering.

4.2.5. Chimeric Approach

In addition to the studies discussed above, an emerging strategy to create synthetic core promoters and synthetic variants is to generate chimeric promoters through the engineering and combination of UAS, URS, core promoters and chromatin remodeling sequences [133, 134]. In this approach, constitutive promoters exhibiting a wide dynamic range are used as starting point, as well as several well-known *cis*-elements (Fig. **2**). The main goal of this approach is to combine useful features of different promoters into a single expression system. In this regard, a hybrid expression system for *S. cerevisiae* was rationally designed using the strong GPD core promoter with tandem repeats of different UAS elements. The resulting synthetic promoter displayed a 50-fold increase in activity when compared to the wild-type GAL promoter [135]. The same strategy was also applied to construct hybrid promoters for *Yarrowia lipolytica*. In this case, strong TEF core promoter was combined with 1 to 32 UAS termed as UAS1B and LEU2. This resulted in an in-

crease of 17-fold when compared to the wild-type TEF promoter [119]. In both works, the approaches used allowed the construction of hybrid promoters with a higher activity than native sequences under different growth conditions, demonstrating its potential to expand the dynamic range of natural expression systems. Another useful strategy for promoter engineering is to modulate chromatin accessibility using nucleosome-disfavoring sequences. In this sense, Iyer and Struhl (1993) have shown that poly(dA:dT) sequences, which are highly prevalent in eukaryotic promoters, are able to increase the accessibility to the nearby TF-binding site for the activator Gcn4 and thus stimulate gene expression [136]. Using this information, Raveh-Sadka and colleagues rationally designed 70 synthetic promoters based on HIS3 yeast promoter, with modulated identity and affinity of the binding site to the transcription factor Gcn4 and flanked by poly(dA:dT) tracts with variations in length and compositions [137]. As a result, the authors demonstrated that Poly(dA:dT) tracts significantly affect the transcriptional outcome of the system, providing thus one additional tunable part to be used for synthetic promoter engineering. Interestingly, those synthetic promoters also displayed a more stochastic expression behavior [138], which could be a drawback of such type of engineering strategy. Using the same principle of engineering chromatin accessibility, Curran and co-workers re-designed endogenous yeast promoter sequences and designed *de novo* synthetic promoters modulated by nucleosome disfavoring sequences [139]. This study used a mathematical algorithm to generate promoters with minimal nucleosome affinities. For this, several rounds of optimization over promoter sequences were performance avoiding the generation or destruction of well-known transcription factors binding sites. With this approach, it was possible to achieve an improvement between 1.5 to 3.2 fold of the synthetic variants from the native yeast promotes CYC1, HIS5, HXT7 and TEF1, when the constructions were tested in a plasmid context. However, these increments were improved up to 16 fold when the constructions where tested in a chromosomal context. This work demonstrates the relevance of computational models to improve promoter sequences and therefore gene expression. All together, these approaches increase the availability of tools and methodologies to design synthetic promoters to be used in synthetic genetic circuits in eukaryotes.

4.3. Synthetic Biology in Metabolic Engineering

As presented before, several approaches have been developed to construct new expression systems for the use in circuit engineering. Along with the rising of these approaches, many works have engineered promoters to construct recombinant strains for specific application. For instance, Wang *et al.*, (2014) have developed a promoter collection for the expression of alkaline cellulose genes in *T. reesi* [140]. This was carried out through the modification of the pPK2 vector to construct different expression systems based on several promoters and terminators of *T. reesei*. This study produced the most efficient enzyme expression system for bio-stoning, which relied on the action of two enzymes synergistically working to modify the fabric surface. Also, Wang *et al.,* (2014) was able to improve the engineering of promoters trough the generation of a light-mediated gene expression system to control heterologous gene expression in *T. reesei* [141]. Similarly, Hong and colleagues created a cellulolytic yeast with enhanced cellobiohydrolase (CBH) activity for efficient cellulose degradation through the integration of three CBH-encoding genes driven by a more efficient TPI (triose phosphate isomerase) promoter [112].

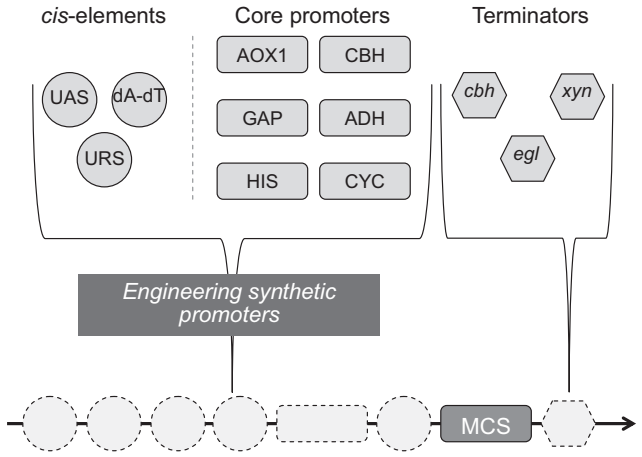

Fig. (2). Engineering synthetic promoters in fungi. The combination of functional regulatory elements has a tremendous potential for the construction of new expression systems for the applications of interest. As summarized in the figure, a pool of such elements with well-characterized functions can be used as a start point. At first, the category of *cis*-regulatory is formed by upstream activation sequences (UAS), upstream repression sequences (URS) and nucleosome destabilizing sequences (dA-dT). Next, core promoters from different systems (AOX1, GAP, HIS, etc.) provide the elements recognized by the basal transcriptional machinery, such as the TATA-box. Finally, strong termination signals are used to isolate the transcriptional unit constructed. Once assembled, multicloning sites (MCS) can be used to insert the gene of interest, generating thus the final functional system.

On the same direction, a pioneering work was carried out to optimize multiple gene expression in a heterologous metabolic pathway based on the metabolic background of the host strain bearing. For this, Du *et al.*, (2012) used an efficient and programmable approach named "customized optimization of metabolic pathways by combinatorial transcriptional engineering", also known as COMPACTER (Fig. **3**, [142]). Originally, introduction of heterologous metabolic pathways into a host required the avoidance of overexpression of some genes or imbalance of others proteins that could impair the heterologous metabolic flux [143]. The classical way employed to avoid cross-talk was to overexpress a key metabolic gene and/or improving certain catalytic enzymes by protein engineering favoring heterologous expression [143, 144], as well as the deletion of competing pathways. However, those approaches have limited success rate during the optimization of multi-step pathways [143]. Additionally, recent approaches to improve metabolic balance, such as multiplex automated genome engineering [145] and promoter modification, have proved to work for laboratory strains, even though these improvements not always were easily transferable to industrial production strains [146]. Therefore, COMPACTER was able to simultaneously

optimize multiple genes in a metabolic pathway, but mainly, it allowed the adaptation of that pathway to the strain of interest. In this sense, COMPACTER consists in the introduction into a host strain of a single-copy vector composed with each gene of the metabolic pathway under the control of distinct promoter/terminator pairs. With this approach, the resulting engineered cells were available to consume xylose 69% faster than the control strain. All together, this method was efficient to construct a library of different combinations of gene expression of a metabolic pathway. In this sense, it could be expected that the usage of a pool of promoters for each gene of the pathway would allow an easy optimization of the metabolic flux of interest (Fig. **3**).

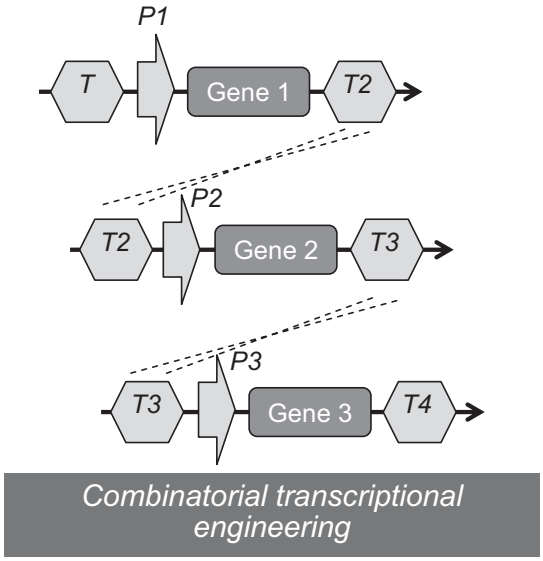

Fig. (3). Construction of complex circuits and pathways. The COMPACTER approach is represented as an example. In this strategy, series of expression systems with flanking homologous regions (in this case, termination signals) are recombined to assemble complex networks *in vivo* [142]. This approach can be used in cycles to generate systems with increasing complexity, as long as sufficient unique regulatory elements are available [142].

Another alternative to avoid cross talk between regulatory elements and endogenous pathways is the generation of orthogonal synthetic circuits. Orthogonal systems are intent to operate with minimal interference of the surrounding media, i.e., the circuit must display the same performance independently of the other pathways of the organism [143, 147]. Blount *et al.*, (2012), reported one of the first works focused on the design of orthogonal synthetic circuit. In this novel work, authors use microarray expression data from more than 20 experimental conditions together with bioinformatics approaches to identify the promoter sequence of the Profilin-encoding gene PFY1 with a relatively short sequence and with minimal regulatory elements. Promoter activity was analyzed using a vector carrying a yeast enhanced Green Fluorescent Protein (yEGFP) coding sequence as a reporter and a CYC1 terminator [103]. Using this basic scaffold, authors developed a synthetic promoter library by the circular polymerase extension cloning (CPEC) approach to construct and characterize 36 promoter variants. Additionally, rational engineering of the promoter logic was performed through the introduction of tandem TetR operator sequences at the core promoter region. This approach was enough to generate a regulated synthetic promoter with an output of more than 75% of the PFY1 wild-type sequence. However, since it only offers one *wire* for gene network engineered, additional *wires* to construct more sophisticate orthogonal synthetic circuit were obtained using the TALEN system [148]. The incorporation of TALEN system allowed the introduction of glucose or galactose as inputs to the system, reinforcing the notion that complex and orthogonal synthetic circuits could be constructed using engineered regulatory elements.

In addition to the work presented above, Vogl *et al.*, (2014) were the first to describe a fully synthetic core promoter for *P. pastoris* [149]. To achieve this goal, Vogl *et al.* used the well-known core promoter sequences of AOX1, GAP, HIS4, and ScADH2 promoters and used sequence alignment to identify a general minimal consensus. Next, the minimum core promoter (Core1) was re-engineered *in silico* by matrix-based models to add putative transcription factors binding sites. The different variants of synthetic core promoters generated were tested *in vivo* in this yeast. This work shows that functional synthetic core promoter could be obtained using *in silico* approaches. Yet, this study provides the basis for the design and construction of a next generation library of fully functional core promoters in order to achieve completely orthogonal regulatory networks.

5. MODULATING THE STOCHASTIC RESPONSE ON SYNTHETIC PROMOTERS

5.1. Biological Decision-making and Phenotypic Diversification

In most of the works directed to the study of mechanisms controlling gene expression, one often-neglected aspect is the behavior of the expression system at the level of individual cells. In fact, even cells from an isogenic population thriving on the same environment can display very different phenotypes when analyzed individually [150, 151]. This phenomenon is the result of the noise inherent to biochemical reactions occurring between components found in small numbers, and while this can be deleterious to the cell [152-154], many organisms have exploited noise for some useful purposes [155-157]. From a practical point of view, the analysis of the stochasticity levels during gene regulation at single cells allows their general categorization into two groups. In the first group, the system is considered as having a *deterministic* or *graded* response when all cells become simultaneously active when exposed to a given signal (Fig. **4**). As the concentration of the signal increases, all individual cells become more and more active. On a different scenario, the second group refers to systems displaying a *stochastic* or *all-or-none* response, where exposure to the inducer makes some cells to become fully active while others remained inactive. As the inducer concentration increases, more cells switch to the active state, so the inducer directly controls the switching rate of the system (Fig. **4**). In naturally occurring systems, examples of both situations have been reported [155, 158, 159] and several network modifications could be implemented to switch its response from deterministic to stochastic and vice versa [138, 156, 160-162].

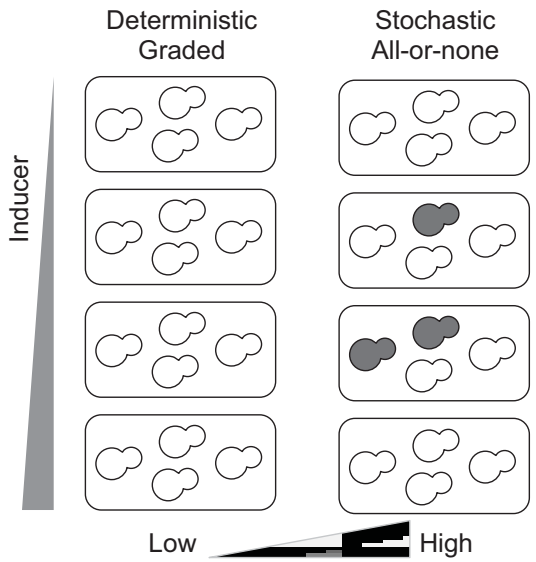

Fig. (4). Single-cell behavior of expression systems. In the deterministic (also called graded) expression behavior, cells respond simultaneously to a given signal, and the expression level in each cell increase as the signal gets stronger. In a stochastic (alternatively known as all-or-none) behavior, cells randomly switch from OFF to ON state when the signal is present. Thus, the switching rate of cells is directly proportional to the concentration of the inducer.

5.2. Stochastic Response in Yeast Synthetic Networks

Due to the importance of the stochastic processes in gene regulation, understanding the role of noise in gene expression is crucial in order to enhance the performance of the circuits for biotechnological applications. In order to investigate the source and effect of noise on gene expression in eukaryotic cells, *Blake et al.*, (2006) rationally designed mutant promoters with modifications in the TATA box and evaluated the level of variability in gene expression in *S. cerevisiae*, as well as its phenotypic consequences. Therefore, speed and variability of promoter response within a cell population were analyzed through combined experimental and computational approaches. For this, the reporter gene GFP was placed under the control of a GAL1 promoter with mutations of the TATA box, and the promoter was also subjected to the control of a TetR repressor variant. For the modeling part, a stochastic model of gene expression from GAL1 promoter was constructed taking into account transitions between various states of promoter occupancy prior to transcription initiation, transcript elongation, and translation. The result form these analysis demonstrated that cell-cell variability could dramatically affect the ability of a cell population to respond rapidly to an acute stress. Additionally, these results demonstrated that increasing variability could provide a phenotypic benefit for the organism after a severe change in its environment [163].

Another pioneer work has significantly contributed with both the design of synthetic circuits and the concepts to understand stochastic gene expression in eukaryotes [164]. In this elegant work, different strategies were used to determine the source of noise in gene expression in yeast. At first, diploid yeast strains were constructed where two different gene reporters (GFP and YFP) under control of the same promoter (PHO5) were integrated at the same locus on homologous chromosomes. The analysis of these constructions allowed the demonstration of both *intrinsic noise* (i.e., the noise resulting from the stochastic fluctuations in the biochemical reactions occurring during gene expression) and *extrinsic noise* (which is the noise resulting from the inherent cell-to-cell heterogeneity of the basic components required for gene expression) [158]. In this sense, extrinsic noise was further confirmed by using two different gene reporters (CFP and YFP) and two additional promoters (PHO8 and GAL1), revealing that extrinsic noise is not promoter-specific [164]. Yet, by using promoters regulated by the same transcriptional activator (PHO5 and PHO8) and the GAL1 promoter regulated by another transcription activator, measurement of intrinsic noise demonstrated that, contrary to extrinsic noise, this process is promoter-specific and does not depend on the expression rate of the system [164]. Finally, a mathematical model of stochastic gene expression was constructed that incorporates two distinct promoter states: an inactive state not permissive for transcription and an active state that is competent for transcription. This model was contrasted with the results of synthetic constructions carrying PHO5 promoter plus different variants of UAS as well as TATA-box mutations, all assayed into strains carrying deletions of components of chromatin-remodeling complex. This experimental set up allowed the demonstration that modulation of the stochastic response at the promoter level is intrinsically dependent of the basic *cis*-regulatory elements existing in the promoter. By the same token, it is easy to extrapolate that the level of stochasticity during gene expression can be tuned using synthetic promoters with modified *cis*-regulatory structures. Also, this work demonstrates the power of mathematical modeling to get further insight into the molecular mechanisms associated with gene expression and noise.

Since the level of noise resulting from promoter activity can strongly affect the performance of the regulatory network (and globally, of the whole organism), understanding how changes in the promoter structure can affect its stochasticity level is fundamental for Synthetic Biology application. Similarly, synthetic combination of *cis*-elements for multiple transcriptions factors in a promoter could result in an unpredictable behavior, making it extremely necessary to understand how combinations and multiplicity of regulatory sequences might affect gene expression. In this way, two very relevant works aimed to investigate the behavior of synthetic complex promoters through the modification of its architecture were performed. In the first work, the GAL1 wild-type promoter was rational combined with one, two, or three *tetO* operators at different positions between the TATA-box and the TSS, and the behavior of the resulting constructions were assayed using a yEGFP reporter gene [161]. Next, the stochastic responses of the different architecture of promoters were quantified at single cell level. Interestingly, it was found that the architecture of the promoter is highly relevant to the level of stochasticity of the resulting system, since placing an operator closer to the TATA-box increases its stochasticity level [161]. Additionally, a mathematical model was developed to describe the correlation between architecture of promoters and stochastic gene expression (i.e., the dynamic characteristic of promoter). Quite remarkably, while the model was able to predict the behavior of dual syn-

thetic promotes containing a single *tetO* element, the promoter with a more complex architecture (T123 promoter, which has three *tetO* sequences) presented an unpredicted output and higher level of noise [161].

In a second example, the effect of multiple operators in the final response of the synthetic promoters was further assayed using promoter variants with one (*1xtetO*) or seven (*7xtetO*) *tet* elements, in the presence or absence of a feedback loop [165]. The main goal of this work was to investigate if the configurations of the system could lead to unimodal (graded) or bimodal (all-or-none) behaviors. This work demonstrated that, while promoters without feedback displayed a graded response, the complex *7xtetO* promoter presented a bimodal response when placed under feedback control. More importantly, this bimodal response was not associated with bistability, but rather was the result of stochastic fluctuations in the regulator level trigged by the multiple binding sites [165]. Finally, even though tremendous progress has been made toward the understating of the interplay between promoter architecture and the level of stochasticity of the system, there are still many gaps that should be filled in order to generate the technology necessary to engineer new, robust synthetic regulatory networks for biotechnological applications.

CONCLUDING REMARKS

The fields of Systems and Synthetic Biology of Fungi have experienced a great development in the past decade. While omics-based approaches have provide a huge amount of large-scale data related to the cell response to cellulose exposure/degradation, new engineering attempts have addressed the construction and characterization of novel expression systems for fungi with enhanced performance. It is worth to notice that, even though much progress have been achieved in Synthetic Biology of model organisms such as *E. coli*, similar works on filamentous fungi are only starting to appear. For instance, when dealing with the engineering of synthetic promoters for fungi, little attention has been placed on the effect of changing physical composition of promoters on the level of stochasticity of the final system. The fully understanding of this scenario (i.e., the architecture /stochasticity relationship) will still require many further studies using novel techniques for single-cell analysis of gene expression in fungi. Perhaps, within another decade or so, we could finally have the consolidated technology to generate engineered fungal strains suitable for large-scale use in the biofuels and cellulose-based industries.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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