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Combinatorial Alkali-Responsive Hybrid Promoters as Tools for Heterologous Protein Expression in *Saccharomyces cerevisiae*

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ABSTRACT

The demand for strong and easily inducible promoters to produce heterologous proteins in *Saccharomyces cerevisiae* has attracted considerable attention in the last years. In this organism, alkalinisation triggers a wide and well-characterised transcriptional response that includes activation of the calcium–dependent calcineurin-Crz1 and the phosphate-responsive PHO pathways. Here, we present the construction of random libraries containing multiple combinations of Crz1- and Pho4-binding sequences, and we show that these elements are able to promote efficient expression of GFP by simple addition of KOH to the medium. The expression in Crz1 or Pho4-deficient cells allowed us to define the relative contribution of these elements to GFP production. We also show that the addition of a single copy of a 60-bp fragment of the *ENA1* promoter containing an Stp1/2 site further enhances expression. Finally, we demonstrate that these constructs drive strong expression of secretable laccase, an enzyme of industrial interest in processing lignin biopolymers, and that the level of expression can be adjusted by modifying the pH of the medium. In conclusion, our work presents a novel expression system whose induction is simple, cheap, and easy to monitor, and that could be an attractive alternative to current expression platforms for both research and industrial protein production purposes.

1 | Introduction

The yeast *Saccharomyces cerevisiae* is among the most used eukaryotic expression systems. This is due to several advantages, including easy handling, their status as a GRAS (Generally Regarded as Safe) organism, rapid growth up to rather high cell densities in inexpensive culture media, the capability to accomplish (to some extent) many eukaryotic posttranslational modifications, and the capacity to efficiently secrete the proteins synthesised intracellularly to the medium. Consequently, substantial advances have been made concerning the fine-tuning of key steps in the expression process, including re-designing the transcriptional machinery (promoters and terminators), re-wiring of the glycosylation patterns, and improving the secretion mechanisms (Gündüz Ergün et al. 2019). Concerning transcriptional machinery, industrial production of recombinant proteins (rP) requires the use of strong transcriptional promoters. Although constitutive promoters have occasionally been used, it is considered preferable to use inducible promoters. In these cases, mRNA is only produced in substantial amounts at the desired time, usually in response to a change in the conditions of the culture or the addition of a specific inducer.

Examples of inducible promoters used for rP production in *S. cerevisiae* are *GAL1-10*, *MET3*, *PHO5*, *CUP1*, or *ARG1* promoters (He et al. 2023).

However, most inducible promoters are not well suited for industrial purposes, since in many cases they require either a change in the carbon source (*GAL1-10*), which is impractical at the industrial scale, limitation of nutrients (*PHO5*), with a negative impact on growth, or the addition of relatively toxic inducers (*CUP1*). Therefore, there is a need for the identification of strong, tightly controllable, and inexpensive inducible options for industrial production of rP in *S. cerevisiae*. In recent years, several studies have explored modifications and optimisations of natural promoters based on different strategies, such as nucleosome removal (Curran et al. 2014), screening after random mutagenesis (such as error-prone PCR) (Alper et al. 2005; Yuan and Zhao 2013; Hubmann et al. 2014), or the hybrid promoter engineering approach. The latter is based on taking advantage of the modularity of promoter design by combining distinct upstream activating sequences (UASs), core promoters, and tailored terminators, as pioneered by Alper's laboratory (Blazeck et al. 2012; Curran et al. 2013). Such an approach has been generalised to yeasts other than *S. cerevisiae* (Blazeck et al. 2013; Yan et al. 2022).

Work for the past 20 years has demonstrated that *S. cerevisiae* responds transcriptionally to a moderate alkalinization of the medium. Such response is mediated by several signalling pathways, including the calcineurin-Crz1, Rim101, Snf1, PKA, PKC1-Slt2, and PHO pathways (Serra-Cardona et al. 2015). Thus, alkalinization triggers an almost immediate burst of intracellular calcium that activates the phosphatase calcineurin, promoting the dephosphorylation of the transcription factor Crz1, followed by its entry into the nucleus and rapid binding to CDRE (Calcineurin Dependent Response Element) sequences (Viladevall et al. 2004; Ruiz et al. 2008; Petrezsélyová et al. 2016; Roque et al. 2016). Similarly, alkalinization initiates a response alike to that observed upon phosphate starvation, activating the expression of diverse genes, some of them exclusively through activation of the Pho4 transcription factor, such as *PHO84* and *PHO12* (Serrano et al. 2002), and others, such as *PHO89*, by combination of Pho4, Crz1, and other regulatory inputs (Serra-Cardona et al. 2014). This body of knowledge was the starting point to conceive that alkaline pH-responsive promoters could be a source of components (*cis* regulatory modules or CRMs) for designing hybrid promoters that could be activated by the simple increase of pH. Following the concepts developed by Alper and collaborators, in this work, we present a random combination strategy that allowed the construction and isolation of multiple alkaline pH-responsive cassettes based on CDRE or Pho4-binding (PHO) elements, or combinations of both. These regulatory components were chosen because they are complementary, as they differ in their sensitivity to external pH (Serrano et al. 2002) and in the timing of their response (Crz1-mediated response is faster than that of Pho4) (Serra-Cardona et al. 2014). We show that some of these combinations can drive strong, pH-dependent expression of GFP. We also show that the addition of a recently discovered STP element present in the *ENA1* gene can further boost expression in response to alkalinization. Finally, we demonstrate that our approach is suitable for the expression of enzymes of industrial interest, taking the production of laccase as an example. Altogether, our findings pave the way for the design and construction of *S. cerevisiae* expression platforms that can be regulated by simple alkalinization of the medium.

2 | Experimental Procedures

2.1 | Yeast Strains and Culture Media

Yeast cells were incubated at 28°C in YP medium (1% yeast extract, 2% peptone) supplemented with 2% glucose, or in synthetic medium (SC) lacking uracil (Adams et al. 1998). Strains used in this work are BY4741 (*MAT**a*** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) and its isogenic *crz1::kanMX4* and *pho4::kanMX4* derivatives (Brachmann et al. 1998; Giaever et al. 2002), and CEN.PK2-1C (*MAT* ***a*** *leu2*-3112 *ura3*-52 *trp1*-289 *his3*Δ1 *MAL2*-8C *SUC2*) (Entian and Kötter 2007).

2.2 | Recombinant DNA Techniques and Reporter Construction

Escherichia coli DH5α cells were used as plasmid DNA hosts and were grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented, when required, with 50 μg/mL ampicillin. Transformations of *E. coli* and standard recombinant DNA techniques were performed as previously described (Green and Sambrook 2012). *S. cerevisiae* cells were transformed by a variant of the lithium acetate method (Gietz et al. 1995).

Construction of the basic vector pCYC, which contains a 269 bp fragment of the basal *CYC1* promoter region followed by the

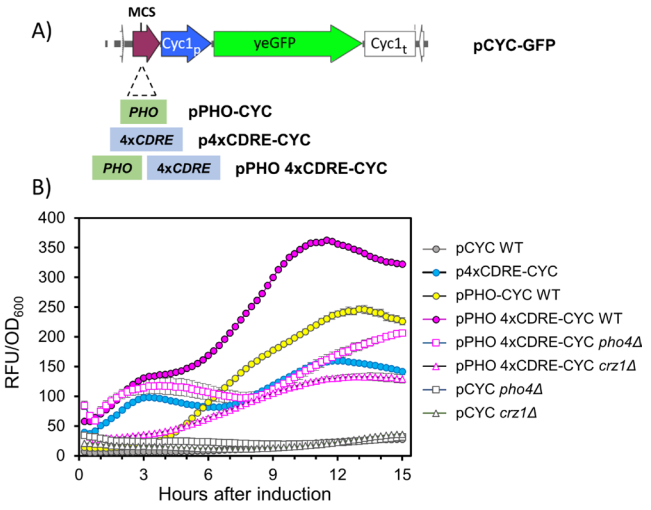

FIGURE 1 | Additive effect of the combination of Pho4 and 4xCDRE transcriptional elements. (A) The cartoon depicts the structure of the basic pCYC vector and that of the three variants tested in panel (B) for GFP expression. (B) Wild type strain BY4741 and its *crz1Δ* and *pho4Δ* derivatives were transformed with the vectors shown and cells grown as indicated in Materials and Methods. The fluorescence and cell density (OD_{600}) values were measured every 15 min after shifting the pH to 8.0 for 15 h. Data are expressed as relative fluorescence units (RFU) divided by the OD_{600} value and the mean ± SEM from four independent cultures (SEM bars are not visible in several cases due to their small size).

yeGFP ORF and the *CYC1* terminator (pCYC-GFP, Figure 1), as well as of p4xCDRE-CYC and pPHO-CYC was described in (Zekhnini et al. 2023). Construct pPHO 4xCDRE-CYC was made by cloning into the BamHI and EcoRI sites of p4xCDRE-CYC the 53 bp fragment derived from the *PHO84* promoter described in (Zekhnini et al. 2023). This fragment contains the consensus Pho4 elements C and D reported in (Ogawa et al. 1995). Expression of GFP from the *GAL1* promoter (pGAL-GFP) was accomplished by digestion of pCYC-GFP with KpnI and XhoI to release a 992 bp insert containing the yeGFP ORF followed by the *CYC1* terminator. The purified fragment was cloned into the p426 GAL (episomal, *URA3* marker) vector (Mumberg et al. 1994) previously digested with KpnI and XhoI.

2.3 | Construction of Random Combinatorial Libraries

TE (*TE*rminator) duplexes were constructed by hybridisation of synthetic oligonucleotides. To this end, complementary oligonucleotides (30 μg each) were mixed, heated at 95°C for 10 min, and allowed to cool down to room temperature to favour the formation of dsDNA. Oligonucleotides Generic_HdIII-NO-STRE_5′/Generic_HdIII-NO-STRE_3′ were used for HindIII-containing TE duplexes, whereas EcoRI internal sites were created with oligonucleotides Generic-RI-End-5′ and Generic-RI-End-3′ (oligonucleotides used in this work can be found at Table S1). *R*esponse *E*lement-containing duplexes (RE) were made by PCR. These DNA fragments contained a consensus binding sequence for a given transcription factor, flanked by a type IIS restriction enzyme consensus sequence (BsaI). The RE duplex carrying the Pho4 binding site was generated from plasmid pCYC-PHO with oligonucleotides amp-pho4-BsaI-5 and amp-pho5-BsaI-3. The 4xCDRE duplex (4 tandem repeats of the CDRE element present in the *FKS2* gene) was obtained from the plasmid pCYC-4xCDRE with oligonucleotides 4xCDRE-5′ (A) and 4xCDRE-3′ (A). The STP-MIG duplex was generated from the plasmid pCYC-MIG described in (Zekhnini et al. 2023) using the oligonucleotides Stp2-Mig BsaI-Fw and Stp2-Mig BsaI-Rev. PCR reactions were carried out using Q5 high-fidelity DNA polymerase (New England Biolabs). The structure of these fragments can be found in Table S2.

Combinations were created by mixing TE and RE duplexes (300–500 ng total DNA), 2 μL of BsaI FastDigest, 1 μL of T4 ligase (5 U/μL, ThermoFisher), 6 μL of T4 ligase 5× buffer (ThermoFisher) and completed with water up to 30 μL. The digestion/ligation reactions were carried out by setting 25 cycles of incubation for 5 min at 37°C, followed by 5 min at 22°C. Then, the mixture was incubated at 80°C for 10 min to inactivate the enzymes. Different relative amounts of TE and RE duplexes were tested in the combinatorial experiments. As an example, the 10.1N and 10.2N libraries used for cell sorting experiments were made using TE:RE1:RE2 ratios of 1:50:50 and 1:100:100, respectively. Finally, 1 μL of FastDigest HindIII or EcoRI enzymes was added to the mixture and the incubation was prolonged for 30 min at 37°C. At the end of the incubation, the mixture was heated at 80°C for 10 min. The reaction products were mixed with 1 μL of T4 ligase (5 U/μL), 1 μL of T4 ligase 5× buffer, 200 ng of vector p*CYC1*-GFP (previously digested with the appropriate restriction enzyme and treated with FastAP alkaline phosphatase (ThermoFisher)), and milliQ water was added up to a final volume of 40 μL. The mixture was used to transform competent *E. coli* DH5α cells. Transformants (> 1000 clones/plate) were pooled with 1 mL of LB medium plus ampicillin 50 μg/mL. Half mL of the suspension was used to inoculate 5 mL of LB plus ampicillin; the culture was grown for 4 h and used to isolate the plasmids.

2.4 | Plasmids for Expression of Laccase

For expression of laccase driven by our hybrid promoters, vector p10.1N.21-laccaseHR was constructed as follows. A 6366 bp fragment was amplified by PCR using Q5 DNA polymerase (New England Biolabs), p10.1N.21-GFP as template, and oligonucleotides pRS_10.1N.21-GFP-Fw and pRS_10.1N.21-GFP-Rev. This fragment contained the entire p10.1N.21-GFP vector except the yeGFP ORF. In parallel, an 1844 bp fragment was generated by PCR using plasmid pJRoC30-αopt86T/87N-CLeB (from now on pJRC30-CleB) as template, and oligonucleotides pRS_laccase-Fw and pRS_laccase-Rev_V2. The amplified fragment contained the C-LeB variant of the PM1 basidiomycete laccase (Rodríguez-Escribano et al. 2022) preceded by an optimised α-factor leader (Aza et al. 2021), flanked by 40 bp overlapping the ends of the 6366 bp PCR fragment described above. Both fragments were purified and used to transform BY4741 yeast cells to allow homologous recombination. Clones able to grow on SC-ura$^-$ plates were recovered, and plasmid extracted (Robzyk and Kassir 1992) and used to transform *E. coli* cells. Once purified from bacteria, the relevant region was sequenced to confirm correct recombination.

Vectors pCYC-laccaseHR, p10.2N.5-laccaseHR, pE4-1-laccaseHR, and pE6-1-laccaseHR were generated as follows. p10.1N.21-laccaseHR was digested with HindIII to give pCYC-laccaseHR. Then, the regulatory regions from p10.2N.5-GFP, pE4-1-GFP, and pE6-1-GFP were recovered by digestion with SpeI and ClaI and transferred to pCYC-laccaseHR, previously digested with the same enzymes.

2.5 | Fluorimetric Determination of yeGFP Production

Yeast cells carrying the constructs of interest were grown overnight in SC medium lacking uracil (SC-ura$^-$) at 28°C. Exponential cultures were prepared in SC-ura$^-$ medium to an OD_{600} of 0.6. One mL of the cultures was centrifuged at 1300*g* for 5 min and resuspended with the same volume of fresh medium SC-ura$^-$ supplemented with 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) and adjusted to pH 5.5 (control cultures) or 50 mM N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) for induced conditions. Fifty mM of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was also used as a buffer for pH dose–response experiments. Then 285 μL of the cultures were transferred into opaque 96-well plates (ThermoFisher, #165305) with 15 μL of 0.5 M KOH to adjust the pH to 7.9–8.0. Fifteen μL of a 0.5 M KCl solution was added to the control cultures. The plates were transferred to a Varioskan LUX Multimode equipment (ThermoFisher), and cells were cultured at 28°C with intermittent shaking. Fluorescence was measured in relative

fluorescence units (RFU) at 488 nm excitation and 520 nm emission wavelengths. The growth of the cultures was monitored by determining the optical density at 600 nm, and this data was used to normalise the fluorescence values. Readings were taken every 15 min for 15 h. All fluorimetric determinations presented in this work were done with the same equipment. According to the literature, eGFP fluorescence is not affected by the pH shift used for induction (dos Santos et al. 2020).

2.6 | Flow Cytometry Analysis and Cell Sorting

BY4741 yeast cells were transformed with the plasmid libraries (one μg of DNA/library) and transformants (> 1000/plate) were collected with 1.4 mL of SC-ura$^-$ medium. One hundred μL of the suspension was used to inoculate 50 mL of SC-ura$^-$ containing 50 mM TAPS at pH 5.0, and cultures were grown for 10 h until an OD_{600} of ≈0.6. Cultures were made in 25 mM KOH, thus reaching pH 7.95–8.0, and growth was resumed for 4 h. Cells were then diluted with SC-ura$^-$ 50 mM TAPS (pH 8.0) to 10^6 cells/mL and used for immediate sorting. Cell sorting was carried out in BD FACSJazz cell sorter (Becton Dickinson, San Jose, CA) equipped with blue (488 nm) and red (640 nm) lasers, a 100 μm nozzle and working at 27 psi of pressure. GFP was detected with the FITC detector and collected through a 530/40 nm bandpass filter. Instrument setup and performance were optimised by using standard 8-peaks Rainbow beads (Sphero Rainbow Calibration Particles). All parameters (FCS/SSC/GFP) were collected in log mode and data analysed with the BD FACSSort Software (Becton Dickinson, San Jose, CA). Single-cell sorting of yeasts was made with the "one drop single" sort mode with index sort. The flow rate was adjusted to 100 events/s. Cells were seeded in a modified sorting matrix designed to seed 64 separated drops in 100 mm SC-ura$^-$ plates.

2.7 | Expression of Laccase and Determination of Laccase Activity

Yeast cells carrying the appropriate alkaline pH-inducible expression vectors were cultured in 20 mL of SC-ura$^-$ (plus 2% glucose) medium until OD_{600} ≈3–4. Cultures were centrifuged at 1300*g* and resuspended in 20 mL of SC-ura$^-$ medium containing 50 mM MES and adjusted to pH 5.5 (control cells) or SC-ura$^-$ medium containing 50 mM TAPS adjusted to the desired alkaline pH (7.6–8.2) by addition of the appropriate volume of 3 M KOH (induced cells). Cells were grown for 8 h. Then, 1 mL of culture was taken, centrifuged at 1300 xg, and the supernatant stored at −20°C. The pH of induced cultures was measured, re-adjusted to pH 8.0 with 3 M KOH, and growth was resumed up to 24 h, when a 1 mL sample was taken and processed as above. Then, 2 mL of a 20% glucose solution and half mL of 10× SC-ura$^-$ were added to the remaining culture and growth was resumed for 8 additional hours, when the pH was readjusted again to 8.0. A final 1 mL sample was taken after 48 h of the initial induction and processed as above for laccase activity determination.

For expression of laccase from the *GAL1-10* promoter (plasmid pJRC30-CleB), cultures were grown until OD_{600} ≈3–4 and centrifuged at 1300*g* as above. Cells were then resuspended in 20 mL of SC-ura$^-$ medium containing 100 mM MES and adjusted to pH 6.0 (Rodríguez-Escribano et al. 2022). Control cells were grown in 2% raffinose-containing media, whereas the expression of laccase was achieved by removing the medium by centrifugation and resuspension of the cells in medium containing 2% galactose. After 24 h, 2 mL of a 20% galactose (or raffinose, for control cells) solution plus 0.5 mL of 10× SC-ura$^-$ were added to the culture. Samples (1 mL) were taken after 8, 24, and 48 h of induction, as described above.

Because laccases are multicopper oxidases that require the presence of copper ions for activity, aliquots of the medium were made with 1 mM $CuSO_4$ before the assay. This was preferred to the addition of copper salts to the medium during culture due to their negative effect on cell growth. Laccase activity was measured essentially as in (Rodríguez-Escribano et al. 2022). Six μL of the sample (diluted 1:6 with water for samples taken after 48 h of induction) were mixed in 96-well plates with 354 μL of a reaction mixture containing 3 mM ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) in 50 mM citrate phosphate buffer at pH 3. The increase of absorbance of the oxidised products was monitored spectrophotometrically (λ 405 nm) at 37°C. The activity was calculated according to equation 6 described in (Baltierra-Trejo et al. 2015), using a molar absorbance for ABTS (ε418) of 36,000 $M^{-1}cm^{-1}$ and assuming an optical pass length of 1 cm.

2.8 | Other Techniques

Prediction of nucleosome positioning was carried out using the NuPoP software (Chem option) (Xi et al. 2010), running the R package (version 2.10.0) under Windows. The relevant regulatory regions were extended 200 nt upstream (vector sequences) and downstream (*CYC1* basal region) to provide a common sequence context. The cartoons depicting the structure of the regulatory regions are drawn to scale from the specific sequences and were created in Excel with a Visual Basic for Applications program developed in-house.

3 | Results

3.1 | The Alkaline pH Response of a Hybrid CDRE-PHO Promoter

We recently reported the construction and testing of two different alkali-sensitive GFP reporters (Zekhnini et al. 2023), one based on the presence of two Pho4-binding sites (pho dyad) from the *PHO84* gene, and the other consisting of a cluster of four CDRE elements (4xCDRE) derived from the *FKS2* gene (Stathopoulos and Cyert 1997), in both cases driving expression from a *CYC1* basal promoter. Because both CRMs are known to transcriptionally respond to alkalinization through independent pathways, we considered creating a hybrid promoter containing both elements. As shown in Figure 1, a construct containing the 4xCDRE motif (p4xCDRE-CYC) shows a faster activation than the one harbouring the Pho4-responsive element (pPHO-CYC), as it can be deduced from the peak observed after 3 h of induction. In contrast, expression from the Pho4 element was slower but persistent, and after 12 h of growth, it exceeded by 40% the GFP levels obtained with the 4x-CDRE construct. The Pho4-CDRE hybrid (pPHO 4xCDRE-CYC) combined the fast

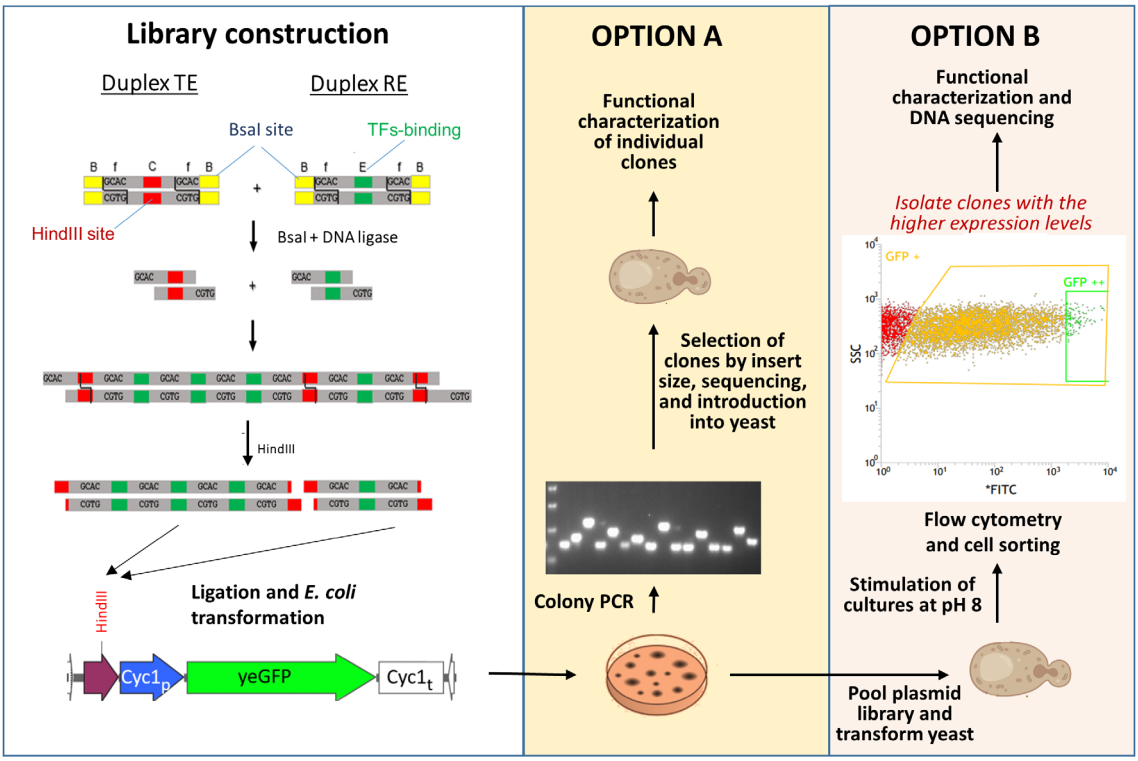

FIGURE 2 | Schematic depiction of the process to create random combinatorial libraries and the options tested to recover functional clones. The left panel depicts the process leading to the expression libraries. Upon concatenation of the monomeric TE and RE duplexes the mixture was digested with HindIII and cloned into the pCYC-GFP vector. Individual clones were analysed to identify the size of the insert (central panel) and selected plasmids introduced into yeast cells for further evaluation of GFP expression. Alternatively, libraries were used to transform yeast cells (right panel). Yeast cultures were stimulated at pH 8.0 to trigger GFP expression, and cells showing the highest fluorescence level (GFP++) were recovered by cell sorting. See the main text for details.

response of the calcium-responsive Crz1 transcription factor and the persistence of the phosphate-inducible element and resulted in higher GFP expression (Figure 1). Expression from this vector in a *pho4* deletion mutant yielded a profile very much like to that obtained with p4xCDRE-CYC in the wild-type strain, while the lack of Crz1 eliminated the characteristic early response of the 4x-CDRE element. These results suggested that careful design of artificial promoters combining diverse alkali-responsive CRMs could allow the integration into a single transcriptional unit of different signalling pathways that respond to environmental alkalinization. Thus, we considered that this might lead to extremely simple and economical platforms for expression in yeast of heterologous proteins of industrial interest.

3.2 | Combinatorial Generation and Testing of Libraries of Pho4 and CDRE Elements

The generation of synthetic hybrid promoters combining CRMs can be achieved by methods such as direct cloning according to a rational design or by random combination of the CRMs. We soon realised that generating multiple combinations of CRMs by standard cloning methods would be a cumbersome task, due to the high number of alternative possibilities. Therefore, we devised a method to generate libraries of random combinations of different CRMs by mixing two kinds of DNA duplexes (Figure 2). One of them, named RE (for *R*esponse *E*lement), contained a consensus binding sequence for a given transcription factor, flanked by type IIS restriction enzyme consensus sequences (in our case, BsaI). The other, designated duplex TE (for *TE*rminator) was characterised by a central type II restriction enzyme sequence, also flanked by BsaI restriction sites. The duplexes were mixed at specific molar ratios and subjected to cyclic digestion-ligation reactions. Because after each digestion-ligation cycle, the resulting junctions no longer contained BsaI sites, this procedure yielded a random number of RE duplexes occasionally separated by TE blocks. Final digestion with the selected type II enzyme (HindIII in Figure 2) allowed cloning of the randomly generated fragments into the GFP expression vector upstream of the *CYC1* basal element, thus creating libraries of random promoters. To note that while Figure 2 depicts the multimerization of a unique RE duplex containing a single TF consensus sequence, random hybrid promoters could be generated by using mixtures of RE duplexes carrying different TF consensus sequences.

In an initial step, we created three different libraries, one with Pho4 dyads in the RE duplex, one with the 4x-CDRE block, and a third one with a combination of both RE blocks, using in all cases a HindIII TE block. We isolated a total of 56 *E. coli* transformants from each library and probed the size of the insert by colony PCR. In this way (option A in Figure 2), we identified 14 different constructs that, once introduced into *S. cerevisiae* cells for functional characterisation, demonstrated in some cases improved levels of GFP expression (i.e., clone 5.19, Figure 3). However, the percentage of constructs resulting from religation at the BsaI site of two TE elements

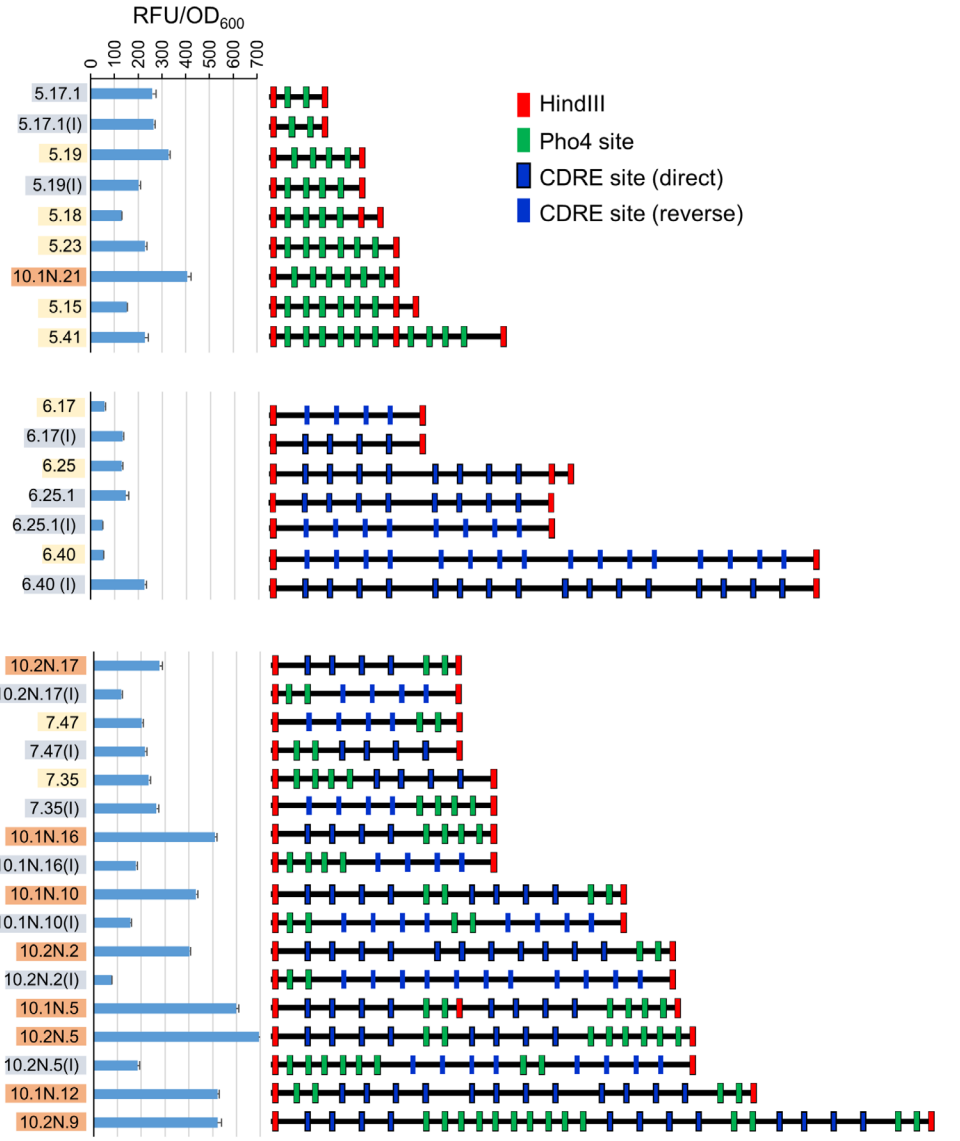

FIGURE 3 | Structure and maximal activity of the different variants constructed in this work. Wild-type strain BY4741 cells were transformed with the different constructs, and their capacity for GFP production tested as in Figure 1. The highest activity determined (usually obtained after 12 to 15 h of growth) is represented here. Values are mean ± SEM from at least 6 independent cultures. The background colour denotes how the specific construct was obtained: Initial screen (option A in Figure 2) in yellow, isolated by cell sorting are in dark orange, and those made to generate opposite orientations (or to remove artefactual HindIII sites) are in a grey background. The basal *CYC1* promoter starts right after the downstream HindIII site. Because the CDRE site is not palindromic, the cartoon differentiates the possibilities: Direct (GTGGCT) or reverse (AGCCAC).

and subsequent cloning into the HindIII site, thus lacking any TF-binding sequence, was unacceptably high (often > 50% of analysed clones) even using high RE/TE ratios (50 to 100-fold excess of RE duplexes over the TE duplex). Therefore, we resorted to a different strategy, based on the direct transformation of yeast cells with the pooled library of plasmids, and the isolation by flow cytometry and cell sorting, upon stimulation of the cultures at pH 8.0, of highly GFP-expressing yeast clones (option B in Figure 2). In this way, we examined two new libraries (10.1N and 10.2N) prepared by combining the HindIII TE block with REs for PHO and 4xCDRE at 1:50:50 and 1:100:100 ratios, respectively. We isolated a total number of 288 clones of which 53 were further analysed by examining the kinetics of GFP expression and the DNA sequence of the insert. This approach provided us with 11 novel promoter structures. The structure and activity of the most relevant promoters are presented in Figure 3 and the sequence of all promoters created can be found in Table S3.

As shown in Figure 3, the concatenation of the Pho4 dyad (clones 5.19 and 10.1N.21) could increase the production of GFP, although an excess of repeats was suboptimal (compare 10.1N.21 and 5.41). In addition, it became clear that the orientation of the regulatory architecture was important. Thus, 10.1N.21 and 5.23, both carrying three copies of the Pho4 dyad, only differ in the orientation of the insert, but the former is nearly 1.8-fold stronger than the latter. For this reason, we selected several representative promoter sequences and, upon digestion with HindIII and re-ligation, we isolated clones representing the opposite orientation (denoted with "(I)"). This raised the number of different promoters investigated to 39 (Figure 3 and Table S3 for sequence). We observed that a single Pho4 dyad

produces GFP with equal effectiveness irrespective of the orientation of the insert. However, the relevance of the orientation in the combinations could be confirmed, as 5.19, which carries 2 copies of the Pho4 dyad in the same orientation as 10.1N.21, was ~1.6-fold more active than its equivalent 5.19(I). Note the presence in some constructs of an internal HindIII site, likely formed by concatenation of two independent blocks during ligation after HindIII digestion of the concatemers. Figure 3 also shows that constructs harbouring only CDRE sites are in general less active (ranging from 1.4- to 6.5-fold) than those carrying Pho4-binding sequences, and that even a version carrying four repeats of the 4xCDRE block (i.e., 16 copies of the Crz1-binding sequence) barely matches a single Pho4 element (compare 6.40(I) with 5.17.1). Thus, concatenation of the 4xCDRE cluster resulted in a very moderate increase in GFP expression. The effect of the orientation in the CDRE constructs was also evident, being the one found in clones 6.17(I), 6.25.1 and 6.40(I) from 2- to 4-fold more efficient than the opposite one.

The analysis of the 19 clones carrying both CDRE and Pho4-binding sequences indicates that, for the same number and orientation of the regulatory elements, when Pho4 elements are proximal to the *CYC1* basal promoter, the response is about 1.3 to 2.2-fold stronger (compare 10.2N.17 with 7.47(I) and 10.1N.16 with 7.35 in Figure 3). The differential effect of the orientation of the inserts is evident in these combinations. Thus, 7.47(I) is more active than 10.2N.17(I), and 10.1N.16 is more active than 7.35(I). In this set of experiments, the most effective construct was 10.2N.5, carrying one 4x-CDRE and one Pho4 dyad, followed by one 4x-CDRE and three Pho4 dyads, closely tailed by 10.1N.5, with one Pho4 less in the downstream region and an artefactual HindIII block roughly in the middle of the sequence. The strength of 10.2N.5 was roughly 2.6-fold and 5.2-fold higher, respectively, than that of 5.17.1 (single Pho4 dyad) and 6.17(I) (single 4xCDRE). The more complex construct identified (10.2N.9, with nearly 800 nt of insert) displayed around 75% of the strength of 10.2N.5, indicating that the mere accumulation of responsive elements does not improve expression. Therefore, our results indicate that the expression of GFP from our alkaline pH hybrid promoters is affected by the number of copies of the elements, their orientation, and their distance to the basal promoter.

3.3 | The Influence of the *crz1Δ* and *pho4Δ* Mutations on the Expression From Complex Alkaline pH-Inducible Hybrid Promoters

To analyse the contribution of the different CDRE and Pho4 elements present in the constructs, we selected 24 of them and determined their ability to produce GFP in wild-type cells as well as *pho4Δ* and *crz1Δ* deletion mutants. Figure 4A shows four examples of constructs whose inserts only contain one or two Pho4 dyads. As expected, expression from all of them was substantially reduced by the deletion of the *PHO4* gene, yielding nearly values equivalent to that of the pCYC construct (20–40 RFU/OD_{600}) and was barely altered by the removal of *CRZ1*. As already shown in Figure 3, the orientation of the insert is not relevant when only one Pho4 dyad is present (5.17.1 and 5.17.1(I)), but it becomes important when there are two of them (5.19 and 5.19(I)). When a similar analysis was done for 4xCDRE-containing constructs (Figure 4B), deletion of *PHO4* had essentially no effect, but elimination of *CRZ1* resulted in virtually no production of GFP. Interestingly, the comparison of nucleosome occupancy predictions for equivalent constructs in which the insert has the opposite orientation was consistent with their differences in GFP expression (Figure S1A–C). Therefore, from these results, it is deduced that in this set of constructs, expression is driven solely from the expected regulatory elements (thus eliminating the possibility of unexpected spurious binding sequences) and that the differences in expression due to the orientation of the inserts could be attributed, at least in part, to differences in nucleosome occupancy.

Fourteen constructs containing different combinations of both 4x-CDRE and PHO elements were also evaluated in *crz1Δ* and *pho4Δ* mutants (Figure 5). The constructs 10.2N.17, 10.2N.17(I), 7.47, and 7.47(I) provide an example of one Pho4 dyad and one

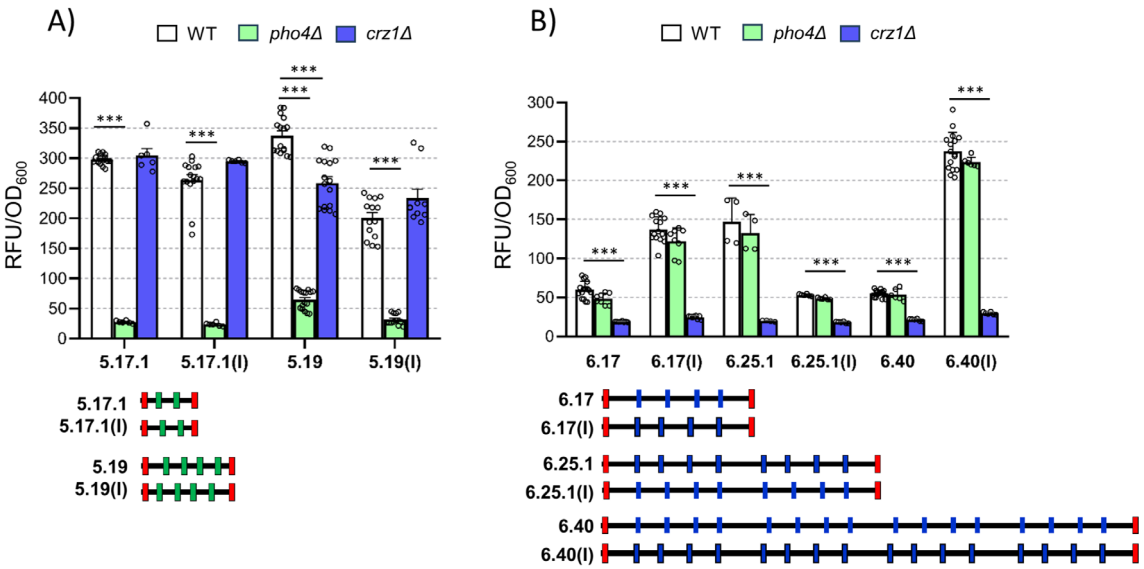

FIGURE 4 | Activity and Crz1 and Pho4 dependence of simple PHO (panel A) or CDRE (panel B) combinations. The indicated constructs were introduced into wild-type strain BY4741 and its *crz1Δ* and *pho4Δ* derivatives and grown as in Figure 1. The histogram represents the peak of GFP production. Data are means ± SEM from at least 6 independent cultures. The cartoons at the bottom represent the different regulatory architectures.

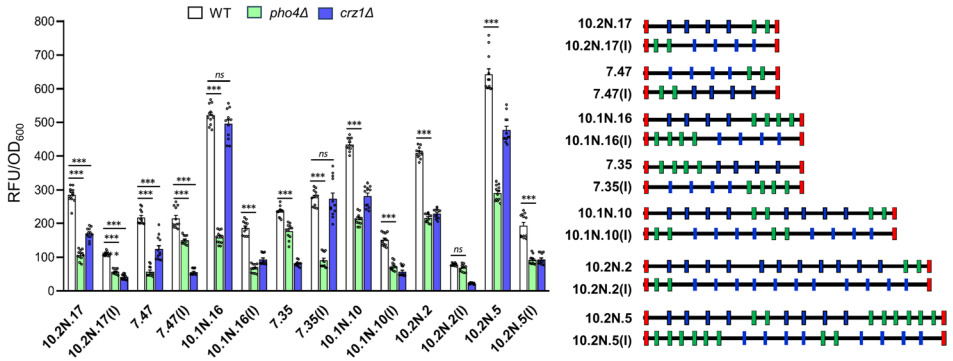

FIGURE 5 | Influence of orientation on the activity and Crz1 and Pho4 dependence of complex CDRE and PHO combinations. Data was obtained as in Figure 4 (mean ± SEM, $n = 6$). The cartoons on the right represent the structure of the different regulatory components.

4xCDRE block in the two positions relative to the *CYC1* basal promoter and both possible orientations. It can be observed that when the Pho4 dyad is proximal to the basal promoter, the activity is higher (compare 10.2N.17 and 7.47(I) for the same orientation). Interestingly, in the former, the response is mainly mediated by Pho4, whereas in the latter, it depends mostly on Crz1. This suggests that the proximal element plays a more important role in the response. This is also observed when the regulatory block is placed in the opposite orientation, albeit the overall response is 30 to 50% lower in this case. The relevance of the position with respect to the basal promoter is also observed for the set of constructs 10.1N.16, 10.1N.16(I), 7.35, and 7.35(I), which contain two Pho4 dyads and a single 4xCDRE block. The addition of a proximal Pho4 element has an important positive effect (nearly 2-fold) on the activity of the promoter (compare 10.1N.16 with 10.2N.17) and leads to a promoter almost exclusively Pho4-dependent. The same expression pattern (albeit with lesser activity) is seen in 7.35(I), which also displays two proximal Pho4 dyads, but with the entire regulatory insert in the opposite orientation. Therefore, in these two constructs, the contribution of Crz1 seems negligible. Placing the 4xCDRE element proximal to the basal *CYC1* promoter increases the Crz1-dependence of the response, although the overall intensity is lower. Construct 10.1N.10 can be considered a duplication of 10.2N.17 and exhibits the same profile but with a 50% higher activity. Again, the same segment in the opposite orientation becomes much less efficient in driving GFP expression (~3-fold less). The construct 10.2N.2 consists of a proximal Pho4 dyad and three upstream 4xCDRE blocks (one incomplete). Both Crz1 and Pho4 contribute equally to its activity, which is drastically reduced in the construct with the opposite orientation (and becomes fully Crz1-dependent). Finally, clone 10.2N.5, which shows the 5′-3′ sequence 4xCDRE-Pho4-4xCDRE-3xPho4 (roughly 500 nt), exhibits the highest activity, and this is mainly (about 65%) mediated by Pho4. Reversal of the entire fragment had a very negative effect on activity (nearly 4-fold decrease), which then became equally affected by the lack of Czr1 or Pho4.

3.4 | A Single Insertion of the *ENA1* STP-MIG Promoter Region Enhances Expression From Various Hybrid Promoters

We recently reported (Zekhnini et al. 2023) that the *ENA1* promoter contains a consensus sequence for the Stp1/2 transcription factors, the downstream components of the amino acid sensing SPS pathway, which plays a positive role in the response of the gene to the alkalinisation of the medium. To assess if the addition of this sequence could affect expression from our hybrid promoters, we amplified the specific *ENA1* region (that includes a nearby Mig1/2 site and is thus a STP-MIG element) adding BsaI sites and carried out multimerisation reactions using a terminating block that included an EcoRI site. The products were then cloned upstream of the regulatory modules present in 10.1N.21 (only PHO elements) or 10.1N.16 and 10.2N.5 (containing both CDRE and PHO elements). As shown in Figure 6, the addition of a single STP-MIG element to the 10.1N.21 combination, as in clones E6-1 and E6-2, clearly increased GFP expression by 60% and 35%, respectively. Similarly, constructs E5-1 and E5-4, which are the equivalent variants generated from the 10.1N.16 construct, also yielded higher GFP production than the original clone (38% and 28%, respectively). In both cases, the most effective orientation was the inverse of that found in the *ENA1* promoter. Interestingly, the versions containing two STP-MIG units were less efficient in promoting GFP production than those containing a single unit. Specifically, the E3-1, E2-3, and E2-1 versions were 9.6%, 27.2%, and 24.8% less effective than their counterparts (Figure 6). The positive effect of a single STP-MIG sequence was confirmed in E4-1, a variant of 10.2N.15. Once again, repetition of the block eliminated the positive effect (E1-2 and E1-3), whereas inclusion of four repeats (in the orientation found in *ENA1*) dramatically decreased expression. These results indicate that while a single STP-MIG element placed upstream of diverse hybrid promoters has a beneficial effect, preferably in the orientation opposite to the one found in the *ENA1* promoter, multiple copies of this element could even have a negative effect.

3.5 | Expression From the Hybrid Promoters Can Be Modulated by Adjusting the pH of the Medium

To assess if external pH could modulate the expression from our constructs, we selected five representative examples and tested them by growing at four different initial pHs (7.2, 7.5, 7.7, and 8.0), thus providing a pH range appropriate for activation of the CRMs included in the constructs (Serrano

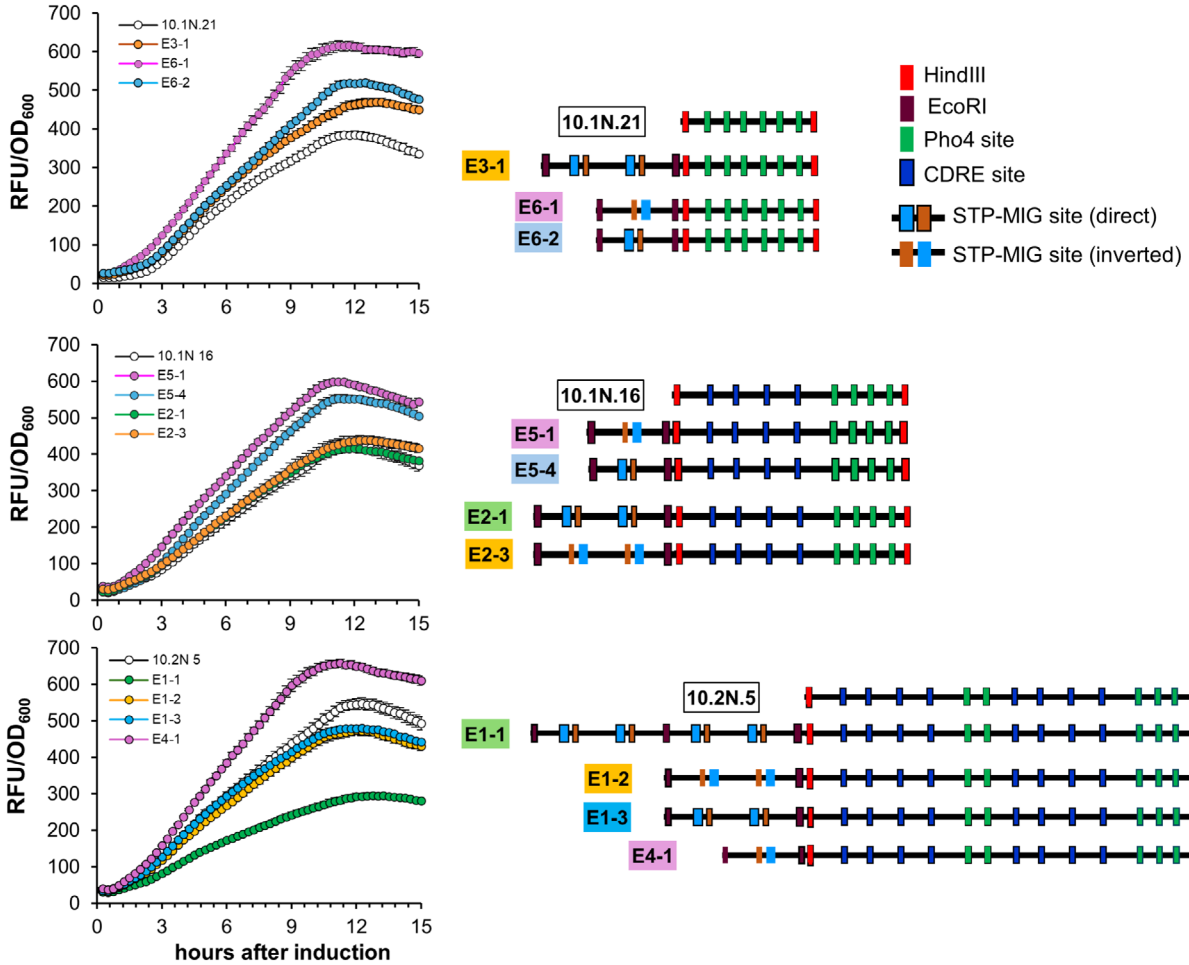

FIGURE 6 | Effect of the insertion of single or multiple *ENA1* STP-MIG promoter fragments. Constructs containing the regulatory components shown on the right were introduced into wild-type BY4741 cells, and these processed for GFP production as in Figure 1B. Data are means ± SEM from 4 independent cultures.

et al. 2002; Viladevall et al. 2004). Because of the wide pH range, the experiments were performed in parallel using two different buffers, TAPS (effective pH buffering range 7.7–9.1) and HEPES (6.8–8.2). Figure 7 shows the results using TAPS, whereas data using HEPES is shown in Figure S2. From Figure 7A, it can be observed that pPHO-CYC was activated nearly to its maximum even at the lowest pH tested (7.2), whereas p4XCDRE-CYC appeared to require higher pHs (in particular when HEPES is used, Figure S2). Compared to pH 8.0, the most complex constructs (p10 2N.5 and p4E-1) already exhibit about 70% of their activity at pH 7.2. Interestingly, the profiles at pH 7.7 and 8.0 are almost identical, indicating that a consistent regulatory output can be obtained under less stressful growth conditions. The dynamic range, in terms of RFU/OD_{600}, and the value of pH equivalent to the EC_{50} for each hybrid promoter are presented in Figure 7B,C respectively. It is worth noting that the effect of alkalinisation on growth resulted mostly in an extended lag phase. This effect was slight for pH 7.5 and increased equally for pH 7.7 and 8.0 (Figure S3). Therefore, these experiments indicate that the intensity and the kinetics of the response can be modulated by properly adjusting the pH of the medium within a range of moderate alkalinisation.

3.6 | Alkaline pH-Driven Promoters Allow Effective Expression and Secretion of Laccase

Figure S4 shows that the GFP expression levels from several of our alkaline pH-driven promoters exceeded 2.6 (E4-1) to 2.0-fold (E6-1) the amounts obtained with the same vector bearing the galactose-inducible promoter *GAL1-10*, which is considered among the strongest regulatable systems in *S. cerevisiae*. However, a proper comparison ought to be made by attempting to express an enzyme of industrial interest that could be secreted into the media. To this end, we replaced the GFP protein with an engineered laccase (an enzyme employed in the degradation of kraft lignins from biomass) and carried out production experiments in Erlenmeyer flasks. The expression levels achieved using BY4741 cells were comparatively very low (not shown), so we tested other genetic backgrounds. Specifically, tests carried out using the CEN.PK2-1C background showed a stronger response (about 20-fold higher). As shown in Figure 8, at the usual induction at pH 8.0, the activity of the enzyme recovered from the medium was barely detectable when laccase was expressed from pCYC. In contrast, after 8 h of induction, the activity was readily detected for any of the four alkaline pH-inducible constructs tested,

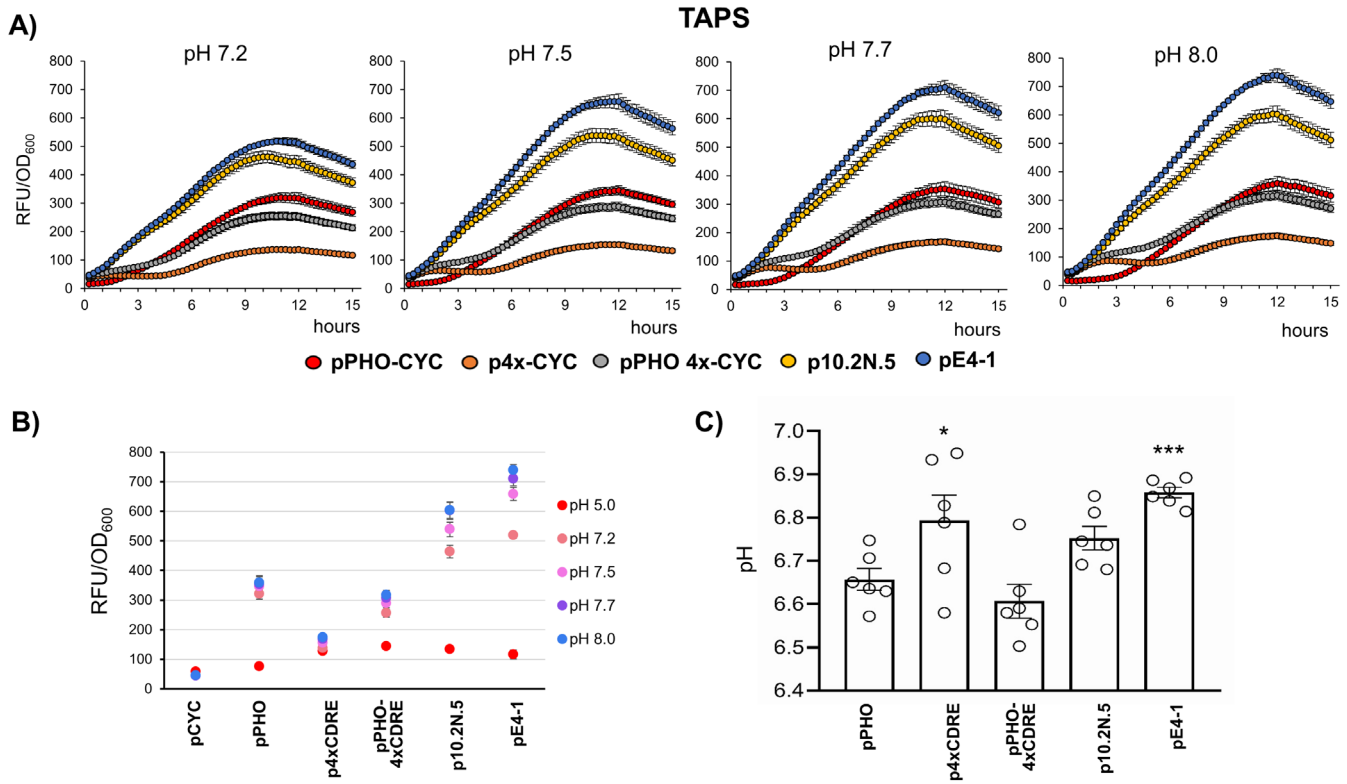

FIGURE 7 | pH-dependent response for selected alkaline inducible vectors. (A) The indicated constructs were introduced in BY4741 cells and grown in SC-ura$^-$ as indicated for Figure 1B except that the media contained 50 mM TAPS adjusted to the indicated pHs. (B) The dynamic range deduced from data presented in panel (A) was plotted. Values correspond to the highest value at each pH. (C) EC_{50} values for the different constructs. Significance was calculated by paired Student's *t*-test by comparison with the pPHO construct. *$p < 0.05$; ***$p < 0.001$. In all cases data are means ± SEM from 6 independent cultures. p4x denotes p4xCDRE.

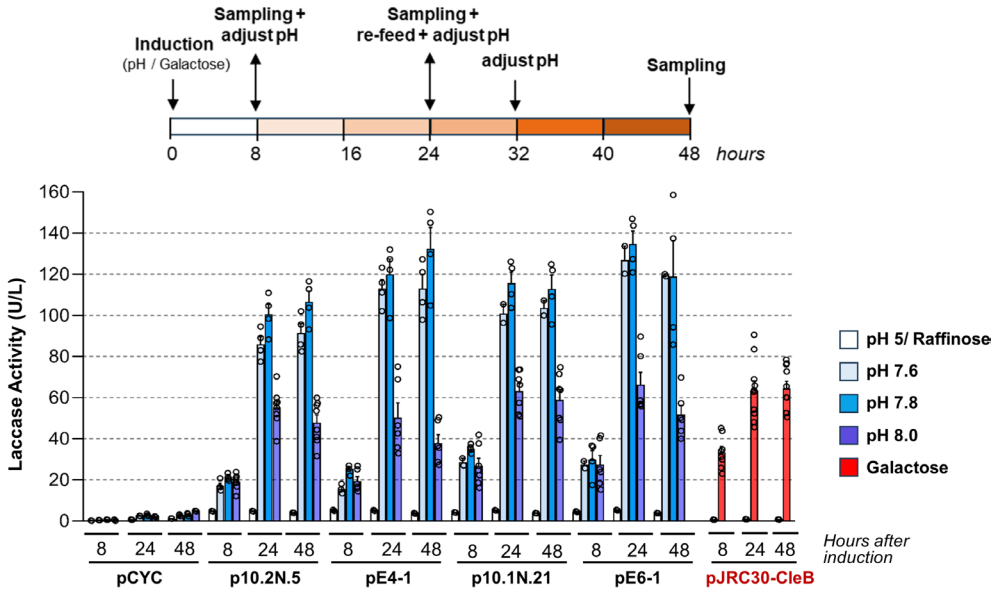

FIGURE 8 | Production of secreted laccase from alkaline pH-inducible vectors. CEN.PK2-1C cells were transformed with the indicated vectors. Cultures (OD_{600} ≈3–4) were subjected to the procedure outlined in the cartoon (see Materials and Methods for details). For pJRC30-CleB induction was achieved by resuspension in SC-ura$^-$ medium containing 100 mM MES (pH 6.0) with 2% galactose (2% raffinose for control, non-induced cells). For the rest of the vectors, induction was triggered by resuspension in SC-ura$^-$ (plus 50 mM TAPS) adjusted to the indicated pHs, or 50 mM MES, adjusted to pH 5.5 (for non-induced cells). Data are means ± SEM from 4 (pH 7.6 and 7.8) or 8 (pH 8.0 and pJRC30-CleB) independent experiments.

reaching a maximum at 24 h and remaining stable after 48 h. At this pH, the amount of laccase secreted to the medium by cells harbouring two of the pH-driven constructs (p10.1N.21 and pE6-1) was comparable to that obtained from the *GAL1-10* based expression vector (pJCR30-CleB). Importantly, whereas increasing induction pH to 8.2 yielded equal or lower laccase production than at pH 8.0 (not shown), the recovered laccase activity increased by at least 2-fold when induction was carried out at slightly lower pHs (7.6 and 7.8). These results demonstrate that the hybrid promoters developed in this work could satisfactorily compete with a well-established, carbon source-inducible promoter system, and that production can be improved by properly adjusting the intensity of the induction signal.

4 | Discussion

The ability of yeast promoters to respond to changes in the environment has been exploited in the past to drive the expression of heterologous proteins. Examples are the *CUP1* promoter, inducible by moderate amounts of copper (Butt and Ecker 1987; Peng et al. 2015), the *MET25* promoter, active under methionine limitation (Solow et al. 2005), or the *HSP12* and *HSP26* promoters, responsive to high temperature and acetic acid stress (Xiong et al. 2018). However, natural promoters, albeit useful, show limitations when used in the industrial production setting. This has led to the development of synthetic promoters, based on the combination of selected transcriptional regulatory elements (Tang et al. 2020; Nguyen et al. 2024). Examples are the combination of diverse upstream activating sequences to confer sensitivity to galactose to the constitutive endogenous promoters (Blazeck et al. 2012) or the potentiation of the weak low pH inducibility of the *YGP1* promoter by modification of the upstream regulatory architecture (Rajkumar et al. 2016).

In response to moderate alkalinisation, *S. cerevisiae* develops a wide transcriptional response that is mediated by multiple signalling pathways, including the calcineurin-dependent Crz1 and the Pho81-Pho4 mediated pathways (Serrano et al. 2002; Viladevall et al. 2004; Ruiz et al. 2008). In this work, we present the generation and characterisation of a collection of randomly assembled Crz1 (CDRE) and Pho4-binding CRMs upstream of a basal *CYC1* promoter, and we show that these constructs efficiently drive expression by simply raising the pH of the medium to around 8.0 with KOH.

From this study, we extract several conclusions. On one side, we observe that induction from the Pho4 element is more effective than from the 4xCDRE tandem (Figure 1). In addition, the kinetics of expression from CDREs is faster than that from Pho4-containing CRMs. This is in agreement with the previous observations that alkalinisation triggers an almost immediate peak of intracellular calcium (Viladevall et al. 2004) and that Crz1 binds to many CDRE-containing promoters within few minutes (Ruiz et al. 2008; Petrezsélyová et al. 2016; Roque et al. 2016), as well as with the kinetics of alkaline pH-induced expression of *PHO84* (fully Pho4-dependent) and *PHO89* (mostly Crz1-dependent) (Serra-Cardona et al. 2014). On the other hand, the Pho4 element is almost fully induced even at pH 7.2 (Figure 7), while activation of CDREs requires higher pH. This behaviour fits well with the early observation that the *PHO84* promoter is markedly activated even at pH below 7.0, whereas the *PHO89* one requires a higher pH (Serrano et al. 2002).

Our work also shows that the mere accumulation of CRMs upstream of the *CYC1* basal promoter does not necessarily increase expression. Thus, construct 10.1N.16 shows the same level of expression as 10.2N.9, even though the latter triples the number of CDRE and PHO elements of the former. Instead, the relative position of the CRMs and, in some cases, their orientation, can be essential for their function. For instance, we observe that when CRMs are proximal to the basal *CYC1* promoter they are more effective than when placed more distantly. This is clearly seen when comparing constructs 10.2N.17 and 7.47(I), which carry one 4xCDRE and one Pho4 dyad in the same orientation but with exchanged positions. The former, with a proximal Pho4 site, is more active than the latter and, as deduced from the study with mutant strains (Figure 5), the contribution of Pho4 is larger than that of Crz1. Inversely, the loss of activity in the construct 7.47(I) is more marked in the *crz1Δ* mutant, indicating a major contribution of the 4xCDRE CRM. The same behaviour is observed for the pair 10.1N.16, with two proximal Pho4 CRMs and one distal 4xCDRE, whose activity is largely dependent on Pho4 and higher than that of construct 7.35, which in turn depends on Crz1. A similar pattern is observed in the equivalent pairs in which the 4xCDRE module is in opposite orientation (i.e., 10.2N17(I) vs. 7.47, and 10.1N.16(I) vs. 7.35(I)). These results are not surprising, since the relevance of the position and orientation of CRMs in their regulatory capacities, and hence in gene expression, has been reported for different organisms, including bacteria (Westmann et al. 2018), yeasts (Lin et al. 2010; Lai et al. 2013), plants (Lis and Walther 2016), and humans (Georgakopoulos-Soares et al. 2023; Duttke et al. 2024). It is expected that the wealth of data collected will allow us to develop predictive models that could be used to shape and improve the alkaline pH-inducible expression vectors presented in this work.

An additional factor that influenced expression was the orientation of the insert carrying the CRMs. In general, reversing the orientation of the original CDRE-containing sequences taken from the *FKS2* promoter (Stathopoulos and Cyert 1997) had a strong negative effect (Figure 3). Reversal of the single Pho4-containing fragment had no effect (compare 5.17.1 with 5.17.1(I)). However, when the insert contained two or three Pho4 dyads, a change in the orientation did affect the activity of the promoter. This may appear surprising because the Pho4 binding sequences contained in the Pho84 promoter fragment (sites C and D) are palindromic (5′-CACGTG-3′) and it was shown that reversing the orientation of a fragment containing site D had no effect on expression (Ogawa et al. 1995). Interestingly, as shown in Sup. Figure 1, the effect on promoter activity that accompanied the change in the orientation was concordant with the computational prediction for nucleosome occupancy. Thus, for 5.17.1 and 5.17.1(I) activities, nucleosome occupancy prediction is virtually identical, while for the rest of Pho4 and CDRE-containing inserts, the prediction for nucleosome-free regions corresponds with a higher activity. This relationship is not surprising since there is plenty of evidence supporting the role of nucleosome positioning on the regulation of yeast promoters (Sharon et al. 2012; Kornberg and Lorch 2020; Lin et al. 2020). Specifically, it has been proposed that nucleosomes

function mainly to decouple the threshold of induction from dynamic range, and that changes in the chromatin state would explain why under moderate Pi shortage Pho4 is more recruited to *PHO84* than to *PHO5*, even though both promoters contain a combination of high and low-affinity Pho4-binding sites (Lam et al. 2008). Moreover, it has been demonstrated that nucleosome architecture can be modified to finely adjust gene expression in *S. cerevisiae* (Raveh-Sadka et al. 2012; Sharon et al. 2012), including the design of synthetic promoters (Curran et al. 2014).

ENA1 is an alkaline pH-activated gene (see (Ruiz and Ariño 2007) and references therein). We recently reported that the upstream region of the *ENA1* promoter (−553/−544) contains a Stp1/2 binding site that contributes to the response of the gene to alkalinisation, and that the combination of a 60 bp fragment (nt −508/−567) containing the Spt1/2 site with single Pho4 or 4xCDRE blocks had a positive effect on expression (Zekhnini et al. 2023). We show here that the same effect can be obtained by adding this region upstream of diverse complex promoters created in this work, indicating that the Stp1/2 site can function in complex transcriptional scenarios. This is important because these results open the way to expand the set of elements suitable for the construction of hybrid synthetic promoters with additional alkaline pH-responsive CRMs. Interestingly, alkalinisation triggers an oxidative stress response, whose transcriptional component appears mainly to be mediated by the transcription factor Yap1 (Viladevall et al. 2004). Similarly, shifting cells to high pH results in alterations in copper and iron homeostasis, which triggers an increase in the mRNA levels for multiple genes encoding iron and copper transporters (Lamb et al. 2001; Serrano et al. 2002, 2004). Because most of these genes are under the control of the Aft1 or Mac1 transcription factors, these CRMs could also be included for the construction of combinatorial libraries.

An intriguing observation was that multiple copies of the Stp1/2 element did not further potentiate expression, but it often had null or even negative effects (Figure 6). In any case, these effects were identical irrespective of the orientation of the concatemer (compare E2-1 with E2-3 and E1-2 with E1-3). It must be emphasised that this fragment also contains a canonical Mig1/2 site (5′-GCGGGG-3′) five nt downstream of the Stp1/2 site, and that Mig1 is generally considered a transcriptional repressor (Shashkova et al. 2015). Therefore, a possible explanation for the lack of increase in expression would be that the accumulation of Mig1/2 sites counteracts the positive effect derived from the presence of the Stp1/2 consensus sequences.

Our work also demonstrates that alkaline pH-inducible hybrid promoters can be useful to produce enzymes of industrial interest with a relatively minor impact on cell growth. Thus, we show the ability of four of our promoter combinations to drive expression of an engineered laccase at levels that surpass those produced from the *GAL1* promoter upon induction with galactose (Figure 8). It is worth noting that the enzyme was effectively secreted to the medium, thus showing that the conditions of expression do not interfere with the secretory mechanisms. This is relevant because it has been reported that the external pH could affect the ability to secrete rPs (Wentz and Shusta 2008). Of course, additional work will be needed to transfer our expression systems to the bioreactor scale. This would allow us to properly monitor and adjust pH at will during the process, but it might introduce additional problems, such as shear stress or pH homogeneity. On the other hand, while the use of moderate alkalinisation to induce expression would be an additional advantage when producing secreted proteins that are unstable at the acidic pH that characterises most fermentation processes (Hu et al. 2014; Jallouli et al. 2017), it might become a limitation when the protein being secreted is unstable at pH above 7.5–8.0. Still, if this were not an irreversible process, it could be overcome by acidifying the medium at the end of the process.

5 | Conclusion

We describe here a novel concept for the expression of rPs based on the use of hybrid promoters that are potently activated by moderate alkalinisation of the medium, and we show its usefulness in the yeast *S. cerevisiae*. It is well known that the methylotrophic yeast *Komagataella phaffii* (formerly known as *Pichia pastoris*) has become a standard organism for heterologous protein production, offering advantages such as growth at high cell densities and a very efficient secretory pathway (Barone et al. 2023; Vijayakumar and Venkataraman 2024). In this regard, we recently showed that alkalinisation also induces a wide and strong transcriptional response in *K. phaffii* (Albacar et al. 2023), and that selected alkaline-responsive promoters can be harnessed to produce a recombinant phytase enzyme at levels matching those of the powerful, methanol-requiring *AOX1* promoter (Albacar et al. 2024). Therefore, we propose that the concepts developed here could be extended to *K. phaffii* (and likely to other fungi of interest) based on the signalling pathways involved in the alkaline pH response for the specific organism. Since expression can in principle be induced in cells growing on any carbon source, the induction method requires inexpensive chemicals (such as KOH), and pH is a very simple parameter to monitor in any bioreactor; alkaline pH-based expression platforms could provide a safe and inexpensive alternative to existing recombinant expression options.

Author Contributions

Abdelghani Zekhnini: investigation, formal analysis, writing – review and editing. **Antonio Casamayor:** funding acquisition, conceptualization, investigation, supervision. **Joaquín Ariño:** conceptualization, funding acquisition, formal analysis, supervision, visualization, project administration, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data not presented in this article will be made available upon request to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1.** Nucleosome occupancy prediction for selected upstream regulatory combinations. **Figure S2.** pH dependent response for selected alkaline inducible vectors in HEPES buffered media **Figure S3.** Effect of the pH of the medium on cell growth. **Figure S4.** Comparison between *GAL1*-driven and alkaline pH-driven GFP expression. **Table S1.** Oligonucleotides used in this work **Table S2.** Monomeric regions employed for concatemer formation in this work. **Table S3.** Sequences of regulatory regions described in this work.