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RESEARCH ARTICLE

Screening of 2A peptides for polycistronic gene expression in yeast

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One sentence summary: This work characterized various 'self-cleaving' 2A peptides to determine suitability for metabolic engineering applications in yeast.
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ABSTRACT

A complexity of pathway expression in yeast compared to prokaryotes is the need for separate promoters and terminators for each gene expressed. Single transcript expression and separated protein production is possible via the use of 2A viral peptides, but detailed characterization to assess their suitability and applications is needed. The present work aimed to characterize multiple 2A peptide sequences to determine suitability for metabolic engineering applications in *Saccharomyces cerevisiae*. We screened 22 peptides placed between fluorescent protein sequences. Cleaving efficiency was calculated by western blot intensity of bands corresponding to the cleaved and uncleaved forms of the reporter. Three out of the 22 sequences showed high cleavage efficiency: 2A peptide from Equine rhinitis B virus (91%), Porcine teschovirus-1 (85%) and Operophtera brumata cypovirus-18 (83%). Furthermore, expression of the released protein was comparable to its monocistronic expression. As a proof-of-concept, the triterpene friedelin was successfully produced in the same yeast strain by expressing its synthase with the truncated form of *HMG1* linked by the 2A peptide of ERBV-1, with production titers comparable to monocistronic expression (via separate promoters). These results suggest that these peptides could be suitable for expression and translation of multiple proteins in metabolic engineering applications in *S. cerevisiae*.

Keywords: *Saccharomyces cerevisiae*; ERBV-1 2A peptide; yeast metabolic engineering; 'self-cleavage'; 'stop-carry on'; multi-gene expression; 'polycistronic'

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INTRODUCTION

The production of fine chemicals, therapeutic natural products or biofuels in microbial cell factories has promising industrial applications (Borodina and Nielsen 2014; Krivoruchko and Nielsen 2014; Breitling and Takano 2015). The yeast *Saccharomyces cerevisiae* is the most common eukaryotic cell factory (Krivoruchko and Nielsen 2014). Its advantages include good tolerance to low pH and fermentation inhibitors, robust industrial performance, reduced susceptibility to phage contaminations, genetic tractability and ability to express functional enzymes with post-translational modifications or membrane anchoring (Borodina and Nielsen 2014; Krivoruchko and Nielsen 2014). *Saccharomyces cerevisiae* is currently the chosen cell factory for production of biofuels such as Farnesene (commercialized by Amyris) and isobutanol (Gevo and Butamax), biopolymers building blocks such as lactic acid (Cargill) and succinic acid (Bioamber and Corbion), as well as higher value molecules such as plant natural products artemisinic acid (Amyris), resveratrol (Evolva) and nootkatone (Evolva).

The production of many industrially interesting compounds in a heterologous host such as *S. cerevisiae* demands the insertion of whole or partial biosynthetic pathways (Mikkelsen *et al.* 2012; Ongley *et al.* 2013; Krivoruchko and Nielsen 2014). Recent years have seen a rise in the availability of synthetic biology tools aimed at expediting yeast metabolic engineering (Fletcher, Krivoruchko and Nielsen 2016). This includes characterization of new parts such as promoters (Redden and Alper 2015; Rajkumar *et al.* 2016) and terminators (Curran *et al.* 2015), new episomal and integrative expression systems (Mikkelsen *et al.* 2012; Jensen *et al.* 2014) and the emergence of CRISPR/Cas9 for rapid strain modification (Jakočiūnas *et al.* 2015). Still, one disadvantage of engineering yeast compared to prokaryotic systems is the absence of a polycistronic gene expression system. As a result, a separate promoter and terminator are required for each gene, resulting in larger constructs. Furthermore, due to the efficient homologous recombination system of *S. cerevisiae*, dual-usage of the same promoter and terminator can result in gene loss, while using different promoters/terminators can result in differential expression of pathway genes. This can be especially problematic when assembling complex pathways consisting of many different genes.

In the present paper, we aimed to overcome these disadvantages by creating and characterizing a polycistronic-like expression system in yeast using 2A peptides. In addition to significantly reducing construct size, such a system would allow for stoichiometric expression of the different proteins since only one promoter and terminator are used and no *cis*-acting element is needed for the expression (Ahier and Jarriault 2014; Daniels *et al.* 2014; Liu *et al.* 2017). Furthermore, the small size of 2A peptides allows for easy amplification together with the gene of interest, reducing the number of cloning parts of one cassette and significantly reducing pathway construction time.

A high separation efficiency is crucial to effectively use 2A peptides for metabolic engineering applications. Cleavage functionalities of various 2A peptides have been characterized since the 90s (Ryan, King and Thomas 1991; Ryan and Drew 1994; Ryan *et al.* 1999). The so-called mechanism of 'self-cleavage' promoted by 2A peptides actually involves ribosomal stalling and peptide bond skipping recoding protein translation in a 'stop-carry on' or 'StopGo' way (Donnelly *et al.* 2001; Atkins *et al.* 2007). That is possible because of the characteristic C-terminal DXEXNPGP motif (Donnelly *et al.*, 2001; Sharma *et al.* 2012). The ribosome pauses after glycine transfer to the nascent peptide chain and translocation of the glycine and proline codon to P and A sites, respectively. In the predicted model, peptide bond to the prolyl-tRNA^{Pro} is hampered by the 2A shifted conformation in the ribosomal exit tunnel and due to the following poor nucleophile proline (Doronina *et al.* 2008; Sharma *et al.* 2012). The situation is solved by releasing the upstream protein linked to the 2A peptide and translation of downstream protein continues with proline as the first residue (Donnelly *et al.* 2001; Atkins *et al.* 2007; Doronina *et al.* 2008). Although the separation of the proteins occurs by a ribosomal recoding, terms such as 'cleavage' or 'self-cleavage' are still frequently applied to 2A peptides (Liu *et al.* 2017).

The cleavage efficiency of 2A peptides with different reporter proteins in the ribosome of *in vitro* or different *in vivo* system has been extensively studied (Halpin *et al.* 1999; Provost, Rhee and Leach 2007; Doronina *et al.* 2008; Luke *et al.* 2008; Kim *et al.* 2011; Gao, Jack and O'Neill 2012; Sharma *et al.* 2012; Odon *et al.* 2013; Daniels *et al.* 2014; Geier *et al.* 2015; Roulston *et al.* 2016). 2A peptides have been used for the production of monoclonal antibodies (Fang *et al.* 2005; Chng *et al.* 2015), gene therapy (Szymczak *et al.* 2004) and production of β-carotene in plants (Ha *et al.* 2010). This system has also been shown to work for metabolic engineering applications in yeast (Beekwilder *et al.* 2014; Geier *et al.* 2015). However, it was not extensively characterized in these studies, with limited studies of the cleavage efficiencies of the peptides used or expression and functionality of the resulting proteins compared to monocistronic expression.

The aim of the present work was to further characterize the 2A system for metabolic engineering applications in the yeast *S. cerevisiae*. Since cleavage efficiency tends to differ between different organisms, and because high cleavage efficiency is a key to getting similar expression levels from all proteins in the construct, we evaluated twenty two 2A peptides using two fluorescent proteins. Out of those evaluated, three 2A peptides showed acceptable 'cleavage' efficiencies in yeast and potential to be used in the construction of pathways to be inserted/modified in *S. cerevisiae*.

MATERIAL AND METHODS

Strains, media and growth conditions

Molecular biology procedures and cultivations followed standard literature (Sambrook and Russell 2001). For cloning purpose, we used *Escherichia coli* DH5α strain (*fhu*A2Δ (*arg*F-*lac*Z) U169 *pho*A *gln*V44 Φ80Δ (*lac*Z)M15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17) cultivated in Luria-Bertani medium supplemented with ampicillin 100 μg/mL, at 37°C. *Saccharomyces cerevisiae* strain CEN.PK113-5D (*MAT***a** *MAL2-8^c SUC2 ura3-52*; kindly provided by P. Kötter, University of Frankfurt, Germany) was used for *in vivo* expression and translation system. Yeast pre-inocula were maintained in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium and transformants were selected on synthetic dextrose (SD; 0.67% yeast nitrogen base, 2% dextrose) medium without uracil, at 30°C after lithium acetate/single-stranded DNA heat shock transformation. Experimental cultivations were conducted in the same SD-uracil medium, at 30°C for overnight.

Plasmid constructions

Plasmids were constructed based on p416 (Mumberg, Müller and Funk 1995), a centromeric plasmid carrying the green fluorescent protein (GFP) under control of *TEF1* promoter; *CYC1* as terminator and *URA3* as selection marker gene. To construct

Table 1. General primers utilized in this work.

Primer identification	Primer sequence 5'–3'	Note
P13_fwd	**TGGCATGGATGAACTATACAAATAG**GTAGATACGTTGTTGACACTTCTAA	tADH1 amplification with overlap to p416 (in bold)
P14_rev	**CTATAGGGCGAATTGGGTACCGGCC**GAGCGACCTCATGCTATACCTGAGA	
P11_fwd	GGCCGGTACCCAATTCGCCCTATAG	p416 amplification without tCYC1
P12_rev	CTATTTGTATAGTTCATCCATGCCA	
P15_rev	TTTGTAATTAAAACTTAGATTAGAT	Anneals to P_{TEF1}
P16_fwd	**ATCTAATCTAAGTTTTAATTACAAA**ATGGCCTCCTCCGAGGACGTCATCA	RFP amplification with overlap to p416 (in bold)
P1_fwd	**TGCTTTCTCAGGTATAGCATGAGGTCGCTC**GGCCGGTACCCAATTCGCCCTATAG	Primer forward used with reverse primers in Table S1. Anneals to p416. Overlaps to tADH1 (in bold)
P4_rev	GAGCGACCTCATGCTATACCTGAGAAAGCA	Primer reverse used with forward primers in Table S1. Anneals to the end of tADH1
Fwd_GFP_5seq	GGCAGACAAACAAAAGAATGG	Used for sequencing and colony PCR with P4_rev

the GFP control plasmid, tCYC1 was exchanged for tADH1 using overlapping primers P13_fwd and P14_rev to amplify tADH1 fragment whereas [p416-P_{TEF1}-GFP] (David, Nielsen and Siewers 2016) was amplified using primers P11_fwd and P12_rev with overlap to GFP and the vector as recommended by Gibson protocol (Gibson *et al.* 2009). Primers used in this work are listed in Table 1 and Table S1, Supporting Information. Vector and fragments were amplified by PrimeSTAR® HS DNA polymerase (Takara Bio Inc, Saint-Germain-en-Laye, France) according to the manufacturer recommendations. Amplicons were purified and combined in a 3:1 insert:vector ratio together to Gibson Assembly® Master mix (New England Biolabs, Ipswich, MA, USA). Red fluorescent protein (RFP) control plasmid was constructed in the same way using primers (P11_fwd and P15_rev) to amplify vector and P_{TEF1}. The fragment RFP-tADH1 was amplified from plasmid template p0394 with overlapping primers (P16_fwd and P14_rev).

All the 2A sequences were constructed between GFP and RFP by adding about 45–50 nucleotides into the reverse primer (Table S1, Supporting Information) for GFP-vector amplification pairing with primer forward P1_fwd (Table 1). Primers overlapping and complementing 2A sequence by 45–50 nucleotides were designed into the forward primer (Table S1, Supporting Information) for RFP-tADH1 amplification using reverse primer P4_rev (Table 1). Nucleotides coding the 2A sequences were codon optimized for yeast translation. After 30 min the assembly mixture was transformed into *E. coli* and correct assembly was verified by colony PCR using DreamTaq (Thermo Scientific) according to the manufacturer protocol using primers Fwd_GFP_5seq and P4_rev. DNA sequencing was performed with primer Fwd_GFP_5seq. Reporters were transformed into CEN.PK113-5D and selected in SD–uracil medium.

Mutation of ERBV-1 2A site

The site-directed mutagenesis was conducted using [p416-P_{TEF1}-GFP-ERBV2A-RFP] plasmid as a template and single primer amplification method (Edelheit, Hanukoglu and Hanukoglu 2009). Primers used to promote the substitution of Pro20 to Ala were AlaF (forward): 5'-GAATTGAATCCAGGTgctATGGCCTCCTCCGAG-3' and AlaR (reverse): 5'-CTCGGAGGAGGCCATagcACCTGGATTCAATTC-3', where lowercase indicates the amino acid substitution. Amplifications were performed with Phusion High Fidelity DNA polymerase (2000 U/mL; New England Biolabs). The single-stranded amplification products were mixed and annealed by gradually decreasing the temperature 10°C/min from 98°C to 37°C. Resulting double-strands were treated with *Dpn*I (20 U/μL; New England Biolabs) and transformed into *E. coli*. DNA sequencing was performed with primer Fwd_GFP_5seq to confirm the presence of the desired mutation. Mutant 2A peptide reporter was transformed into CEN.PK113-5D and selected in SD–uracil medium.

Fluorescence microscopy

Yeast strains expressing the reporters were pre-cultured overnight and then diluted to an optical density at 600 nm (OD_{600nm}) of 0.1 in 20 mL of SD–uracil medium. Strains were cultivated at 30°C and samples were taken from mid-exponential phase ($OD6_{00nm}$ 0.5–0.8) and washed once with phosphate buffered saline (PBS). GFP fluorescence was detected with a 525/30 filter and RFP, with 690/50 filter using a Leica AF 6000 inverted fluorescence microscope (Wetzlar, Germany) with a 100× objective. Images were processed with the Leica Application Suite software.

Western blotting

Protein extracts were prepared as described previously (Chen and Petranovic, 2015). Quantification was performed using RC DC Protein Assay (Bio Rad, Hercules, USA) against a bovine serum albumin (Sigma-Aldrich) calibration curve. A 4%–12% Bis-Tris gel (Invitrogen) was used to separate 50 μg of protein of each sample for about 2 h at 90 V in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3; Bio Rad). Transfer of proteins to Polyvinylidene difluoride (PVDF) membrane (Bio Rad) was performed in a semi-dry transfer system (Bio Rad) using a transfer buffer containing 50 mM Tris,

38 mM glycine, 20% v/v methanol for 1 h at 25 V. Membranes were blocked with blocking buffer (Sigma-Aldrich) for 1 h and then incubated with the primary monoclonal antibody anti-RFP (Life Technologies, Eugene, OR, USA) or anti-GFP (Roche Life Science) for overnight at 4°C. After, membranes were washed 4 times with PBS-0.05% Tween 20, probed with secondary anti-rabbit antibody for 1 h and again washed previous to the detection by luminescence with ECL Prime reagent (GE Healthcare) and ChemiDoc XRS image analyzer (Bio Rad). The band intensities on the membranes were determined using ImageJ software. 'Cleavage' efficiency was calculated as follows: cleavage efficiency = 100 × (cleaved RFP form)/(cleaved RFP form + uncleaved form).

Evaluation friedelin production using the 2A system

Friedelin synthase coding sequence from *Maytenus ilicifolia* (MiFRS, GenBank accession number KX147270) (Souza-Moreira *et al.* 2016) was synthesized by GenScript with codon optimization for expression in *S. cerevisiae*. MiFRS sequence was subcloned into the yeast expression pSP-GM1 (Partow *et al.* 2010) under control of the *TEF1* promoter using restriction enzymes *Sac*I and *Spe*I (FastDigest, Thermo Fisher Scientific). The truncated form of *HMG1* gene was cloned into the [pSP-P_{TEF1}-MiFRS] plasmid between *BamH*I and *Sal*I (FastDigest, Thermo Fisher Scientific) restriction sites under *PGK1* promoter control. The plasmid [pSP-P_{TEF1}-MiFRS, P_{PGK1}-t*HMG1*] was used as control of friedelin production level in CEN.PK113-5D strain.

To demonstrate the functionality of the 2A peptide for metabolic engineering applications, the friedelin biosynthetic pathway was expressed in yeast using the bicistronic construct [pSP-P_{PGK1}-MiFRS-2A-t*HMG1*]. To construct the plasmid, fragments of each module (i.e. homologous recombination module for up and down parts of the plasmid, promoter, MiFRS, t*HMG1* and *ADH1* terminator) were PCR amplified with overlapping primers (described in Table S2, Supporting Information) using PrimeSTAR® HS DNA polymerase. After gel purification, modules were PCR assembled into one fragment (Zhou *et al.* 2012). pSP-GM1 backbone was PCR amplified and gel-purified. Equal molar of assembled modular fragment and pSP-GM1 backbone were chemically transformed into CEN.PK113-5D. Colony PCR was used to screen the presence of assembled plasmid. Plasmids were then recovered from cell lysates, transformed into *E. coli* and again recovered for sequencing verification of the construct.

Quantification of heterologous friedelin production

CEN.PK113-5D transformed with empty pSP-GM1, [pSP-P_{TEF1}-MiFRS, P_{PGK1}-t*HMG1*] or [pSP-P_{PGK1}-MiFRS-2A-t*HMG1*] were pregrown in SD-uracil medium. For heterologous friedelin production, cells were diluted to a starting OD_{600nm} of 0.05 in minimal medium (Scalcinati *et al.* 2012) and cultivated for 72 h at 30°C with shaking. Then, cells were collected, dried and about 30 mg of dried cell weight were extracted with chloroform:methanol (2:1, v/v; Khoomrung *et al.* 2013) in an ultrasonic bath (2840D, Odontobrás, Ribeirão Preto, SP, Brazil) for 10 min. The organic phase was collected after addition of 0.73% NaCl and centrifugation. The extract was dried and resuspended in 200 μL acetonitrile to be analyzed by gas chromatography associated to mass spectrometry (QP2020C W/O RP230V, Shimadzu, Kioto, Japan) using a HP-5 column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Santa Clara, California, USA). Analysis was performed with inlet temperature of 270°C, heating gradient from 200°C to 290°C (10°C/min), trap temperature of 200°C, interface temperature of 290°C for 18 min, injection volume of 1 μL, split ratio of 1:10, flow gas of 1.0 mL/min, ionization of 70 eV and detection interval of 35–600 m/z. Cholesterol was spiked in as internal standard control at 40 μg/mL before friedelin extraction process. An analytical curve of friedelin standard (Sigma-Aldrich, St. Louis, Missouri, USA) was constructed. The peak of friedelin was observed at retention time of 23.08 min and identity was confirmed by mass spectral detection compared to National Institute of Standards and Technology (NIST) library and standard. Quantification analysis was done in triplicate and statistical significance was analyzed by the Student's t-test (p-value < 0.05).

RESULTS AND DISCUSSION

Reporter structure

Avoiding the use of a separate promoter and terminator for each single gene expressed in yeast cells can make metabolic engineering easier and faster in cases where multiple pathway steps must be expressed. Enabling two, three or more genes to be expressed in one single mRNA molecule would result in stoichiometric co-translation of separate proteins without the need for proteinase cleavage (Ryan *et al.* 1999; Donnelly *et al.* 2001; Lorens *et al.* 2004; Torres *et al.* 2010). As the cleavage efficiency of 2A peptides can vary greatly between different organisms and peptides, we selected 22 different 2A self-cleavage peptides in which the 'self-cleavage' had been described previously. When designing the reporters, we used the *consensus* sequence NPG-P for 'self-cleavage' (Donnelly *et al.* 2001) and the flexible linker Gly-Ser-Gly (Holst *et al.* 2006; Gao, Jack and O'Neill 2012). In addition, the size of the peptides was kept at 18–22 residues since these showed to be the smaller lengths that could still have considerable cleavage efficiency (Donnelly *et al.* 2001; Minskaia and Ryan 2013), which makes easier their design within primers.

We designed primers with about 48 nucleotides of the peptide sequences in the 5' and 30 nucleotides in 3' annealing to the tested proteins, as shown in Table S1, Supporting Information. One of the possible strategies to assemble all the fragments is to use the Gibson assembling kit, so the overlap between both primers carrying the 2A partial sequences should have a melting temperature ≥48°C. This assembling strategy enables a single-step construction of pathways consisting of multiple proteins.

Screening of the best 2A self-cleavage efficiency in *Saccharomyces cerevisiae*

After its discovery and 'cleavage' mechanism elucidation, 2A peptides were widely used in biotechnology (Minskaia, Nicholson and Ryan 2013; Minskaia and Luke 2015; Roulston *et al.* 2016). They were employed for polycistronic expression in different cells, for instance in filamentous fungi (Unkles *et al.* 2014), protozoan (Tang *et al.* 2016), worm (Ahier and Jarriault 2014), insect (Daniels *et al.* 2014; Wang *et al.* 2015), plant (Farré *et al.* 2014), zebrafish (Provost, Rhee and Leach 2007; Kim *et al.* 2011) and mammalian (Szymczak *et al.* 2004; Kim *et al.* 2011; Chng *et al.* 2015; Liu *et al.* 2017) cells. However, use in yeast for biotechnological purposes has been limited, and experiments to date have only used two or three of the most known sequences in yeasts *Saccharomyces cerevisiae* (Sharma *et al.* 2012; Beekwilder *et al.* 2014) and *Pichia pastoris* (Amorim Araújo *et al.* 2015; Geier *et al.* 2015). However, the 2A 'cleavage' efficiency was not

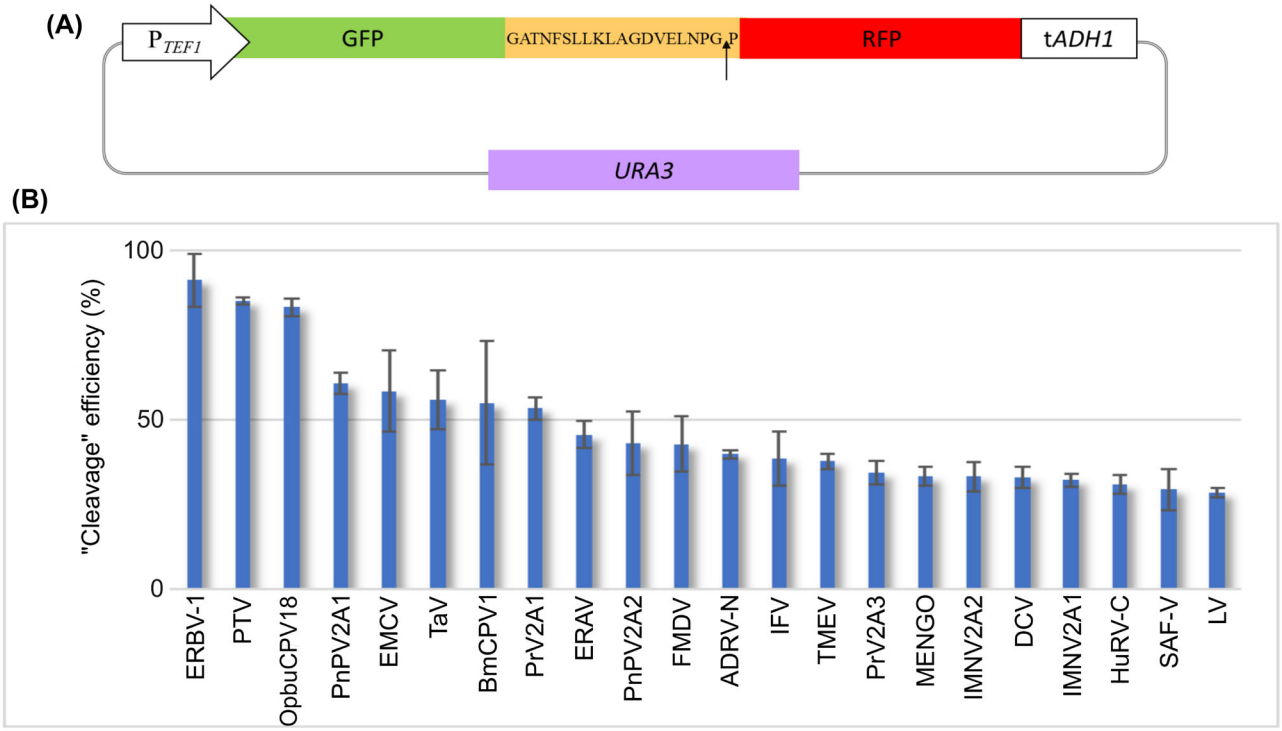

Figure 1. Analysis of 2A peptide 'cleavage' efficiency. (**A**) Structure of the plasmid reporters showing 'cleavage' site in the 2A sequence. (**B**) 'Cleavage' efficiency, as percentage for the 22 different 2A peptides tested. Data shown represent the intensity average values ± standard deviation of western blotting of three independent biological replicates.

carefully determined. To the best of our knowledge, this is the first screening performed among a variety of 2A peptides to determine the sequence with the highest 'self-cleavage' efficiency in *S. cerevisiae*.

Our constructs contained GFP in the first position and RFP in the second position. 'Cleavage' efficiency determination was based on western blots luminescence values relative to RFP bands, following previously reported methodology (Liu *et al.* 2017), since the presence of the cleaved form of the second protein is a clear evidence of the multi-protein separation from a single transcript. The products quantified were the cleaved form of RFP (the sequence starting with the last proline of the 2A peptide) and uncleaved form of GFP-2A-RFP reporter (Fig. 1A), with calculated molecular weight of 25 and 50 kDa, respectively. The production of GFP linked to the 2A sequence was also confirmed as a band of about 25 kDa by western blot. The 'self-cleavage' efficiency of each of the 22 sequences of 2A peptides tested in *S. cerevisiae* was determined as percentage of the ratio between the intensity of the luminescent band of RFP cleaved form (~25 kDa) correlated to the uncleaved form of the reporters (~50 kDa; Fig. 1B). Therefore, the percentage of cleavage efficiency was correlated with the level of the two single fluorescent proteins from just one mRNA.

All the 2A peptide sequences evaluated showed some level of cleavage, ranging from ~33% to 91% efficiency. From all the 22 peptides, three exhibited efficiencies higher than 80% and six showed about 50% (Fig. 1B).

The best performance was achieved using the Equine rhinitis B virus (ERBV-1) in which the uncleaved form GFP-2A-RFP (of 50 kDa) represented less than 10%, followed by the Porcine teschovirus-1 (PTV) (also known as P2A) and Operophtera brumata cypovirus-18 (OpbuCPV18) peptides (Fig. 2A). On the other hand, commonly used sequences such as TaV (or T2A from Thosea asigna virus), ERAV (or E2A from Equine rhinitis A virus) or FMDV (or F2A from Foot-and-mouth disease virus) showed self-cleavage efficiency in CEN.PK113-5D of 56%, 46% and 43%, respectively (Fig. 1B). Furthermore, the level of expression of the RFP cleaved form from the polycistronic construct with the best 2A peptides was comparable with the expression of RFP in the monocistronic construct under control of the same promoter, in the same *S. cerevisiae* strain under same culture conditions (Fig. 2B), indicating that the use of 2A peptide can lead to the production of more than one protein from one mRNA and result in comparable levels of protein produced as the protein levels from the monocistronic translation. To ensure the 2A peptide of ERBV-1 was responsible for the high efficiency in producing the cleaved proteins, we substituted the last proline in the NPGP site for an alanine residue (ERBV-1^{Pro20Ala}). The loss of 'self-cleavage' efficiency by an alanine substitution in that specific position was already reported (Sharma *et al.* 2012) and, as expected, the site-direct mutant decreased the production of the cleaved protein form and accumulated the uncleaved form (Fig. 2A), resulting in 57% of efficiency and RFP detection was half of RFP control (Fig. 2B).

GFP and RFP did not lose functionality due to the amino acids of the 2A peptide from ERBV-1 in the C-teminus of GFP nor to the proline starting sequence of RFP and that can be seen by their fluorescence detected by microscopy with the cells (Fig. S1, Supporting Information). Besides, it is possible to observe a general reduction mainly in the fluorescence of RFP from the cells containing the mutant ERBV-1^{Pro20Ala} reporter as result of the increased level of uncleaved form of the proteins. GFP in the mutated reporter showed higher fluorescence intensity compared to RFP, indicating that functionality is impaired to the protein downstream 2A peptide when cleavage does not occur (Liu *et al.* 2017).

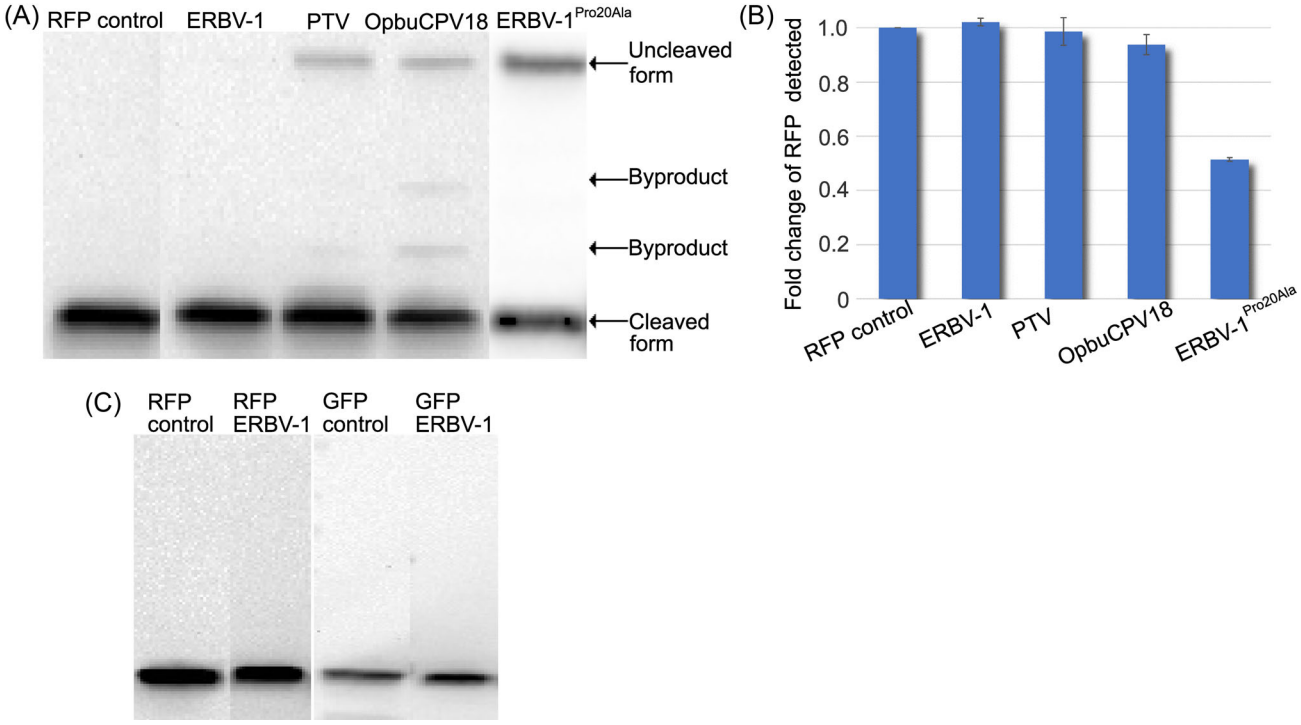

Figure 2. Western blot of RFP from bicistronic expression and its detected level compared to the monocistronic control. (**A**) Western blot representing bands of RFP from control plasmid expression and reporters with the most active 2A peptides in *S. cerevisiae* (ERBV-1, PTV and OpbuCPV18) and reporter mutated ERBV-1^{Pro20Ala}. The presence of byproducts increases as the cleavage efficiency decreases. (**B**) Fold change of intensity of RFP level detected from expression of the most active 2A peptides and mutated ERBV-1^{Pro20Ala} (average values ± standard deviation), compared to the intensity of RFP level detected from expression of the control plasmid (fold change = 1), showing no significant difference in the expression among the different constructs. (**C**) Representative western blot of the 'flipped' reporter, with RFP in first position and GFP after the 2A peptide sequence of ERBV-1. Bands of RFP and GFP in the reporter have similar intensity than control bands and almost none uncleaved band present.

The cleavage efficiency of ERBV-1 was previously tested *in vitro* using reticulocyte lysate (Luke *et al.* 2008). The sequence had 30 amino acids and exhibited 99% cleavage as other sequences from the *Picornaviridae* family, such as FMDV and ERAV. Interestingly, among the 22 sequences tested herein, ERBV-1 was the only one to have a leucine residue in position 16, whereas the conserved residue at this position is a serine or other amino acids with polar side chain such as threonine, glutamate or glutamine (Fig. 3). In previous studies (Donnelly *et al.* 2001; Sharma *et al.* 2012), changing the serine at this position to other amino acids reduced drastically the cleavage activity of FMDV sequence to about 40%. However, in *S. cerevisiae* the variation to the apolar leucine residue in the consensus motif GDVELNPGP (Sharma *et al.* 2012) did not mean loss of efficiency.

PTV 'cleavage' efficiency was measured in different cellular systems (as shown in Table S3, Supporting Information) and it was employed in some bicistronic constructions assayed in different model cells, such as the yeasts *S. cerevisiae* (Beekwilder *et al.* 2014) and *P. pastoris* (Geier *et al.*, 2015), the protozoan *Eimeria tenella* (Tang *et al.* 2016), the worm *Caenorhabditis elegans* (Ahier and Jarriault 2014) and human T-cell lines (Yang *et al.* 2008).

Remarkably, when PTV sequence was assayed in *S. cerevisiae* CEN.PK2-1C no cleavage was observed from GFP-P2A and *LEU2*-HA bicistronic cassettes (Beekwilder *et al.* 2014). However, in the same study, the employment of TaV in the reporter construct did lead to the translation of both cleaved proteins with a slight uncleaved form present (no other 2A peptide was assayed). In our study, PTV showed 85% cleavage efficiency while TaV with 18 or 20 amino acids showed less than 50%, which

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TMEV          1 GYHADY-YKQRLIHDVEMNPGP
LV            1 --YAGG-KFLNQCGDVETNPGP
EMCV          1 --YAGY-FADLLIHDIETNPGP
MENGO         1 --YAGY-FSDLLIHDVETNPGP
SAF-V         1 --HASY-YKQRLQHDVETNPGP
IFV           1 -TRAEI-EDELIRAGIESNPGP
TaV           1 ----EGRGSLLTCGDVEENPGP
PrV2A1        1 ---VGGRGSLLTCGDVESNPGP
PrV2A3        1 ---SGGRGSLLTAGDVEKNPGP
PnPV2A1       1 -QGWV--PDLTVDGDVESNPGP
PnPV2A2       1 -GGGQ--KDLTQDGDIESNPGP
ERAV          1 --QCTNYALLKLAGDVESNPGP
FMDV          1 VKQTLNFDLLKLAGDVESNPGP
ERBV-1        1 --GATNFSLLKLAGDVELNPGP
PTV           1 ---ATNFSLLKQAGDVEENPGP
ADRV-N        1 ---NSSWVRDLTRECIESNPGP
DCV           1 --EAARQMLLLLSGDVETNPGP
HuRV-C        1 --SKFQIDKILLSGDIELNPGP
IMNV2A2       1 ---KEEHTDILLSGDVEENPGP
OpbuCPV18     1 ---KSNYDLLKLCGDVESNPGP
BmCPV1        1 ---RSNYDLLKLCGDIESNPGP
IMNV2A1       1 ---MLPPPDLTSCGDVESNPGP
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Figure 3. Alignment among 2A peptides used in this work showing conserved residues. There is a high conservation in the motif DXEXNPGP important for 'cleavage' efficiency (shown as dark shaded).

could be related to some former observations that the upstream context is sometimes important to the 'cleavage' efficiency (Donnelly et al. 1997). Together with the higher efficiency shown by PTV when employed in other cell systems, it is noteworthy to analyze the more suitable 2A sequence to be used according to the cell system and protein context (Donnelly et al. 2001; Kim et al. 2011; Kuzmich et al. 2013; Minskaia, Nicholson and Ryan 2013). Keeping this in mind, we constructed a reporter with the most efficient 2A sequence from ERBV-1 locating RFP in front of GFP (RFP-ERBV2A-GFP). The 'cleavage' efficiency observed was still higher than 90% (Fig. 2C) corroborating with the use of ERBV-1 2A sequence for co-expression and co-translation of different proteins in *S. cerevisiae* independently, in the present context, of protein position (Rothwell et al. 2010).

OpbuCPV-18 is an insect cypovirus and its 2A peptide showed 99% of 'cleavage' efficiency in an *in vitro* system of reticulocyte lysate (Luke et al. 2008). In the analysis presented in *S. cerevisiae*, it was as efficient as 2A peptide from PTV (Fig. 1). Whereas the 2A peptide sequence of ERBV-1 and PTV share almost 90% homology differing in a leucine in positions 10 and 16 in the first for two polar side chains in the corresponding residues in PTV (Fig. 2), the sequence of OpbuCPV-18 is less than 70% homologous to both.

The FMDV 2A peptide sequence was one of the first 2A/2B protein 'cleavage' peptide described and systematically analyzed (Ryan, King and Thomas 1991). Its employment in *in vitro* and *in vivo* systems resulted in high 'cleavage' efficiency (Table S3, Supporting Information), and *S. cerevisiae* was already used as a model organism to analyze the 'cleavage' mechanism and critical residues for the active peptide (De Felipe et al. 2003; Doronina et al. 2008; Sharma et al. 2012). However, although protein separation by the FMDV 2A peptide was still active, its efficiency in the present study was less than 50%, once more stressing the importance of activity characterization of the 2A sequences in a cell-specific context.

It is interesting to compare the 'cleavage' efficiency of some of the 2A peptides tested in this work to the works with other systems (as described in Table S3, Supporting Information). The peptide displaying the best cleavage efficiency of all 22 peptides so far was achieved with an *in vitro* system, i.e. reticulocyte lysate, and notably for the FMDV 2A peptide. However, when compared to mammalian cells, attention should be given to the decreased efficiency of classic 2A peptides such as FMDV, TaV and ERAV, for example, as also shown in the present work. The only 2A peptide to exhibit high cleavage efficiency in all systems was PTV, being the second with good efficiency herein.

This is the first time that the activity and efficiency of 2A sequences from IFV, SAF or BmCPV1, among others, were assayed in *S. cerevisiae*. All of them showed 'cleavage' activity but this activity is too low to be suitable for metabolic engineering applications (Fig. 1).

Application of the best 2A peptide for heterologous expression in yeast

Friedelin is a unique plant pentacyclic triterpene because it has the higher number of rearrangements during 2,3-oxidosquelene cyclization by the friedelin synthase. Pharmacologically, it has shown promising liver and gastroprotective properties (Sunil et al. 2013; Antonisamy et al. 2015). The compound is also a precursor of the claimed antitumoral quinone methide triterpenoid pristimerin (Corsino et al. 2000).

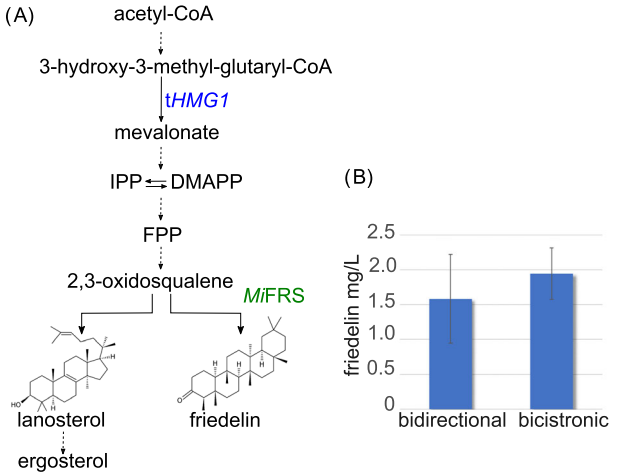

Figure 4. Mevalonate and ergosterol pathway and friedelin heterologous synthesis. (**A**) Biosynthesis of mevalonate by expression of t*HMG1* and friedelin production by cyclization of 2,3-oxidosqualene by MiFRS, deviating the precursor of yeast endogenous production lanosterol. (**B**) Production of friedelin is indicated by the bidirectional plasmid [pSP-P_{TEF1}-MiFRS, P_{PGK1}-t*HMG1*] and the bicistronic [pSP-P_{PGK1}-MiFRS-2A-t*HMG1*].

The friedelin synthase utilized in the present work was characterized from mRNA from the leaves of *Maytenus ilicifolia* and therefore named MiFRS (Souza-Moreira et al. 2016). Its substrate 2,3-oxidosqualene is produced in yeast and cyclized to lanosterol as part of the ergosterol pathway (Corey, Matsuda and Bartel 1994). To improve the precursor production, a truncated form of the catalytic domain of yeast gene *HMG1* coding 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA), a rate-controlling enzyme for isoprenoid production, was also expressed. HMG-CoA is a precursor for mevalonate (Fig. 4A), a precursor of isoprenoids. Squalene accumulation was observed with the overexpression of the truncated form *HMG1* (Polakowski, Stahl and Lang 1998) and it was successfully used in the production of plant sesquiterpenes and triterpenes in yeast (Kirby et al. 2008; Hu et al. 2017).

Friedelin was produced by expressing the two mentioned genes, first, in a bidirectional plasmid, with MiFRS under control of *TEF1* promoter and t*HMG1* under *PGK1* promoter. Second, ERBV-1 2A peptide 'cleavage' was tested by linking MiFRS to t*HMG1* under the control of a *PGK1* promoter.

Expression of the two genes from separate promoters in a bidirectional plasmid resulted in 1.6 mg/L of friedelin, whilst expression via the 2A system led to 1.9 mg/L. While this difference is not statistically significant, these results demonstrate that expression using the 2A system is not detrimental to production, and might even be favorable. This is a proof of the applicability of this peptide in synthetic biology for heterologous production of a compound of interest in *S. cerevisiae* by functional enzymes using a bicistronic expression.

In conclusion, we successfully obtained almost 100% 'cleavage' efficiency when using a codon-optimized sequence of ERBV-1 2A peptide, a neglected 2A peptide. 2A peptides could serve as a valuable tool in synthetic biology strategies for co-expressing multiple proteins. However, the cell and protein context should be considered when using such peptides for metabolic engineering applications. We recognized three 2A sequences with high potential to be used in *S. cerevisiae* among 22 previously described peptides. Friedelin was the first effective heterologous

product produced in yeast using ERBV-1 2A peptide as a step forward to improve pathway cloning and metabolic engineering of yeast. The present analysis of best 2A 'self-cleavage' peptides in yeast could be especially useful for applications involving expression of complex pathways.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declare.

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