



## Compact multi-enzyme pathways in *P. pastoris*†

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**We report for the first time the functional simultaneous expression of nine genes from a single 2A peptide based polycistronic expression construct. The feasibility and arising opportunities for the biosynthetic pathway balancing for chemical production were demonstrated by the co-expression of the violacein and carotenoid biosynthesis pathways.**

Implementing natural and synthetic pathways into microorganisms provides new opportunities for the production of chemical building blocks, food and feed ingredients and active pharmaceutical ingredients (APIs). Thus, an emerging challenge nowadays is not to produce single proteins only, but multiple ones in sufficient amounts and with balanced activities along the pathway in order to avoid accumulation of side products or intermediates.

Commonly, multigene expression is based on co-expression constructs harbouring the pathway genes under the separate control of the (same) promoter and terminator. This usually works sufficiently well for proof-of-concept studies. However, the transformation rates of microbial cells generally decrease with the increasing size of the expression construct, while on the other hand technological difficulties and the costs for labour and materials increase. More importantly, the repeated use of homologous sequences can result in recombination events, and thus in genetic instability.<sup>1</sup> This is of special importance if physiologically problematic proteins or metabolites are produced at high levels and over extended periods. However, the genetic stability of production strains is essential for economically viable industrial processes and to maintain the product quality over extended production times. One strategy to

reduce the loss of genes by homologous recombination is the use of different promoter and terminator sequences for each individual gene of the pathway. In contrast to the repeated use of the same promoter, this strategy also enables us to balance the individual pathway catalysts at the level of transcription in order to avoid bottlenecks in the pathway and in favour of a high direct flux to the desired product. Alternatively, the number of regulatory elements can be reduced by the expression of multiple genes from a single, polycistronic transcript. While this is simple to achieve in prokaryotes, eukaryotes generally do not express polycistronic operons. One way to achieve polycistronic expression is the use of internal ribosome entry sites (IRES).<sup>2</sup> These sequences are able to initiate translation at internal sites within a polycistronic transcript. However, the employment of IRES in biotechnological applications is limited as the sequences are rather large (~500 nucleotides) and, more importantly, result in as much as 10 fold lower expression of the downstream-encoded protein.<sup>3–5</sup> Due to this fact this technology can hardly be applied for multigene expression. An attractive alternative constitutes the use of self-processing 2A sequences.<sup>6</sup> These 2A sequences, also known as *cis*-acting hydrolase elements, are short peptides (up to 20 amino acids) originating from viral polyproteins. 2A peptides act co-translationally causing a ribosomal skip that terminates translation at the final proline codon of its C-terminally located conserved sequence “NPGP”.<sup>4,7</sup> Thus, discrete proteins can be produced from a single transcript. It has to be noted that proteins located upstream to the 2A peptide keep this short stretch of amino acids as a C-terminal extension, while proteins located downstream of it contain an N-terminal proline. 2A peptides have been used for the co-expression of two genes in a variety of eukaryotic systems including yeasts, plants and mammalian cell lines<sup>8–10</sup> and the stoichiometric coproduction of individual peptides was seen as an advantage compared to the IRES technology.<sup>5</sup> However, careful analysis employing *in vitro* translation studies showed imbalances in protein levels for 2A mediated co-translation, too. Proteins that are encoded upstream of the 2A sequence accumulated to higher levels than those encoded downstream, and partial fusion protein formation was observed.<sup>4</sup>

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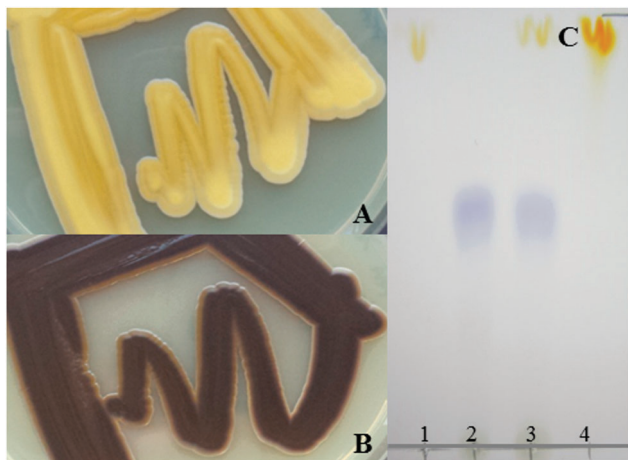
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† Electronic supplementary information (ESI) available: Western blot analysis of the preliminary experiments and experiments including a ubiquitin tag to demonstrate the feasibility to create authentic N-termini as well as experimental details and sequence information. See DOI: 10.1039/c4cc08502g

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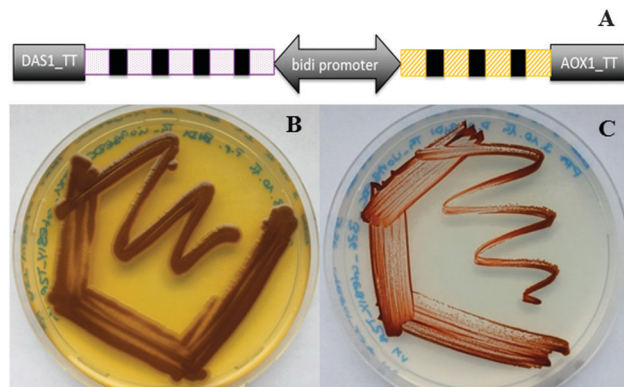


**Fig. 2** Functional expression of nine genes from a single polycistronic 2A peptide based transcript. (A) *P. pastoris* strains expressing a construct in which the carotenoid pathway genes are positioned upstream of the violacein pathway ones. (B) *P. pastoris* strains expressing a construct in which the violacein pathway genes are positioned upstream of the carotenoid pathway ones. The functional expression of both pathways is indicated by brownish coloured cells. (C) Thin layer chromatography of cell extracts obtained from strains expressing the carotenoid (1), the violacein (2) and the violacein/carotenoid pathway (3) based on 2A peptides;  $\beta$ -carotene served as the reference (4).

of individual components of the expression constructs. Whether premature termination of transcription is also a major factor to cause or enhance this positional effect is still under investigation. Nevertheless, for the first time this study shows that efficient polycistronic multigene expression is possible by positioning individual genes at different positions to fine tune the pathway expression. This is an attractive simple alternative to transcriptional regulation by different individual promoters. The short DNA sequences coding for 2A peptide sequences also provide future opportunity to serve as universal linkers for random combinatorial assembly of the individual coding sequences to optimize the order for improved and balanced expression of individual pathway components.

In an unprecedented evaluation of alternative compact pathway construction with even more genes and as an additional innovative strategy to tune the efficiency of individual pathway components we also combined the concept of polycistronic pathway expression with bidirectional promoters, short DNA sequences driving gene expression in both directions.<sup>17</sup> Constructs harbouring the violacein and carotenoid biosynthesis pathways in a bidirectional polycistronic format were generated. Also this new expression strategy resulted in strains successfully producing the target products of both pathways (Fig. 3).

In conclusion, we have successfully expressed nine genes from a single polycistronic construct employing 2A peptides in the yeast, *P. pastoris*. To the best of our knowledge this is the highest number of genes expressed in a coordinated fashion so far. By further combining these 2A peptides with bidirectional promoters this number might easily be exceeded. Thus, the presented expression strategy represents a valuable tool for the quick and simple establishment of multi-enzyme pathways.



**Fig. 3** Combining polycistronic expression based on 2A peptides with bidirectional promoters for pathway expression. (A) Schematic representation of the polycistronic bidirectional expression construct. *P. pastoris* strains harbouring the  $\beta$ -carotene (orange boxes) and violacein (purple boxes) pathways on expression constructs based on 2A sequences (black boxes) in combination with a bidirectional constitutive (B) or inducible (C) promoter.

The corresponding expression cassettes can be designed in a compact manner facilitating not only their construction, but all further molecular biologic steps. A further advantage is that 2A peptides allow the generation of genetically stable strains as the repetitive use of long homologous sequences is avoided. Various factors lead to partial generation of fusion proteins and non-stoichiometric production of the co-translationally produced proteins, and these effects imply useful strategies to optimize and balance pathway efficiencies by different sequential arrangements. Therefore, the 2A based expression concept not only simplifies and speeds up the discovery and feasibility of multi-enzyme pathways, but also their engineering. It remains to be shown whether multiplication of individual protein coding regions can be used for additional improvements. By applying existing recombination techniques, the 2A sequences can also be exploited as linkers to generate shuffled libraries containing the pathway genes in variable order and copies or functional homologs of individual pathway proteins. As the pathway genes are not separated by any additional regulatory sequence elements, the polycistronic expression cassette can directly be subjected to methods for random library generation such as error-prone PCR. However, as one deleterious mutation in one gene can cause the shut-down of the whole pathway, suitable high-throughput screenings are essential.

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