Direct Combinatorial Pathway Optimization

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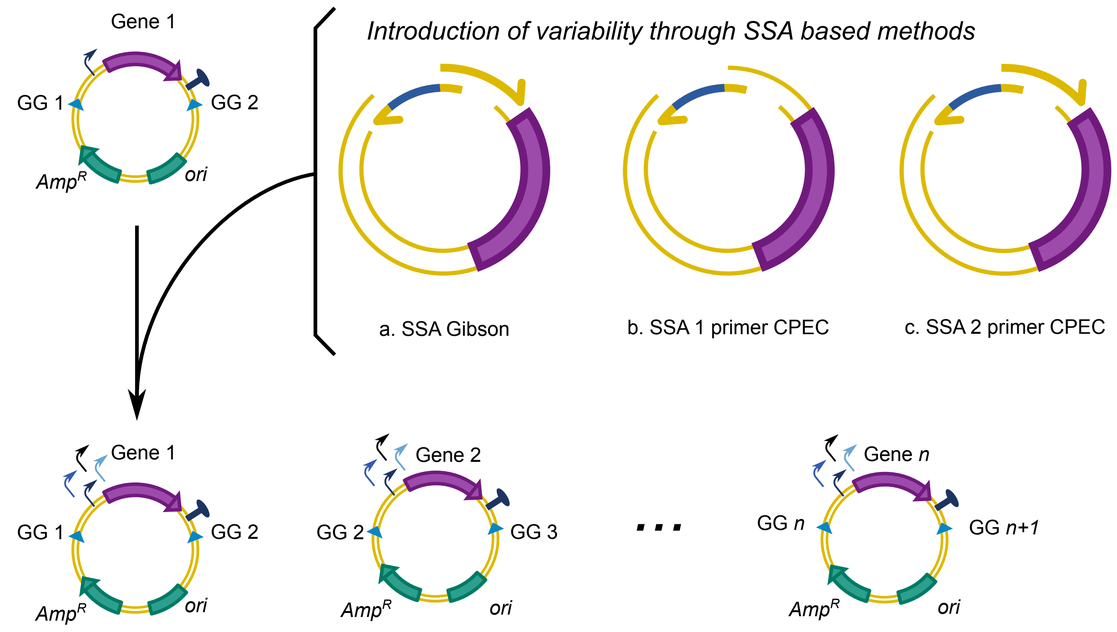
Introduction

Combinatorial engineering apporaches are becoming increasingly popular, yet they are hindered by the lack of specialized techniques for both efficient introduction of sequence variability and assembly of numerous DNA parts, required for the construction of lengthy multigene pathways. As a solution, we present the **Direct Combinatorial Pathway Optimization** workflow which combines the strenghts of Single Strand Assembly (SSA) methods[1] and Golden Gate Assembly (GGA)[2].

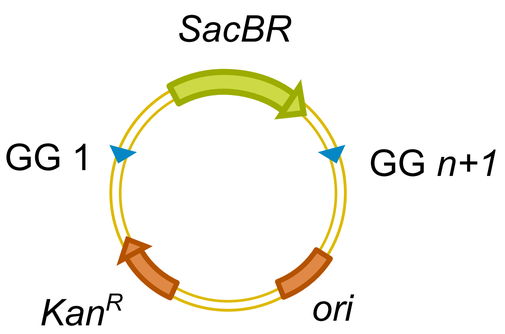
Carrier Plasmids



A set of carrier plasmids (pCPs; high copy *ori* and a Golden Gate proof amp^R selection marker) contain an operon flanked by 2 unique BsaI cut sites, designed for ordered GGA into an expression plasmid.
SSA techniques introduce variability in each carrier plasmid based on (a) Gibson assembly[3] or (b-c) CPEC[4].

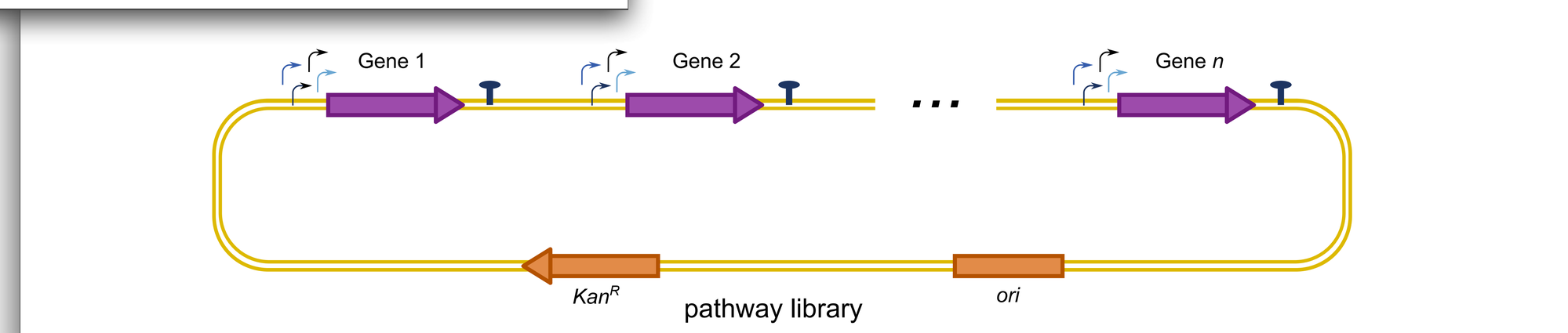
Expression Plasmids

Two sets of expression plasmids (pEXs; low and medium copy) were designed containing:
(i) a different selection marker,
(ii) a *sacBR* counter selection marker and
(iii) a set of inverse oriented BsaI cut sites for assembly.



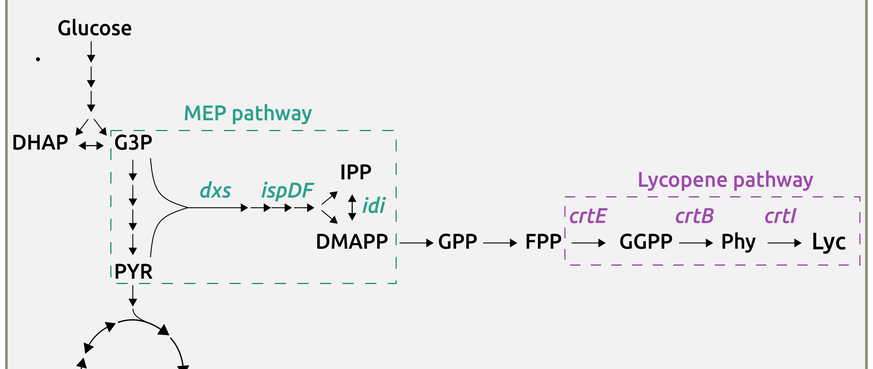
Golden Gate Pathway Assembly

Using GGA, the final multigene and combinatorial pathway is assembled. The BsaI created parts starting from the carrier plasmids are assembled in the appropriate expression plasmid(s). Library screening is performed by forward selection (*kanR*) and counter selection (*sacBR*).

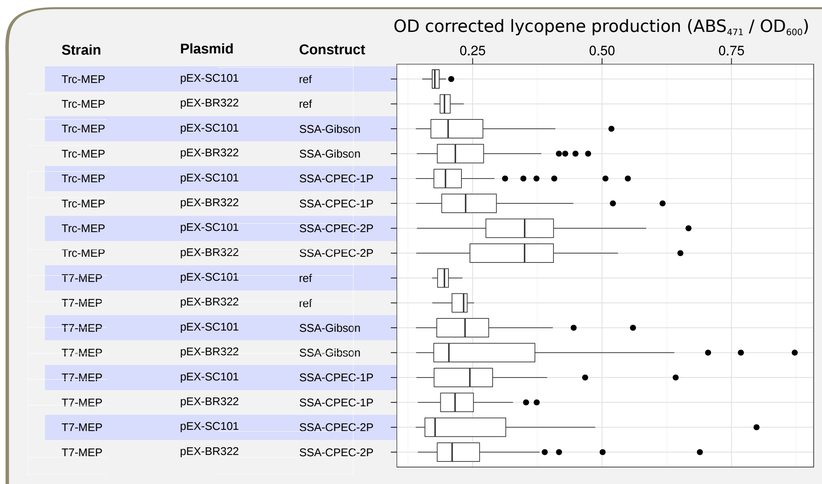


Lycopene test case

The **lycopene biosynthetic pathway** was chosen as proof-of-principle. This pathway of 3 enzymes (CrtE, I, B) leading up to the red carotene **lycopene**, which is used as food colorant and antioxidant.
To allow for sufficient precursor supply, a Trc-MEP (*dxs-idi- spdDF)* and a T7-MEP overexpressed strain were used[5].



crtE, *crtI* and *crtB* were introduced in pCP(1-2), (2-3) and (3-4) respectively and 3 individual promoter libraries, using the SSA methods, were introduced.

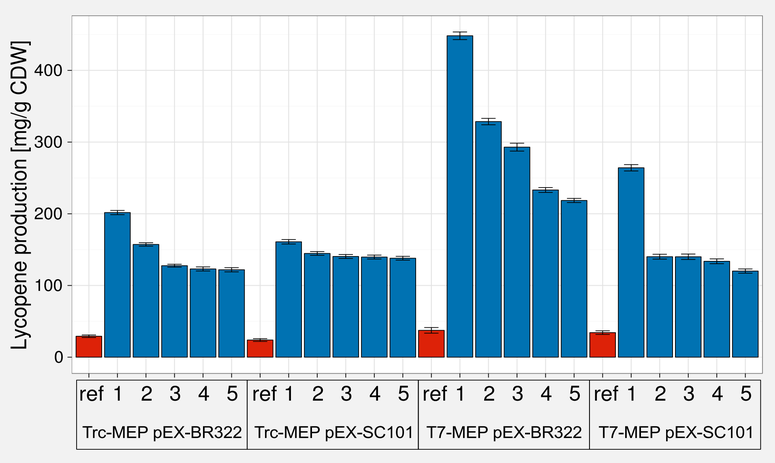


The individual gene libraries were assembled into pEX-SC101(1-3) and pEX-BR322(1-3) with copy numbers of 5 and 20, respectively, and cloned in both of the MEP overexpressed strains.

Outcome of the screening of 288 randomly picked colonies for each of the 12 generated strain libraries and 4 reference strains (left).

Consistencies between the individual libraries could not be observed, yet huge differences were observed between individual transformants.

The 5 best ranked strains for every expression host-plasmid combination (right). The best lycopene producer produces up to 448 mg lycopene/g CDW, twice as much in comparison with Rad et al. (198 mg lycopene / g CDW)[6].



Acknowledgments

David Bauwens was supported by a fellowship of Flanders Innovation & Enterpreneurship (VLAIO), Pieter Coussement by a fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT- Vlaanderen). This research was also supported by "Nano3Bio" (FP7/2007-2013, 613931), "Condex" (FWO - G.0321.13N) and "ML4SB" (BOF16/IOP/040).

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