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In vivo evolution of metabolic pathways by homeologous recombination in mitotic cells ☆

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ABSTRACT

We describe a rapid and highly efficient method for the assembly, recombination, targeted chromosomal integration and regulatable expression of mosaic metabolic pathways by homeologous recombination in DNA repair deficient yeast cells. We have assembled and recombined 23 kb pathways containing all the genes encoding enzymes for the production of flavonoids, a group of plant secondary metabolites of nutritional and agricultural value. The mosaic genes of the pathways resulted from pair-wise recombination of two nonidentical (homeologous) wild-type genes. The recombination events occurred simultaneously in the cell. Correctly assembled mosaic gene clusters could only be observed in DNA repair deficient strains. Thus, libraries of intragenic mosaic pathways were generated. Randomly isolated clones were screened for their ability to produce flavonoids such as kaempferol, phloretin and galangin. Thus, the functionality of the recombinant pathways was proven. Additionally, significant higher concentrations of metabolites such as naringenin, pinocembrin and dihydrokaempferol were detected. Further analysis also revealed the production of different aromatic compounds such as styrene, hydroxystyrene, phloretic acid and other molecules. We show that the *in vivo* homeologous recombination strategy can generates libraries of intragenic mosaic pathways producing a high diversity of phenylpropanoid compounds.

1. Introduction

Synthetic biology comprises the re-design and assembly of naturally existing parts and processes for the generation of cells which are able to produce chemicals of commercial interest such as nutraceuticals and fuels, polymers or therapeutic drugs from renewable feedstock. Some biomolecules of interest can be obtained through a single enzymatic step in a host cell; however, in the majority of cases the synthesis of compounds demands a cascade of enzymatic reactions. There are numerous reviewed examples which describe the successful intergenic assembly and generation of functional metabolic pathways (Khalil and Collins, 2010; Quin and Schmidt-Dannert, 2011). Nonetheless, most of these engineered pathways express solely wild-type enzymes derived from different organisms and they often demonstrate production yields which are not of economic interest compared to existing petrol based processes. The ability to improve enzyme activities is therefore pivotal for practical applications in the chemical and pharmaceutical industry and the interest to implement biocatalysts in large scale reactions is not new (Huisman and Gray, 2002; Schoemaker et al., 2003). Directed protein evolution has emerged as a powerful technology platform in protein engineering, in which libraries of variants can be conceived by computational design and/or searched experimentally for clones possessing the desired properties (Raillard et al., 2001; Turner, 2003; Yuan et al., 2005; Khersonsky et al., 2010). Applications of directed evolution are widely reviewed and described by academic and industrial laboratories to improve protein stability, enhance the performance of enzymes and organisms, alter enzyme substrate specificity and design new activities and biocatalysts (Eijsink et al., 2005; Parales and Ditty, 2005; Dougherty and Arnold, 2009; Quin and Schmidt-Dannert, 2011; Urvoas et al., 2012).

Various *in vitro* techniques are used to generate protein mutants and variants by changing coding sequences of their genes and selecting hits with desirable functions. Library diversity is obtained through random or site directed mutagenesis (Pirakitikulr et al., 2010; Esvelt et al., 2011). Accumulated improvements are usually obtained through iterative mutation/shuffling and screening. DNA shuffling allows the direct recombination of beneficial mutations

☆In this work, two DNA sequences or genes are considered homeologous if they share 75–99% identity. In contrast, homologous DNA sequences are considered strictly identical.

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from multiple sources, and family-shuffling allows exchanges of blocks between related genes. Screening and selection then permits the isolation of new genes encoding improved enzymes (Fujii et al., 2006; Gupta and Tawfik, 2008; Khersonsky et al., 2009; Romanini et al., 2012). Since *in vitro* shuffling protocols can only be conveniently applied to relatively short DNA fragments (<5 kb), it is then difficult to apply *in vitro* shuffling to pathway evolution (>10 kb). Nonetheless, several combined methods have been developed to shuffle and evolve genomes, or part of them, to improve and select accumulated and beneficial properties to the whole organism (Schmidt-Dannert et al., 2000; Patnaik et al., 2002; Wang et al., 2009; Weng et al., 2012).

In vivo evolution (directed evolution in living cells) has the advantage that DNA fragments are recombined under physiological conditions and selection for properties can be performed directly in the host cell. As an example, wild-type yeast cells were shown to recombine similar but nonidentical (homeologous) DNA sequences (Mézard et al., 1992). Moreover, when two genes sharing 89.8% of DNA sequence identity are mutated by PCR and transformed into wild-type yeasts, a chimeric library is created through *in vivo* homeologous recombination, showing crossovers throughout the two genes (Swers et al., 2004). Noteworthy, short length genes were used in these reports to demonstrate the efficacy of *in vivo* shuffling. *in vivo* recombination between genomes or chromosomes of different origin with up to 30% diversity was earlier described by shuffling of two homeologous DNA in meiotic (Rayssiguier et al., 1989; Elefanty et al., 1998; Smith and Borts, 2005) and mitotic (Datta et al., 1996) mismatch repair (MMR) deficient prokaryotes and eukaryotes cells. These cells cannot correct discrete heteroduplexes and/or unmatched structures during DNA replication (Hoffmann and Borts, 2005), which significantly enhances recombination frequencies between diverged homeologous sequences and generates richer gene mosaic libraries.

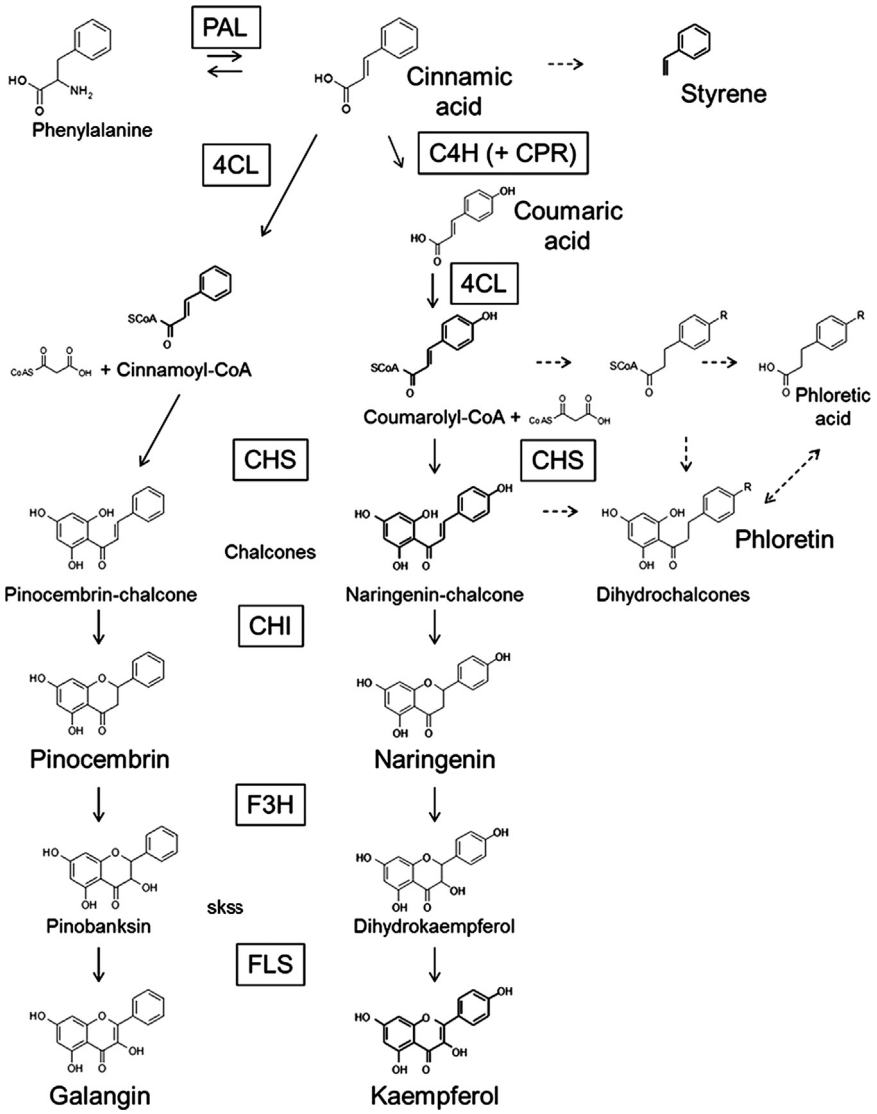

Fig. 1. Schematic representation of the flavonoid biosynthesis pathway. Genes encoding enzymes involved in the pathway are: phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavanol synthase (FLS). Coumaroyl-CoA is a key branching point for the biosynthesis of different polyphenols such as stilbenes and flavonols and is the precursor of a reaction cascade in which the CHS, CHI, F3H and FLS produce flavonols such as kaempferol. The NADPH-cytochrome P450 reductase (CPR) allows electron transfer from NADPH to C4H and F3H enzymes. Cinnamic acid is converted in cinnamoyl-CoA by 4CL which is the precursor of flavanones like pinocembrin and galangin. Dihydrochalcones such as phloretin result from a deviation of naringenin-chalcone in which CHS and others enzymes are involved. Phloretic acid is another derivative from the main flavonoid cascade as a degradation product from phloretin or by sequential reactions involving 4CL. Styrene results from a side transformation of cinnamic acid.