PRODUCTION OF ANTHOCYANINS FROM *ESCHERICHIA COLI* CONTAINING AN ARTIFICIAL GENE CLUSTER

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During the last ten years Metabolic Engineering has emerged as the discipline that is involved with (among other things) the introduction of novel pathways in prokaryotic and eukaryotic species (5). Several examples of such successful modifications, especially in the area of secondary metabolites, have been reported in the past and have been reviewed elsewhere (3). In the present study, we demonstrate for the first time that the expression of an artificial gene cluster of four genes involved in anthocyanin biosynthesis in plants allows the production of a stable anthocyanin, pelargonidin 3-O-glucoside, in *E. coli*. The artificial cluster contained the flavanone 3-hydroxylase (FHT) gene from *Malus domestica* , the dihydroflavonol 4-reductase (DFR) gene from *A. adraeanum,* the anthocyanidin synthase (ANS) gene from *Malus domestica* and the UDP-glucose:flavonoid 3-O-glucosyltransferase (3-GT) gene from *Petunia hybrida.* The present study comes as a natural continuation of an extended amount of work on the biochemical characterization of reactions involved in anthocyanin biosynthesis in plants. Our success in pelargonidin 3-O-glucoside biosynthesis from recombinant *E. coli* is based on the ability of *E. coli*, demonstrated in the present study, to uptake naringenin, as well as its ability to functionally express a DFR that can utilize dihydrokaempferol as a substrate.

In the past, engineering entire metabolic pathways on a single operon has proven to be successful in prokaryotes (4). In fact, our initial cloning strategy was based on the construction of an artificial operon containing all four genes under the control of a single Ribosome Binding Site (RBS), mimicking the genetic organization of many prokaryotic metabolic pathways. However we decided to proceed with the use of individual promoters and ribosome binding sites for each one of the four genes after it was demonstrated that the construction of the flavanone biosynthesis pathway on a single operon did not lead to efficient translation and flavanone production yields (2). That study demonstrated the importance of separate RBS as well as that of the mRNA amount. However, despite following a similar approach, the amount of pelargonidin 3-O-glucoside produced from the recombinant strain was relatively small, about an order of magnitude lower from the amount of flavanones produced from the recombinant *E. coli* strain in the presence of tyrosine.

Increasing the anthocyanin production yield would involve the elucidation of the process of flavanone uptake from the culture medium and a more thorough understanding of the kinetic properties and protein-protein interactions for the four enzymes involved in anthocyanin biosynthesis starting from naringenin. Such data, together with a good quantification of the pathway's intermediate metabolites, would allow a better manipulation of the pathway and even selection of enzymes from different plant or other species with more desirable properties. Nonetheless, in our opinion, the most significant step to follow is the expression of the gene cluster constructed in the present study together with an artificial gene cluster that would allow the conversion of phenylalanine or tyrosine to naringenin. Such simultaneous expression would permit the conversion of the two amino acids that are native to *E. coli* to anthocyanins and later other polyphenolic compounds that are considered specific to plants. It is also important to note that certain metabolic steps involved in flavonoid biosynthesis are catalyzed by cytochrome P450 enzymes, such as flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase. The functional expression of P450 enzymes in *E. coli* has proven to be challenging. In that respect, utilization of other, eukaryotic systems as production platforms for at least some types of flavonoids may prove to be a wiser selection and a better alternative.

Whether *E. coli* or any other host is utilized as a production platform, the possibilities of metabolically engineering high-value anthocyanins and other, high-value flavonoid compounds are vast. In the case of anthocyanins, the combination of metabolic engineering, rational protein design and directed evolution tools seems to be quite promising, due to the natural coloration that these compounds provide. This coloration would provide an easy and low cost screening method for anthocyanin producers derived from the application of various combinatorial techniques. Such techniques could be applied towards improvement of enzyme function, alteration of substrate specificities and introduction of novel catalytic activities and would result not only in better production of natural compounds but also in the generation of novel flavonoids with unique structures and functions.

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