THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Improving flavonoid production in *Saccharomyces cerevisiae* using synthetic biology tools

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Doodle illustrating key research elements explored in this thesis.

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Abstract

Flavonoids are plant secondary metabolites and represent one of the largest classes of natural products. Due to their health-beneficial properties, they have found potential applications in foods, beverages, cosmetics, and pharmaceuticals. Currently, their production is based on extraction from plant material. However, the low abundance of flavonoids in nature hinders efficient extraction and purification, thus inhibiting their market expansion. Chemical synthesis, although possible, relies on the use of harmful chemicals, harsh operating conditions, and high energy consumption.

Metabolic engineering of microorganisms to develop so-called "microbial cell factories" has gained increasing attention as a more efficient and sustainable way to produce a variety of chemicals – including flavonoids. *Saccharomyces cerevisiae* (baker's yeast) is one of the most well-studied and widely applied eukaryotic organisms for this endeavor. The fact that yeast shares cellular similarities to plants makes it a suitable host for the heterologous expression of flavonoid biosynthetic pathways.

In this thesis, I present our efforts to improve the production of flavonoids in *S. cerevisiae* through the development and application of several synthetic biology tools. First, transcription factor-based biosensors for the isoflavonoid genistein and the flavonoid precursor *p*-coumaroyl-CoA were established. The latter sensor was used to devise a dynamic regulation strategy to produce naringenin, a central flavanone and precursor for many flavanone derivatives. Cell growth was improved and naringenin titers were increased significantly.

Next, a malonate assimilation pathway was implemented in yeast to enhance the supply of malonyl-CoA, an important precursor for all flavonoid compounds. By expressing a heterologous malonate transporter and malonyl-CoA synthetase, I constructed strains able to grow on externally supplied malonate. The malonate transporter was further evolved through targeted *in vivo* mutagenesis and beneficial mutations were identified through growth-based enrichment under selective conditions.

Lastly, the production of the dihydrochalcone phloretin was explored. Its biosynthesis was accompanied by substantial byproduct formation and product degradation in the yeast cultivation medium. Different strategies, including enzyme scaffolding and antioxidant supplementation, were investigated to improve yeast-based production.

Taken together, I addressed some significant challenges within microbial flavonoid production and showcased how synthetic biology tools may overcome these obstacles.

Keywords: genistein, naringenin, phloretin, biosensor, *in vivo* directed evolution, enzyme scaffolding

List of publications

This thesis is based on the work contained in the following papers and manuscripts, referred to by their Roman numerals in the text:

Paper I: Liu D, Sica MS, Mao J, Chao LFI, Siewers V. A *p*-coumaroyl-CoA biosensor for dynamic regulation of naringenin biosynthesis in *Saccharomyces cerevisiae*. *ACS Synthetic Biology*. 2022; 11(10), 3228-3238.

Paper II: Chao LFI*, Liu D*, Siewers V. A highly selective cell-based fluorescent biosensor for genistein detection. *Engineering Microbiology*. 2023; 100078.
*Authors contributed equally

Paper III: Skrekas C*, Liu D*, Sun C, Brack Y, Bornscheuer UT, Siewers V, David F. *In vivo* evolution of malonate transport in *Saccharomyces cerevisiae*.
Manuscript
*Authors contributed equally

Paper IV: Liu D, Siewers V. Addressing challenges in yeast-based phloretin production.
Manuscript

Publications not included in this thesis:

Otto M, Liu D, Siewers V. *Saccharomyces cerevisiae* as a heterologous host for natural products. In: Skellam E. (eds) Engineering natural product biosynthesis. *Methods in Molecular Biology*. 2022; vol 2489. Humana, New York.

Contribution summary

Paper I: Designed and performed most of the experiments, analyzed the results, and wrote the manuscript.

Paper II: Designed and performed experiments on *in vivo* genistein detection, analyzed part of the results, and wrote part of the manuscript.

Paper III: Designed and performed experiments on growth evaluation of transporter strains, yeast library construction, and reverse engineering of enriched mutants, analyzed part of the results, and wrote part of the manuscript.

Paper IV: Designed and performed the experiments, analyzed the results, and wrote the manuscript.

Preface

This dissertation serves as partial fulfillment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The Ph.D. studies were carried out between February 2019 and August 2023 at the Division of Systems and Synthetic Biology under the supervision of Verena Siewers and the co-supervision of Yun Chen and Eduard Kerkhoven. The thesis was examined by Jens Nielsen. The research was funded by the European Union's Horizon 2020 research and innovation programme under grant agreement no. 814650.

Dany Liu

August 2023

Table of contents

Abbreviations

4CL	4-coumarate:CoA ligase
AAA	Aromatic amino acid
ACC	Acetyl-CoA carboxylase
ATP	Adenosine triphosphate
C4H	Cinnamate-4-hydroxylase
CHI	Chalcone isomerase
CHIL	CHI-like enzyme
CHS	Chalcone synthase
CoA	Coenzyme A
CPR	Cytochrome P450 reductase
CRISPR	Clustered regularly interspaced short palindromic repeats
DAHP	3-Deoxy-D-arabino-heptulosonic acid 7-phosphate
DBD	DNA-binding domain
DBTL	Design-Build-Test-Learn
dCas9	Catalytically deactivated Cas9
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
E4P	Erythrose 4-phosphate
epPCR	Error-prone PCR
F1,6P	Fructose 1,6-biphosphate
F6P	Fructose 6-phosphate
G3P	Glyceraldehyde 3-phosphate
G6P	Glucose 6-phosphate
GEM	Genome-scale metabolic model
GFP	Green fluorescent protein
GOI	Gene of interest
HID	2-Hydroxyisoflavanone dehydratase
IFS	Isoflavone synthase
LBD	Ligand-binding domain
nCas9	Cas9 nickase
NLS	Nuclear localization signal
PAL	Phenylalanine ammonia-lyase
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
R5P	Ribose 5-phosphate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Ru5P	Ribulose 5-phosphate
S7P	Sedoheptulose 7-phosphate
SELEX	Systematic evolution of ligands by exponential enrichment
SV40	Simian virus 40
TAD	Transactivation domain
TAL	Tyrosine ammonia-lyase
TF	Transcription factor
UV	Ultraviolet
X5P	Xylulose 5-phosphate

"I produced a detailed tribute to my wrongness."
"That is science."

– Nathan W. Pyle, Strange Planet

Chapter 1. Introduction

Plant natural products hold tremendous potential and significance for human health, playing a crucial role in drug discovery and drug development.[1] However, their production predominantly relies on extraction from plant material, which is inefficient and insufficient to satisfy increasing global demands. This has caused the endangerment of various plant species and unstable product supplies due to seasonal or climatic changes.[2] The use of genetically engineered microorganisms for fermentation-based compound production would not only alleviate the consumption of plant biomass but also enable more sustainable and efficient natural product synthesis. Biotechnological production processes thus have the potential to positively impact both our environment and our economy.

In this thesis, I focus on the yeast-based production of a specific class of natural products – flavonoids. These plant secondary metabolites have versatile functions in the food, beverage, cosmetic, and nutraceutical industry. Furthermore, due to their health-promoting effects, they have great potential for pharmaceutical applications. In **Chapter 2**, I will lay out several biological, disease-targeting activities of flavonoids, to give the reader a general idea of their economic value and explain our motivation behind improving their production. In **Chapter 3**, I will provide an overview of the two disciplines that this thesis is centered around, metabolic engineering and synthetic biology. The development process of a microbial production strain will be described, putting a special focus on flavonoids. The results of my original research will then be described in **Chapter 4**. This includes the development and application of metabolite biosensors for screening or pathway regulatory applications (papers I and II), the use of an *in vivo* directed evolution strategy for substrate transporter engineering (paper III), and an enzyme scaffolding approach for metabolic flux improvement (paper IV). Finally, the main conclusions of these results and my outlook on the future of microbial flavonoid production will be summarized in **Chapter 5**.

Chapter 2. Flavonoids – what are they good for?

Flavonoids are plant secondary metabolites, derived from two aromatic amino acids (AAA), L-phenylalanine and L-tyrosine. Structurally, they are characterized by their 15-carbon (C6-C3-C6) skeleton, which is assembled into 2 benzene rings, A and B, connected by a 3-carbon bridge, which usually forms a heterocyclic ring C (Figure 1).[3,4] Currently, more than 7000 flavonoid compounds have been identified in nature.[4] They are structurally classified into different subgroups according to their functionalization pattern, the saturation and oxidation state of ring C, and the positioning of ring B. Besides aglycones, flavonoids commonly occur as glycosides in nature.[5] Other functionalizations such as hydroxylations, methylations, and prenylations also exist.[6] Exemplary compounds of each subclass and main dietary sources[7–9] are given in Table 1.

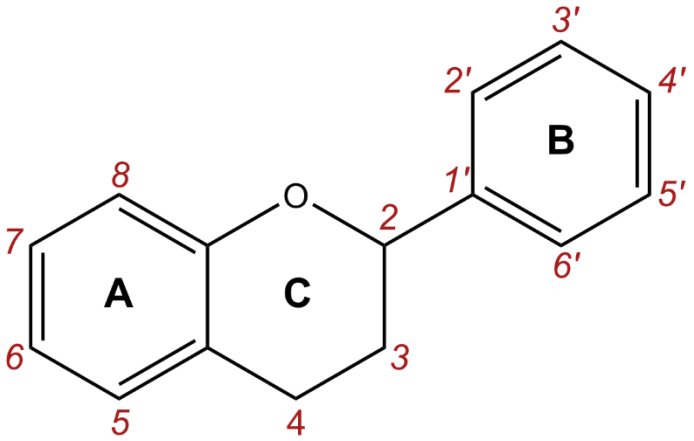
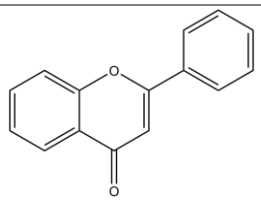
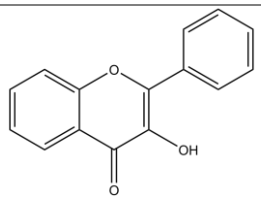
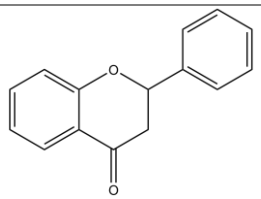
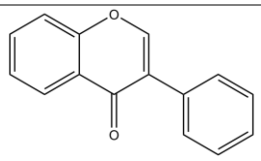
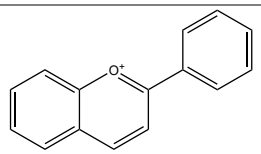
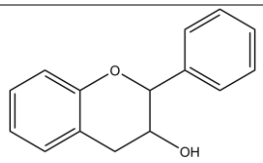
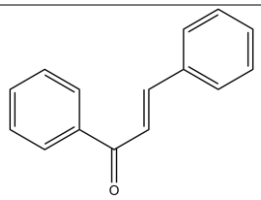


Figure 1. General molecular structure of flavonoids. Two aromatic rings A and B are connected by a 3-carbon chain, usually forming a heterocyclic ring C. Flavonoids are further classified into subgroups, depending on the position of ring B, saturation at the C2-C3 position, and additional functionalization patterns (hydroxy groups, methoxy groups, glycosyl groups, etc.).

2.1 Roles in plants

In plants, flavonoids act as protective agents against environmental stresses, such as microbial pathogens, UV radiation, and oxidative stress. They also function as signaling molecules, for instance in pollen germination.[10,11] Anthocyanins, which are a subclass of flavonoids, also serve as visual attractors to promote pollination.[11] Isoflavonoids, predominantly occurring in leguminous plants, are used as signaling molecules to promote symbiotic relationships with nitrogen-fixing rhizobial bacteria.[11]

Table 1. Structural classification of flavonoids, exemplary compounds, and major dietary sources.

Flavonoid subclass	Structure	Examples	Major dietary sources
Flavones		apigenin, luteolin	cereals, grains, parsley, rosemary, thyme, celery, chamomile tea
Flavonols		quercetin, kaempferol	onions, broccoli, apple, grape, tea, red wine
Flavanones		naringenin, hesperitin	citrus fruits, e.g., oranges, grapefruit
Isoflavones		genistein, daidzein	legumes, e.g., soybean, chickpeas
Anthocyanidins		cyanidin, delphinidin	natural pigments in berries, e.g., cranberries, black currants, blueberries, red grape
Flavan-3-ols		catechin, epicatechin gallate	cocoa, dark chocolate, apples, grape, red wine, green tea
Chalcones		phloretin, phloridzin	fruits, e.g., apples, pears

2.2 Roles in humans

While important for ecological interactions and defense mechanisms in plants, flavonoids have also been a part of human health for centuries.[12] Flavonoid-rich propolis or "bee glue" for instance was used in ancient Greece for its antimicrobial activity to treat sores and ulcers.[12] In traditional Chinese medicine, baicalein-containing huangqin extracts (the root of *Scutellaria baicalensis*) were used to treat oral infections.[12] However, the molecular structure of flavonoids was not elucidated until the late 1800s.[13] Initially discovered as plant pigments,[13] they were only linked to health-promoting effects in 1936, when two compounds, hesperidin and eriocitrin, were isolated from Hungarian red pepper and lemon juice.[14–16] These plant extracts were found to improve capillary resistance and restore vascular permeability. At the time, they were referred to as "vegetable dyes" and "vitamin P" (P for permeability) due to their vitamin-like properties and promising biological activity.[14,15,17] Although this term did not carry on due to a lack of evidence for the essential functionality of flavonoids,[18] the initial discovery led to the identification of additional compounds in the following years.[13,19,20] By 1955, over 30 physiological activities had been reported for various flavonoids.[21] However, this was followed by several decades of only moderate academic progress,[22,23] while some flavonoids were even suspected of carcinogenicity.[24] Finally, in the 1990s, several epidemiological studies which reported inverse associations between dietary flavonoid intake and major causes of disease and death[25–27] in developed countries, fostered increasing interest and a rapid surge in the number of scientific publications.[13] A brief timeline of flavonoid discoveries made since 1936 is depicted in Figure 2.

As humans, we take up a variety of flavonoids through our diet, in the form of vegetables, fruits, spices, beverages, etc.[8] The daily intake of total flavonoid content is estimated to range between 50-800 mg/day.[8] This is 10-20x higher than the average intake of vitamins C, E, and carotenoids.[10] Many *in vitro* and *in vivo* studies suggest that flavonoids have antioxidant, anti-inflammatory, and antimicrobial activities[3,8,12,28] and that they may exert positive effects against chronic diseases, such as cardiovascular diseases, diabetes, cancer, and neurodegenerative diseases.[17,29,30] In the following, different potential biological activities are considered.

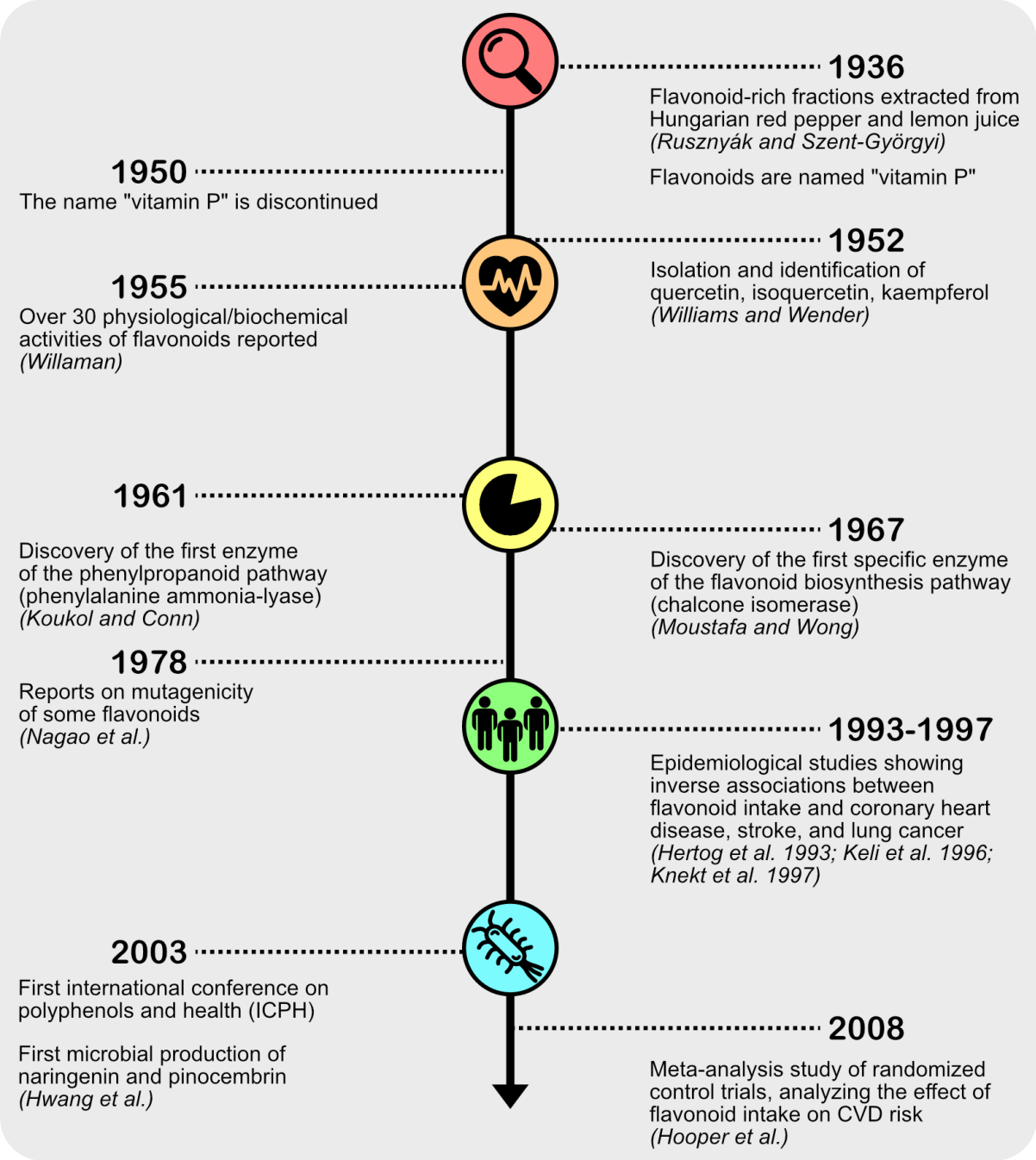


Figure 2. Timeline of selected discoveries made on flavonoids, their biological activities, and biosynthetic pathways over the last century.

2.2.1 Antioxidant activity

Oxidation, the transfer of electrons from one atom to another, is an essential process in living organisms.[8] It can lead to the generation of unpaired electrons in the form of free radicals and more specifically, reactive oxygen species (ROS). These molecules play crucial roles in cellular processes such as gene expression, signal transduction, enzymatic reactions, and more.[31] However, ROS are also linked to the aging process and degenerative diseases, as excess ROS can create oxidative stress and cause damage to

cellular material, such as DNA, lipids, and proteins.[8,32] Cells have endogenous defense and repair mechanisms to avert such damage. Exogenous antioxidants such as vitamins A, C, and E can be taken up through diet.[8] Many flavonoids have been shown to exert *in vitro* antioxidative activities that are stronger than those of vitamin C and E.[33] Some flavonoid compounds can inhibit enzymes and chelate to trace elements responsible for ROS generation. Due to their aromatic nature, flavonoids can scavenge ROS and form more stable radical species themselves. Furthermore, they may also upregulate endogenous defenses.[8,33,34] These activities are highly dependent on specific structural properties, such as hydroxylation patterns. A catechol group on ring B enhances the compound's electron donating ability, while a 2,3-double bond, conjugated with a 4-oxo group leads to improved electron delocalization and thus more stable flavonoid radical formation. Both configurations also promote trace metal chelation.[8,33] The flavonol quercetin for instance possesses a higher *in vitro* antioxidant capacity than its precursor kaempferol which lacks the catechol moiety.[8,35] Studies on the *in vivo* antioxidant activity of flavonoids are limited and contradictory in some cases. This is partly ascribed to their low bioavailability in humans.[3,8] Furthermore, at high doses, flavonoids have also been reported to exhibit pro-oxidant effects.[3,33]

2.2.2 Anti-inflammatory activity

Both oxidative stress and inflammation play major roles in the development of chronic diseases and metabolic disorders.[3] Inflammation is a natural and necessary process for resolving infections and injuries.[36,37] However, uncontrolled inflammation due to chronically active signaling pathways is the basis for autoimmune diseases (e.g. rheumatoid arthritis, Crohn's disease) as well as allergic reactions, such as allergic asthma and anaphylaxis.[36] Moreover, excessive inflammatory responses have been linked to the development of diabetes, obesity, cardiovascular diseases, and cancer.[38] Neuroinflammation is suggested to promote the progression of neurodegenerative diseases such as Parkinson's, Alzheimer's, multiple sclerosis, and amyotrophic lateral sclerosis.[39]

Although anti-inflammatory steroids as well as non-steroidal drugs can be used to successfully treat inflammations, these medications often come with unexpected side effects, especially during long-term usage.[40,41] Numerous studies have shown that flavonoids possess anti-inflammatory functions[3] and several mechanisms of action have been proposed. Inflammation is typically accompanied by the formation of reactive oxygen and nitrogen species.[36,38] Radicals not only cause cellular damage but also attract additional inflammatory mediators, exacerbating the inflammatory response.[38] As mentioned above, some flavonoids are strong antioxidants and may scavenge these radicals to limit cell damage. Furthermore, flavonoids have been reported to inhibit pro-inflammatory enzymes, for instance by downregulating arachidonic acid metabolism. Arachidonic acid is a precursor of prostaglandins and leukotrienes, which participate in inflammatory signaling pathways. Flavonoids have also been reported to inhibit the

production of certain cytokines and the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPK), which are responsible for various intracellular inflammatory responses.[3,38,39]

Due to these promising activities observed in cell and animal models, flavonoids present a potential new class of drugs that may allow anti-inflammatory action without causing side effects observed in current medications.[36,42]

2.2.3 Antimicrobial activity

Crude extracts from plants as well as individual flavonoids have been shown to possess antimicrobial properties, despite being less potent compared to antibiotics of microbial origin.[12] Moreover, some flavonoids have been reported to act against fungi (*Candida albicans*) and viruses (human immunodeficiency and herpes simplex virus) as well.[12] Flavonoids have been reported to disrupt membrane function and inhibit DNA synthesis, biofilm formation, and energy metabolism in bacteria.[12,43] Synergistic effects have been observed between flavonoids and other antimicrobial compounds.[43]

These findings could potentially be useful to develop flavonoid-based agents to combat the increasing microbial resistance observed in the public health sector.[12,43]

2.2.4 Potential in the treatment of cardiovascular diseases

Due to their biological activities, flavonoids have been proposed as potential therapeutics for several highly prevalent human diseases, including cardiovascular diseases (this chapter), type 2 diabetes (Chapter 2.2.5), and cancer (Chapter 2.2.6).

Many *in vitro* and animal studies have demonstrated the beneficial effects of flavonoids against cardiovascular diseases (CVD).[44] Epidemiological studies have also been conducted. In 1993, as an extension of the Zutphen Elderly study, Hertog *et al.*[25] reported a significant inverse association between flavonoid (more specifically flavonol) intake and mortality from coronary heart disease and myocardial infarction. Later studies reported similar relations between flavonoid consumption and CVD, albeit to a much lesser extent.[45] Meta-analyses of prospective cohort studies have suggested that flavonoids possess promising therapeutic potential, but more standardized methods and better strategies to assess flavonoid intake are needed to support these findings.[46–48] Multiple potential mechanisms of action have been outlined as well. As previously mentioned, certain flavonoids have strong antioxidant properties and can thus inhibit cellular oxidation processes. They have been shown to improve vascular endothelial functions by preventing the ROS-mediated inactivation of nitric oxide (NO), which is an important vasodilation-promoting compound. Flavonoids have also been reported to inhibit low-density lipoprotein (LDL) oxidation, a risk for atherosclerosis occurrence, by radical scavenging or upregulating endogenous antioxidant enzymes.[44] Some flavonoids,

e.g., naringenin *(explored in paper I)* and quercetin, have been reported to lower blood pressure.[10]

2.2.5 Potential in the treatment of type 2 diabetes

Type 2 diabetes (T2D) is one of the most common chronic diseases worldwide. Diabetes-related deaths have increased by 70% between 2000 and 2019, making it one of the top 10 leading causes of death globally.[49] The disease is caused by dysregulations of the insulin signaling pathway, leading to disruptions in glucose homeostasis in peripheral organs, such as the liver, adipose tissues, and skeletal muscles.[50] Many drugs are available for the treatment of symptoms such as high blood glucose levels, hypertension, and dyslipidemia.[51] However, effective agents to prevent diabetic complications are still needed. As some flavonoid compounds exhibit antidiabetic activities comparable to clinical drugs, they could potentially contribute to existing therapies and lower the risks of diabetic complications.[51] Positive associations between flavonoid intake and reduction of T2D risks have been observed in *in vitro*, animal, and epidemiological studies.[52–54] Publications of clinical studies are still limited but show promising results in diabetic patients.[55–57] Flavonoids are suggested to regulate glucose homeostasis and improve glucose sensitivity through several mechanisms. Some have been shown to promote translocation of the glucose transporter 4 (GLUT4) to the plasma membrane, which leads to improved glucose uptake from the blood.[51] By limiting oxidative damage and inflammation, they may improve pancreatic β-cell viability and thus promote insulin secretion.[51,58] Certain compounds, including luteolin, chrysin, kaempferol, and daidzein, have been reported to inhibit α-glucosidase activity, thereby delaying the digestion of complex carbohydrates into simple sugars and reducing blood glucose levels after meal intake.[51,58]

2.2.6 Potential for cancer treatments

Flavonoids have also been proposed to exert cancer-preventive effects via different biological mechanisms. First of all, their antioxidant properties may counteract DNA oxidation, which likely causes mutations.[59] Secondly, flavonoids have been shown to regulate carcinogen metabolism *in vitro* and *in vivo*.[59] The metabolism of xenobiotics, such as carcinogens, can be described in two phases. Phase I metabolizing enzymes consist mainly of cytochrome P450s, which convert xenobiotics into more polar metabolites, for instance by hydroxylation.[60] Phase II metabolizing enzymes, primarily transferases, catalyze conjugation reactions such as glucuronidations, sulfations, or methylations, which leads to the formation of less active and more hydrophilic, easily excretable compounds.[60] Some flavonols and flavones such as quercetin, kaempferol, apigenin, and galangin have been shown to inhibit cytochrome P450 enzymes, which are involved in the activation of several suspected procarcinogens,[61] while catechins have been reported to promote the activity of phase II metabolizing enzymes.[61] Additionally, quercetin, apigenin, and catechins have been suggested to inhibit cyclooxygenase 2

(COX2), an enzyme involved in inflammation. As COX2 has also been linked to some cancers, its inhibitors are currently being studied as colorectal cancer drugs.[61] Moreover, flavonoids exhibit antiproliferative activities in human cancer cell lines, without showing significant toxic effects on normal cells. Genistein *(explored in paper II)* and quercetin for instance appear to inhibit signal transduction enzymes that promote cell proliferation, such as protein tyrosine kinases.[59,61] Proapoptotic activities of flavonoids have also been observed in cancer cells, e.g., by inhibiting DNA topoisomerase I and II, activating apoptosis-involved caspase enzymes, and inactivating NF-κB, a transcription factor that has been linked to cancer initiation and metastasis.[59] Epidemiologic evidence of anticancer properties of flavonoids is growing,[27,62,63] but clinical trials (mainly phase I and II) have only been performed on a small number of compounds, including flavopiridol, flavone acetic acid, isoflavones, and catechin.[64] Furthermore, their results often show only minimal response or no significant clinical effects.[64] It is possible that the positive effects observed *in vitro* are not found under clinical conditions due to the low bioavailability of these compounds (see Chapter 2.3). Therefore, despite promising initial results, improved drug formulations and more data are necessary to make definitive conclusions on the effect of singular compounds on different cancers.

2.3 The bioavailability of flavonoids

Their generally low bioavailability has long been known as a major challenge for the successful clinical application of flavonoids.[65] Plasma concentrations have been estimated to be in the nM-μM range[50,66] and flavonoid aglycones are hardly detected in either plasma or urine.[66,67] This is one likely reason for the often contradictory observations between *in vitro* flavonoid studies and human clinical trials.[67] The absorption and metabolism of flavonoids have only been studied in recent years. Furthermore, bioavailability varies substantially depending on molecular structure and functionalization.[67] Most flavonoids occur as glycosides in nature. In the small intestine, they are hydrolyzed to their respective aglycones by lactase phlorizin hydrolase (LPH). The more lipophilic aglycones can diffuse into epithelial cells passively.[68] It is suggested that flavonoid glycosides may also enter epithelial cells through transporter proteins and subsequently undergo hydrolysis catalyzed by cytosolic β-glucosidase.[69] Flavonoids are further metabolized in the liver, resulting in the formation of conjugated products, such as sulfates, glucuronides, and *O*-methylated compounds.[66,68,69] These conjugated flavonoids are partly excreted through urine. However, a large proportion remains unabsorbed and can pass into the large intestine, where they are subject to colonic microbial degradation to phenolic acids such as ferulic acid, protocatechuic acid, vanillic acid, etc.[69] These smaller molecules are readily absorbed and may exert certain biological activities.[69] We now know that the colonic microbiota plays an important role in the metabolism of flavonoids. Not only do microbial metabolites have potential health benefits, but flavonoids may also inversely influence the gut microbiota ecology, e.g. by increasing the population of commensal bacteria and inhibiting the growth of pathogenic ones.[69] It has thus been suggested that flavonoids could serve as a form of prebiotic in the

gut.[65] Better understanding of flavonoid metabolism and absorption also leads us to reconsider existing investigations, which predominantly center around aglycones at concentrations much higher than those found physiologically.

To take advantage of the bioactivities of flavonoids observed *in vitro* and to understand whether these observations apply to human subjects, bioavailability needs to be improved considerably. Different strategies exist, such as the use of absorption enhancers to increase intestinal absorption, structural transformation of aglycones to prodrugs that are more stable and soluble, or employing novel drug delivery systems.[70] Popular drug delivery strategies include emulsion or encapsulation of the active drug, e.g. into membranes or nanoparticles, as well as various nanotechnology approaches.[65,67,70]

2.4 Flavonoid production in industry

Due to the large amount of evidence for the health-beneficial effects of flavonoids, there is great interest in their industrial production. Many flavonoid-containing products exist on the market. The dihydrochalcone phloretin *(explored in paper IV)* for instance is used as an ingredient in skincare products for its antioxidant and depigmenting effect. Quercetin, rutin, and fisetin are examples of flavonoids sold as nutraceuticals. Some flavonoids, such as neohesperidine dihydrochalcone,[71] may serve as sweetening agents. Dihydroquercetin can be used as an antioxidant to extend the shelf life of meat products.[72]

Current flavonoid production processes are mainly based on extraction and purification from plant biomass. However, conventional methods such as liquid-liquid or solid-liquid extraction present several drawbacks. They consume large amounts of solvents and energy, provide low product yields, and require long extraction times.[73,74] This leads to overall high production costs. In addition, methods involving heat may result in compound degradation and reduced bioactivity of the final product. Therefore, it will be challenging to meet the increasing flavonoid market demand solely by plant-based extraction.[75,76] Flavonoids can also be produced by chemical synthesis. Depending on the structural complexity of the target compound, this typically requires many reaction steps, harsh reaction conditions, and potentially toxic chemical reagents, rendering this approach unsustainable.[77] Furthermore, some compounds, in particular pure enantiomers, are challenging or impossible to synthesize chemically.[78,79]

With rapid advances in the disciplines of metabolic engineering and synthetic biology,[80] the use of microbes as so-called cell factories for fermentation-based flavonoid production has become a promising alternative. The application of genetically engineered microorganisms allows targeted biosynthesis of a specific compound under scalable, controlled conditions inside of a bioreactor, thereby reducing byproduct and waste formation, and facilitating downstream purification processes.[75] Lower solvent and energy requirements also means that the process becomes more environmentally friendly and cost-efficient. Furthermore, by eliminating the reliance on plant biomass, production will be independent of seasonal, climate, or geographical variations.[77,81]

Compared to chemical synthesis, microbial cell factories present the advantages of working at low temperatures and atmospheric pressures. Furthermore, no chemical catalysts are required.[82] A multitude of molecules, including several flavonoids, have been successfully produced using microorganisms at a laboratory scale.[83–85] Several industrial processes, ranging from bulk to fine chemical production, have been established as well.[86–88] In the next chapter, I will briefly outline the history and progress made in microbial chemical production and summarize some general guidelines and considerations regarding the development of a microbial cell factory.

Chapter 3. Microbial cell factories

Microbial fermentation has been a part of human food production and preservation for millennia. It is essential in bread-baking, beer-brewing, wine-making, and many other processes. However, the mechanisms behind this phenomenon remained a mystery until recent centuries. Only in 1837, was alcoholic fermentation first linked to microbial – mainly yeast – action.[89,90] This was later confirmed through thorough experimentation by Pasteur in 1857.[91] While industrial fermentations have been employed for beer and wine production since the early 18th century, the first chemical fermentation process (acetone production in *Clostridium acetobutylicum*) was not established at large-scale until the first world war.[92] This initial breakthrough was followed by the development of several novel bioprocesses to provide bulk chemicals, such as glycerol, citric acid, gluconic acid, and lactic acid.[89]

After the first antibiotic was discovered by Fleming in 1927, substantial efforts were made to improve its production due to high demands during the second world war. Between 1940 and 1962, over 20 classes of antibiotics were commercialized.[90] Around the same time, significant advances in our understanding of molecular biology were made, such as the elucidation of the 3D structure of DNA (1953),[93] the establishment of the central dogma (1958)[94] and the complete decryption of the genetic code (1961-66).[95,96] In 1973, foreign genetic material was introduced into a bacterial cell for the first time.[97] These and numerous other achievements[98–101] paved the way for the advent of recombinant DNA technology. In 1976, Genentech became the first company to take advantage of this new technology and used it to develop microbial production platforms for the human peptide hormones somatostatin and insulin.[102] The invention of the polymerase chain reaction (PCR)[103] and the automation of Sanger sequencing[104,105] further accelerated the advancement of the field of biotechnology. Several publications in the 1980s/1990s finally proved it feasible to engineer (heterologous) metabolic pathways for microbial production of chemicals from renewable resources.[106,107] In 1991, the term "metabolic engineering" was coined and defined as *the "improvement of cellular activities by manipulation of enzymatic, transport and regulatory function of the cell using recombinant DNA technology"*.[108]

After 30 years since its emergence, the field has evolved tremendously, thanks to the continual development of new engineering and analysis tools. Successes such as the complete sequencing of the *S. cerevisiae* genome in 1996[109] and the construction of its first genome-scale metabolic model in 2003,[110] established yeast as one of the central workhorses in metabolic engineering. New tools including synthetic genetic circuits[111,112] and increasingly facile and modular genetic or genome engineering methods[113–115] illustrate how synthetic biology can promote the development of novel bioprocesses. The commercialization of yeast-based artemisinic acid production[116] presents an encouraging example of how microbial cell factories may benefit our society and economy. The advent of automation for the establishment of biofoundries[117] and machine

learning for better prediction of protein structures[118,119] and cellular behavior is expected to further expedite the expansion of microbial chemical production. Some major discoveries made within the last decades are summarized in Figure 3.

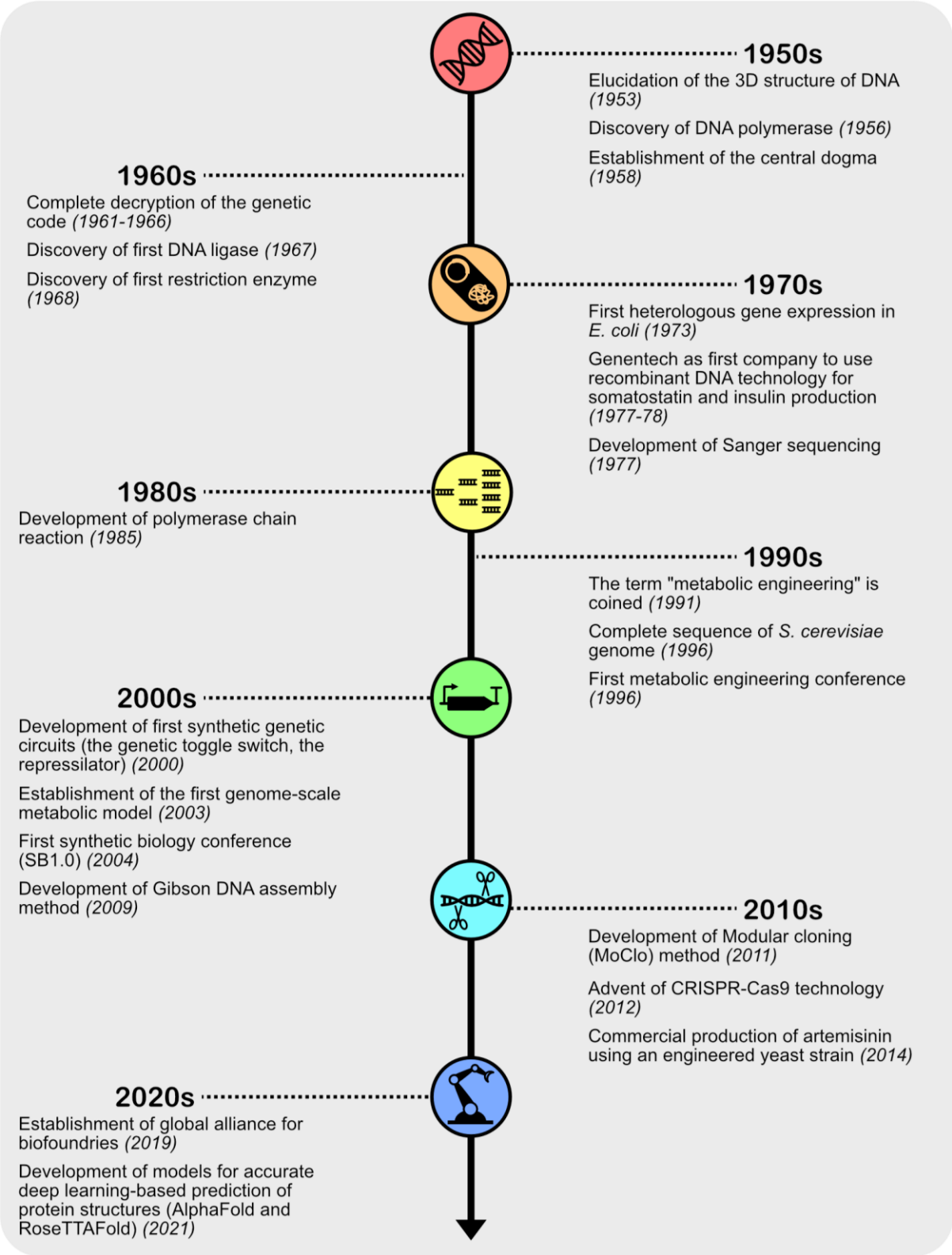


Figure 3. Timeline of selected developments and milestones within the fields of metabolic engineering and synthetic biology (1950s-2020s).

3.1 Metabolic engineering

The overarching goal of metabolic engineering is the conversion of given substrates into desired compounds by modification of the host metabolism using genetic engineering methods. Some common objectives may include the extension of substrate range, an increase of host robustness and tolerance towards toxic intermediates, or products, the elimination of unwanted side reactions, or general improvements of titers, rates, and yields.[120] As biochemical reactions are not isolated but exist within an interconnected metabolic network, it is important to examine such networks as a whole to identify suitable targets for genetic manipulation.[121]

The procedure of developing a new microbial cell factory typically goes as follows:

1. Selection of a product of interest and its corresponding biosynthetic pathway(s)
2. Selection of a suitable host organism
3. Pathway construction to obtain a "proof-of-principle" strain
4. Pathway optimization, typically following iterative "Design-Build-Test-Learn" (DBTL) cycles

In the following, each step will be described in more detail, with a special focus on flavonoids as an example product.

3.1.1 Selection of a product of interest and its corresponding biosynthetic pathway

The product of interest should be in high demand or of high value and have conceivable utility in industry. Several potential pharmaceutical applications of flavonoids are outlined in Chapter 2. Furthermore, flavonoids play an important role in the food and beverage as well as the cosmetics industry.[122] Their global market value amounted to over $1497.7 million in 2020 and is estimated to reach $2717.8 million by 2030.[76] Needless to say, flavonoids are of industrial interest and present a variety of applications in different economic branches.

In metabolic engineering, biosynthetic pathways can be categorized into native-existing (i.e. endogenous to the host organism), nonnative-existing (heterologous to the host organism), and nonnative-created (synthetic pathways for novel compounds).[82] As a result of advancements in the field of functional genomics, knowledge about plant genes responsible for flavonoid synthesis and modification, has increased immensely over the last decades.[123–125] Their pathway implementation in microbes can generally be considered as nonnative-existing in most cases.

Flavonoid aglycones are formed by a series of condensation, isomerization, oxidation, and reduction reactions.[124] Subsequent tailoring reactions, e.g., glycosylations, methylations, prenylations, and acylations, result in additional downstream diversification and contribute significantly to the physicochemical properties and biological activities of the final compound.[124]

The two main pathways necessary for the assembly of aglycones are the phenylpropanoid and the acetate pathway (Figure 4). The phenylpropanoid pathway generates an acyl-CoA thioester which provides the ring B and C components of the flavonoid molecule.[123,126] While different phenylpropanoic acids can be involved in flavonoid synthesis, in this thesis I will focus only on the central precursor, *p*-coumaric acid. The acetate pathway provides the malonyl-CoA units for elongation of the C2 chain, which makes up ring A.[123,126] Malonyl-CoA can be generated by carboxylation of acetyl-CoA via an ATP- and biotin-dependent acetyl-CoA carboxylase (ACC). *p*-Coumaric acid is derived from phenylalanine or tyrosine. In plants as well as microbes, these two AAAs are synthesized via the shikimate pathway.[127] Phenylalanine can be cleaved into ammonia and *trans*-cinnamic acid by phenylalanine ammonia-lyase (PAL). This represents the first committed step in the phenylpropanoid pathway. *trans*-Cinnamic acid can be further hydroxylated to *p*-coumaric acid by the cytochrome P450 monooxygenase cinnamate-4-hydroxylase (C4H). Some plant and bacterial ammonia-lyases take tyrosine as substrate, leading to *p*-coumarate synthesis in a single reaction.[128,129] *p*-Coumaric acid is then activated to its CoA thioester, *p*-coumaroyl-CoA, to participate in subsequent Claisen condensation reactions with 3 malonyl-CoA moieties. This ATP-consuming activation is catalyzed by a 4-coumarate:CoA ligase (4CL). The sequential Claisen condensations are catalyzed by a chalcone synthase (CHS). Upon spontaneous cyclization of the triketide intermediate, naringenin chalcone is formed. The enzyme chalcone isomerase is responsible for the conversion of naringenin chalcone to its flavanone isomer, naringenin. This is a central precursor for many subclasses of aglycones, including flavones, isoflavones, flavonols, flavan-3-ols and anthocyanidins. The synthesis of these subclasses is outside of the scope of this thesis and will not be described in detail.

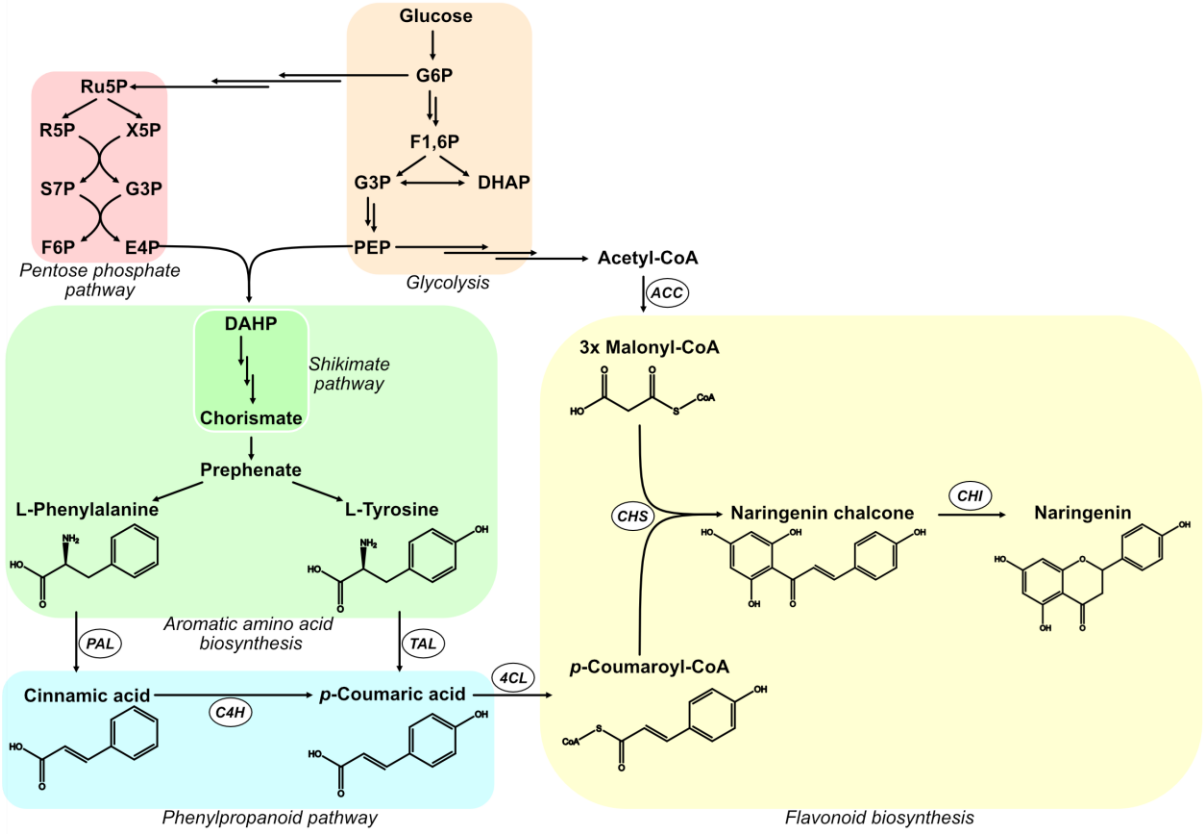


Figure 4. Overview of metabolic pathways involved in flavonoid biosynthesis. The phenylpropanoic acid precursors are derived from AAAs, which are synthesized via the shikimate pathway, using erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) as substrates. Malonyl-CoA is derived from acetyl-CoA carboxylation. Flavonoids are generated by a cyclization reaction of a phenylpropanoid acyl-CoA thioester and three malonyl-CoA moieties. ACC: acetyl-CoA carboxylase; PAL: phenylalanine ammonia-lyase; TAL: tyrosine ammonia-lyase; C4H: cinnamate-4-hydroxylase; 4CL: 4-coumarate:CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase.

3.1.2 Selection of a host organism

The selection of the host organism generally depends on the nature and origin of the product biosynthetic pathway. If an organism produces the compound of interest or its precursor endogenously (and at high efficiency), it makes sense to explore it for further optimization. As flavonoids occur naturally in plants, some studies have focused on modifying these endogenous producers to increase metabolic flux toward flavonoid synthesis.[130–132] However, the development of plant cell factories is hindered by difficulties in the large-scale cultivation of plant cells,[133] low productivity, long production times, variable yields, and fewer genetic manipulation tools.[134,135] Bacteria and yeast may overcome these challenges by providing faster cell growth, efficient genome engineering tools, and robustness in large-scale cultivations.

Some general criteria when selecting a suitable microbial host organism are[136]:

- High growth and production rate
- Ease of genetic manipulation

- High pH, temperature, and salt tolerance
- Robustness
- Tolerance towards pathway intermediates and products
- Broad substrate range
- Extensive knowledge base

Historically, *Escherichia coli* and *Saccharomyces cerevisiae* have been deemed as organisms of choice when it comes to metabolic engineering endeavors.[86] They are considered the best-studied prokaryote and eukaryote, respectively, and have been involved in many discoveries within fundamental biology.[136–138] Moreover, a multitude of genetic engineering tools exist,[139,140] facilitating the construction of production strains. Most attempts to engineer flavonoid production have thus been made in *E. coli* or *S. cerevisiae*. Nonetheless, recent efforts to characterize and develop systems and synthetic biology tools for non-conventional organisms have promoted the expansion to other hosts.[141] *Lactococcus lactis*,[142] *Streptomyces albus,*[143,144] *Corynebacterium glutamicum,*[145,146] and *Yarrowia lipolytica*[147,148] have all been explored for the biosynthesis of flavonoids. While each organism presents benefits and drawbacks, this thesis focuses on the application of *S. cerevisiae*. Hereafter, the use of the term "yeast" will refer to *S. cerevisiae* unless otherwise stated.

With regard to flavonoid pathway expression, yeast presents specific advantages. Due to its eukaryotic nature, it shares some common characteristics with plant cells, including compartmentalization that allows subcellular localization of enzymes and metabolites, similar post-transcriptional and -translational processing systems, and the ease of expressing cytochrome P450s, which are involved in many flavonoid biosynthetic pathways.[81,149] Furthermore, *S. cerevisiae*-based processes typically hold the "generally regarded as safe" (GRAS) status, which may facilitate regulatory approval.[86] Its tolerance toward low pH environments and high salt concentrations and its resistance to phage infection, reduces the risk of contamination at industrial scale. Compared to other eukaryotes, we have an extensive understanding of its physiology, metabolism, and genetics. Moreover, it is easy to modify genetically, and genome engineering is facilitated by its high efficiency of homologous recombination.[150] Compared to *E. coli* (and other bacteria), it has the drawbacks of lower growth and production rates and being less tolerant to higher temperatures.[136] Furthermore, some alternative eukaryotes such as the oleaginous yeast *Y. lipolytica* possess higher fluxes towards malonyl-CoA, which may be beneficial for flavonoid production.[151]

3.1.3 Pathway construction

When designing a cell factory for a new product, one should consider several potential constraints: Which substrates are feasible and economically relevant? Does the product exhibit any toxicity to the host organism? Are products and intermediates stable in solution? Are they subject to degradation by the host organism? Which process conditions (temperature, pH, oxygen levels, etc.) are needed, and do they conflict with

other aspects of the biosynthetic pathway? Once these questions are settled, one needs to identify a suitable precursor in the host's endogenous metabolism to "link" the heterologous pathway to. Central metabolism is highly conserved among all living species. All carbon and energy sources are generally converted into a set of 12 precursor metabolites, which constitute the building blocks for all possible cellular macromolecules and metabolites. This can be illustrated by the so-called bow-tie structure of metabolism (Figure 5).[87] Therefore, it is (in theory) possible to produce almost any heterologous metabolite of a nonnative-existing pathway, if a connecting precursor can be determined.[152] Information on relevant pathway genes/enzymes can then be obtained from literature or databases such as KEGG,[153] MetaCyc[154], and BRENDA.[155] Genome-scale metabolic models (GEMs) serve as predictive tools and provide valuable guidance in cell factory design.[156] Besides reaction stoichiometries, additional constraints, such as thermodynamics[157,158] and enzyme kinetics and abundance,[159] can be introduced to improve the accuracy and predictive power of such models. Various algorithms exist for the selection of feasible synthetic pathways and prediction of gene deletions or expression regulation.[160,161]

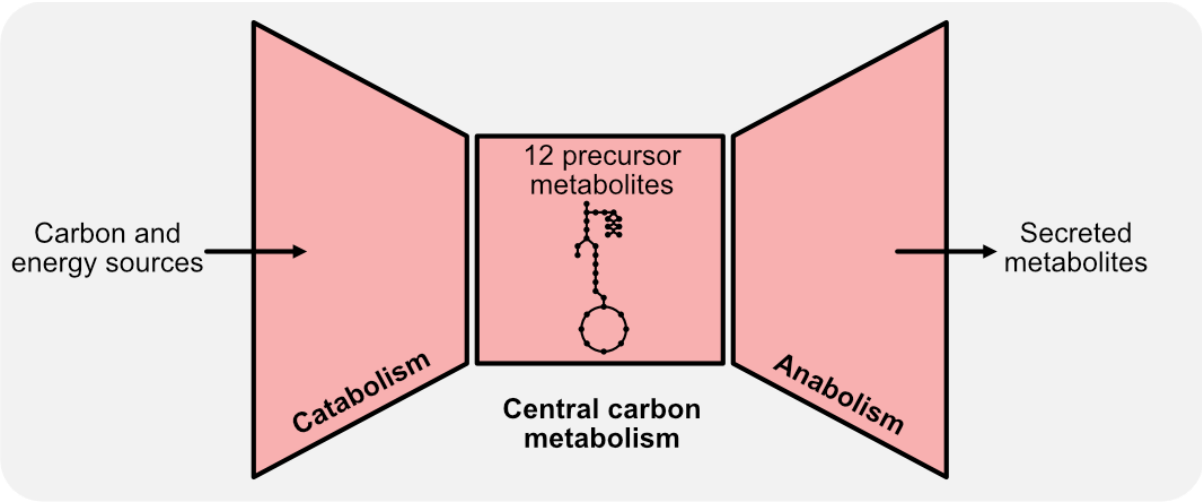


Figure 5. Bow-tie structure of cellular metabolism. Carbon sources such as glucose are broken down into 12 precursor metabolites (e.g., glucose-6-phosphate, erythrose-4-phosphate, acetyl-CoA, oxaloacetate, etc.) via catabolic reactions, which are used for anabolic metabolism to synthesize building blocks (e.g., nucleotides, amino acids, carbohydrates, fatty acids) that constitute macromolecules needed for cell growth.

The last yeast-endogenous metabolites involved in phenylpropanoid/flavonoid biosynthesis are phenylalanine and tyrosine. They thus serve as excellent connection points for the reconstruction of heterologous flavonoid production.[155] In addition, platform strains that readily produce a central precursor with high efficiency are useful, as they may expedite the development of cell factories for the desired product.[162] The production of *p*-coumaric acid for instance has been extensively engineered in yeast. Titers of 12.5 g/L and substrate yields of 155 mg/g from glucose have been realized in fed-batch fermentation.[163] Another ideal platform for various downstream flavonoids is naringenin. So far, maximum *de novo* naringenin production titers of over 1.1 g/L were achieved in *S. cerevisiae* in fed-batch bioreactor fermentations.[164,165] In one study, fatty

acid β-oxidation was upregulated for increased acetyl-CoA and malonyl-CoA supply by expressing a secretory lipase enzyme to hydrolyze supplemented soybean oil into unsaturated fatty acids. Specific genes of the β-oxidation pathway were overexpressed to further improve naringenin production.[164] In another study, 7-*O*-glycosylation of naringenin was engineered to increase product solubility, promoting extracellular secretion and reducing product cytotoxicity. This enhanced naringenin production significantly. The aglycone could be recovered by hydrolysis using hydrochloric acid.[165]

3.1.4 Pathway optimization

Titers, rates, and yields of initial proof-of-principle strains are typically low and unfit for industrial applications. Therefore, several iterations of designing, building, and testing strains, and subsequent learning from obtained data (the so-called DBTL cycle, Figure 6)[87] are usually needed to reach satisfactory results. The development of "market-ready" cell factories has been estimated to require 6-8 years of optimization and over $50 million of investment.[87] Despite progress made in automation, laboratories lacking high-throughput workflows still rely on manual work. A single iteration of the DBTL cycle can thus take up to several weeks or months.[87]

Phenylpropanoid biosynthesis has been engineered extensively. Significant improvements in *p*-coumaric acid production in yeast have been made through various engineering strategies, such as enhancing AAA biosynthesis by overexpressing certain pathway genes and employing feedback-insensitive Aro4/Aro7 mutants, rewiring carbon flux towards E4P by introducing a heterologous phosphoketolase pathway, dynamically controlling the downstream reactions from AAAs to *p*-coumaric acid using the galactose-inducible promoters, optimizing precursor supply by screening a promoter library for regulating PEP and E4P consuming reactions, and reducing fusel alcohol and acid byproducts by knocking out corresponding genes.[163,166] The microbial production of flavonoids at lab-scale was first demonstrated in 2003 by Hwang *et al*.[167] By expressing three pathway-specific enzymes in *E. coli* it was possible to obtain the flavanones naringenin and pinocembrin. The diversification into other flavonoid subclasses (e.g., flavones,[143,168] flavonols,[169,170] isoflavonoids[84,169,171], and anthocyanins[145,172]) and various functionalizations (e.g., glycosylation,[173–176] methylation,[177–179] prenylation[180–182]) have also been realized, although typically at low titers and oftentimes requiring expensive precursors. Despite promising results and initial small-scale successes, low titers, rates, and yields have hindered the transfer from lab to industry.[76,81]

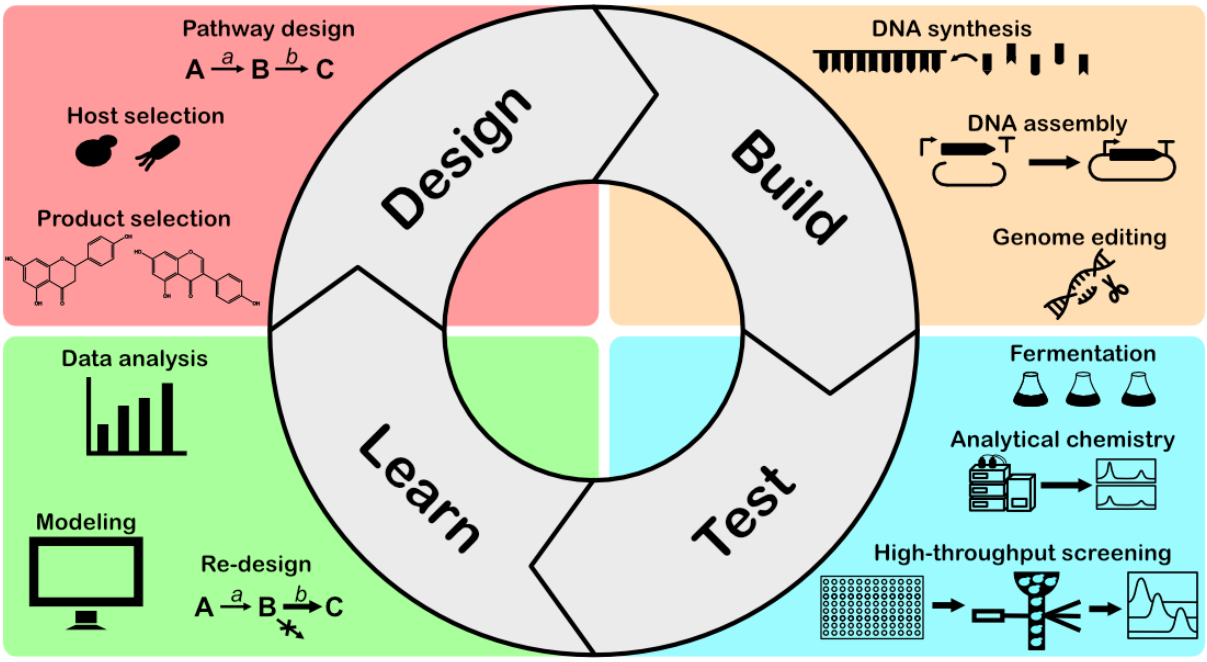


Figure 6. The Design-Build-Test-Learn cycle. To obtain an industrially viable bioprocess, the development of a suitable microbial cell factory usually must go through multiple iterative rounds of designing, building, and testing strains. Depending on the lab infrastructure (e.g., availability of automated processes), a single cycle can take weeks or months, limiting the speed at which a new production process can be established.

3.2. Synthetic biology

Metabolic engineering originated from concepts of chemical engineering, intending to produce compounds using biological rather than chemical systems. Synthetic biology, on the other hand, arose from ideas of electrical engineering. With the progress made in our understanding of living systems and available tools for modifying organisms, it became increasingly feasible to characterize biological components and construct completely novel, synthetic circuits and pathways.[183] Synthetic biology findings could thus be applied to metabolic engineering objectives. The two disciplines are highly intertwined, and one could say that metabolic engineering would not be where it is now without the help of synthetic biology, and vice versa. The construction of microbial cell factories typically requires expertise in both fields to achieve successful outcomes. While no universally accepted definition of synthetic biology exists, it may broadly be described as the application of engineering principles to biology.[184] Analogous to electrical engineering, the goal is to rationally design and build devices and systems, which are made of (standardized) parts encoded in DNA. These can then be employed in various disciplines, including metabolic engineering and healthcare.[185] Due to the complexity of biology, the goals of high-level standardization and modularity within synthetic biology are still far from accomplished. But incremental steps are taken, and new tools are continuously being developed so that biology is gradually becoming more and more “engineerable”.

Synthetic biology may be categorized into top-down approaches, which deal with the engineering of existing living systems, and bottom-up strategies, which are described as

the *de novo* creation of fully artificial living systems.[186,187] The main advantage of the top-down approach is the possibility of leveraging available cellular machinery for energy supply, protein synthesis, etc. Within the bottom-up concept, the motivation of creating "minimal" living systems made of only essential components would eliminate undesired crosstalk between endogenous and heterologous parts, which is frequently observed in top-down approaches, and would reduce the overall energetic burden on the system.

In this thesis, we used both rational and random top-down approaches of synthetic biology, including metabolite biosensors, *in vivo* directed evolution, and enzyme scaffolding methods, to engineer existing yeast strains for improved flavonoid production. Based on my Ph.D. research, I will discuss how the aforementioned tools may aid in overcoming several challenges in the development of microbial flavonoid factories in the following chapter.

Chapter 4. Overcoming challenges in the development of microbial cell factories

This part of the thesis serves as an overview and connection point of the different projects I worked on during my Ph.D. The overarching goals of my project will be outlined, and each sub-project will be put into context. For detailed information on the methodology and experimental results, please refer to the papers themselves.

4.1 Aim of the thesis

The aims and research questions of my Ph.D. were developed as part of the EU-funded project "SynBio4Flav" (https://synbio4flav.eu/). The main objective of this project was to improve the microbial production of specific flavonoid compounds by creating a synthetic consortium of microorganisms, instead of engineering the entire biosynthetic pathway in a single host (Figure 7). The complete heterologous biosynthesis of natural products typically requires the expression of many plant genes and additional fine-tuning of endogenous pathways. Such heavily engineered metabolism often leads to flux imbalances and depletion of cellular resources. This can create a metabolic burden on the cell, which may impede growth and consequently limit production efficiencies.[188,189] By compartmentalizing a pathway into shorter parts implemented in multiple hosts, the burden loaded onto each strain would be reduced. Additionally, this would allow us to engineer each module simultaneously, potentially reducing the time needed to optimize the whole production pathway. In the case of multispecies consortia, each species provides unique cellular environments which can be advantageous for the functional expression of certain heterologous enzymes.[190] Bacterial hosts for instance can provide an efficient supply of precursors, while eukaryotic hosts such as *S. cerevisiae* can be leveraged for the expression of plant-derived genes. Microbial consortia have been explored for the synthesis of a range of flavonoids, demonstrating remarkable improvements and benefits compared to monoculture applications.[191,192]

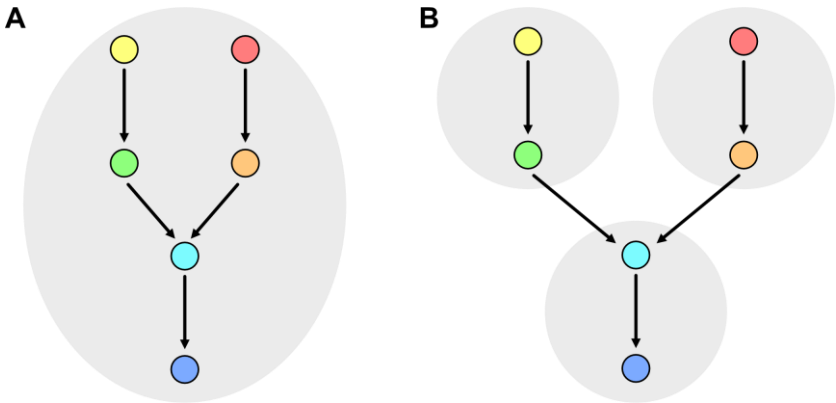


Figure 7. Illustration of single host vs. consortium-based production of a compound. (A) Expression of a biosynthetic pathway in a single host organism. **(B)** Division of a pathway into multiple modules, each expressed in a different host organism.

In the SynBio4Flav project, the flavonoid biosynthetic pathway is divided into three modules: malonic acid production will be engineered in *Pseudomonas putida*, *p*-coumaric acid production in *E. coli*, and both precursors will be taken up by *S. cerevisiae* and converted to various flavonoid compounds. Downstream decorations, e.g., glycosylation reactions, will lead to further diversification of flavonoids.

Within the project, my focus was to improve the yeast-based module for producing flavonoids directly from malonate and *p*-coumarate. We developed and applied several synthetic biology tools to enhance strain performance or to accelerate the strain development process. Three flavonoids were targeted specifically – naringenin (paper I), genistein (paper II), and phloretin (paper IV). The different projects and resulting papers are illustrated in Figure 8.

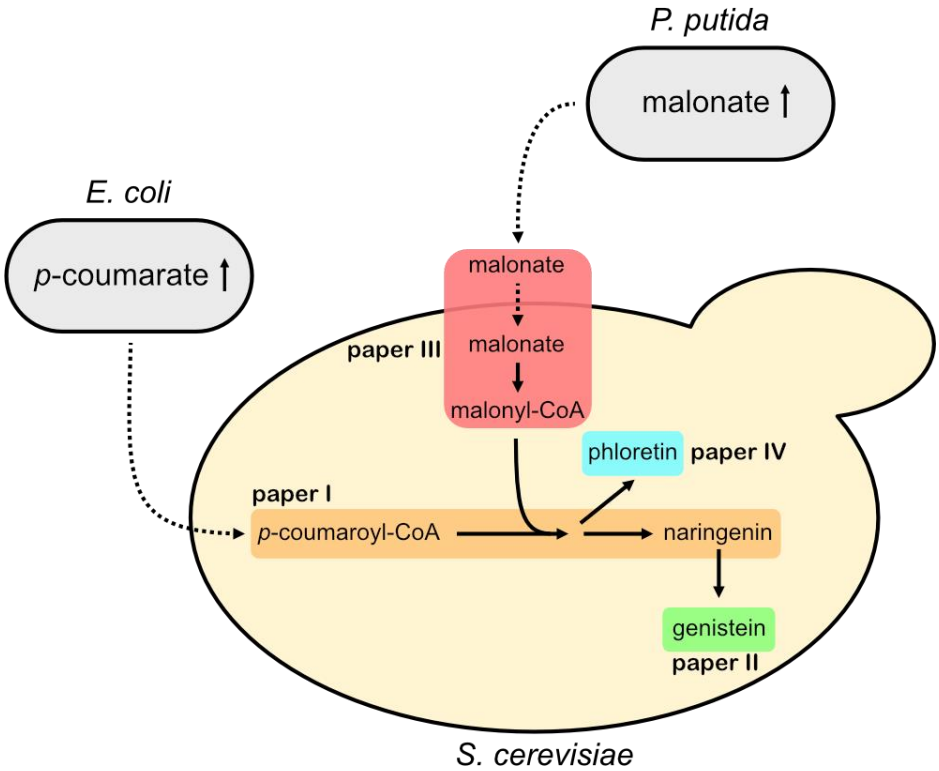


Figure 8. Overview of projects (and related papers) investigated in the thesis. As part of the SynBio4Flav project, *S. cerevisiae* was used as a host organism to assemble the precursors malonate and *p*-coumarate into different flavonoid aglycones, including naringenin, genistein, and phloretin. Different synthetic biology tools were developed to optimize yeast-based flavonoid biosynthesis.

Paper I centers around the flavonoid precursor, *p*-coumaroyl-CoA. While a malonyl-CoA biosensor had already been developed in yeast,[193,194] a sensor for *p*-coumaroyl-CoA detection has not been established. In this project, our goal was to design and validate such a sensor, which could then be employed for screening or regulation purposes. To understand whether this sensor could be used to improve flavonoid biosynthesis through dynamic pathway control, we further designed a genetic circuit that involves the *p*-coumaroyl-CoA sensor to dynamically control *4CL* expression in the naringenin biosynthetic pathway.

A downstream product of naringenin, genistein, was explored in **paper II.** Genistein possesses interesting bioactive, health-beneficial properties and could potentially be applied for hormone replacement therapy or to target certain diseases, including cancer. While genistein has been successfully produced in *S. cerevisiae* and other microorganisms,[171,195,196] current titers, rates, and yields are still low. To improve yeast-based production, we aimed to develop a genistein-responsive biosensor. Such a device could be used to efficiently screen mutant libraries for high-producer strains. Due to the structural similarity of genistein to its precursor naringenin, high ligand specificity is crucial for this purpose. Furthermore, the sensor should operate within a dynamic and operational range that is suitable for its intended applications.

In **paper III**, we focused on the flavonoid precursor, malonyl-CoA. As the synthesis of a single molecule of naringenin (or any other flavonoid) requires three molecules of malonyl-CoA, providing sufficient malonyl-CoA supply is essential to ensure high productivity. In the SynBio4Flav project, *P. putida* was employed to produce additional malonate, which could be utilized by yeast for increased malonyl-CoA supply for flavonoid biosynthesis. However, as *S. cerevisiae* does not possess a native malonate assimilation pathway, this sub-project aimed to construct and optimize a yeast strain to take up and convert malonate to malonyl-CoA in an efficient manner. We investigated a novel *in vivo* directed evolution approach to further improve a heterologous transporter's substrate affinity and functionality at high pH (for co-cultivation of yeast and bacteria).

Finally, in **paper IV**, we investigated the production of phloretin, a dihydrochalcone that exhibits antioxidant effects and can be used for dermatological applications. Although the biosynthesis of this compound had been realized in yeast previously,[197] not many studies on its production optimization exist. In this project, our goal was to identify bottlenecks in the biosynthetic pathway and to explore synthetic biology concepts to improve production titers. One strategy was the spatial assembly of pathway enzymes to enable substrate channeling for improved production and reduced byproduct formation.

Taken together, this work illustrated some of the molecular devices and engineering approaches that could be used to optimize microbial cell factories for flavonoid synthesis. Our main findings will be described in more detail in the following chapters.

4.2 Metabolite-responsive biosensors (paper I & II)

Secondary metabolites like flavonoids typically come with rather long biosynthetic pathways, the heterologous expression of which can create a metabolic burden on the cell factory. Some plant enzymes and metabolites may exert toxicity on the microbial host, which can further diminish cell viability. A growth impediment in turn often leads to reduced productivity. The possibility to dynamically regulate the expression of relevant pathways would allow us to isolate growth from production and potentially improve both in the process. The availability of metabolite-regulated genetic circuits is critical for the implementation of such dynamic control.

The DBTL cycle plays a central role in the optimization of microbial cell factories. Oftentimes, the testing phase poses a major bottleneck in this process. With improved protein engineering strategies and library construction methods, we require efficient screening tools to facilitate the development of better flavonoid cell factories. The testing phase typically relies on low-throughput traditional analysis tools, such as chromatography and mass spectrometry. The number of possible measurements for these methods is estimated to be only 10^3 per instrument per day, insufficient to efficiently evaluate libraries with millions of variants.[198]

A valuable tool for the acceleration of the testing phase as well as the implementation of dynamic metabolic control is the use of biosensors that can modulate the expression of a particular gene as a function of a metabolite's concentration. A crucial component here is the sensing element which specifically recognizes the compound of interest. It can be a transcription factor (TF), a ribozyme, an enzyme producing a colorimetric product, or a receptor protein linked to a transcriptional output via a signaling cascade. Some examples of biosensors for flavonoids or flavonoid precursors are listed in Table 2.

Table 2. Biosensors developed for flavonoids and flavonoid precursors.

Compound	Sensor element	Mechanism	Host	Reference
Malonyl-CoA	FapR (*Bacillus subtilis*)	TF	*S. cerevisiae*	Ref[193]
Malonyl-CoA	RppA (*Streptomyces griseus*)	enzyme	*E. coli*, *P. putida*, *C. glutamicum*	Ref[199]
Tyrosine	TyrR (*E. coli*)	TF	*E. coli*	Ref[200]
3-Dehydroshikimic acid	CusR (*E. coli*)	TF	*E. coli*	Ref[201]
***p*-Coumaric acid**	PadR (*B. subtilis*)	TF	*S. cerevisiae*	Ref[202]
Quercetin, kaempferol	QdoR (*B. subtilis*)	TF	*E. coli*	Ref[203]
Naringenin	FdeR (*Herbaspirillum seropedicae*)	TF	*E. coli*, *S. cerevisiae*	Ref[203–205]
Naringenin, phloretin, genistein	TtgR (*P. putida*)	TF	*P. putida*, *E. coli*	Ref[206,207]
Naringenin	SELEX-enriched aptamer	riboswitch	*E. coli*	Ref[208]

In my research, I focused on the construction of TF-based sensors, as the large diversity of small-molecule specific TFs presents an excellent opportunity for the development of such devices. TF-based sensors consist of three elements: 1) A **TF** as the sensing element that binds to the molecule of interest via its ligand-binding domain (LBD). The TF-ligand interaction leads to a conformational change in the TF protein, which causes a shift in its DNA binding propensity. 2) The **DNA binding sequence** as the transducing element that is recognized by the TF DNA-binding domain (DBD). The TF-DNA interaction is thus influenced by the presence or absence of the ligand molecule. 3) A **reporter** gene/protein as readout, whose change in expression is induced by the binding or release of the TF

from its DNA binding sequence.[209] If the reporter gene encodes an optical output, such as a fluorescent protein, flow cytometry or fluorescence-activated cell sorting can be used to screen for strains with increased metabolite levels (Figure 9A).[199,210] If the reporter gene encodes a protein for antibiotic resistance, auxotrophy complementation or similar, promising candidates can be selected based on faster growth.[211] Biosensors have also been successfully employed for the improvement of flux balances through dynamic pathway control (Figure 9B).[193,212]

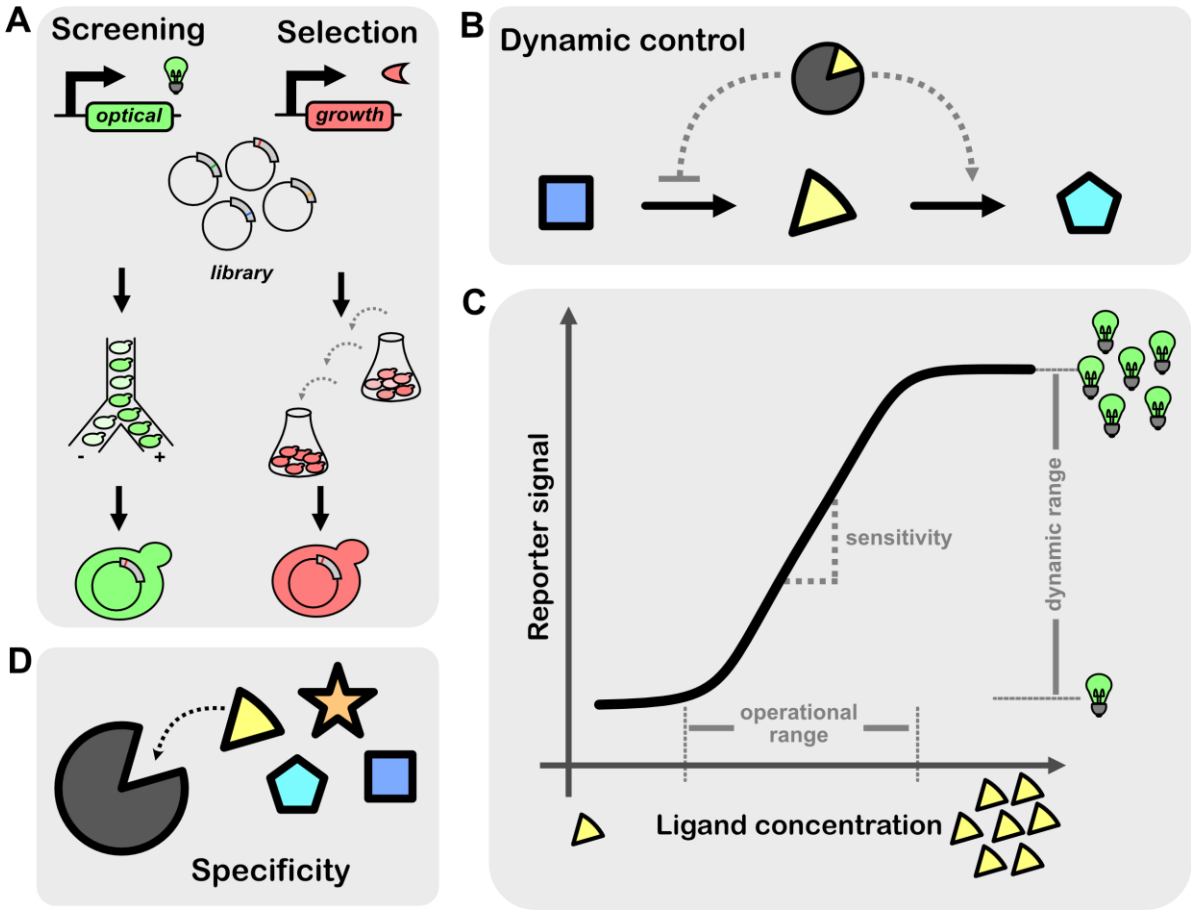


Figure 9. Biosensor applications and criteria. (A) Depending on the type of reporter protein, biosensors can be used for either library screening or selection. **(B)** Biosensors can also be repurposed for dynamic control of a metabolic pathway, e.g., to upregulate or downregulate a reaction in response to concentration levels of a specific metabolite. **(C)** A biosensor can be characterized by its dose-response curve, which indicates its dynamic and operational range and its sensitivity to the ligand. **(D)** High ligand specificity is desirable in most applications.

The dose-response curve of a sensor informs us of its operational range, dynamic range, and sensitivity (Figure 9C). The operational range is defined as the ligand concentration range over which the sensor provides significant changes in the output signal. For proper application, the operational range should cover the expected metabolite concentrations *in vivo*. The dynamic range can be seen as the ratio of the highest divided by the lowest possible output signal. A high dynamic range thus allows better distinguishment of true signal vs. noise. The slope of the dose-response curve represents the sensitivity. A steep slope means that the sensor exhibits a more ON/OFF-like behavior, whereas a gentle

slope corresponds to a more gradual response. Depending on the sensor's application, either may be preferred. Importantly, high ligand specificity is desired, as non-specific TF binding to other compounds will lead to an overestimation of the metabolite concentration (Figure 9D). Nonetheless, if no other potential ligands are present, a less specific biosensor can still find applications.[209]

In papers I and II, we developed two novel biosensors for the detection of a) the isoflavone genistein, which is an interesting phytoestrogen with potential anticancer activities,[213] and b) the flavonoid pathway intermediate, *p*-coumaroyl-CoA. The different steps of sensor development, validation, and application will be outlined below, based on these two studies. In particular, the genistein biosensor exemplifies strategies for sensor optimization, while the *p*-coumaroyl-CoA biosensor demonstrates a potential application for dynamic pathway regulation.

4.2.1 Biosensor development for genistein detection in *S. cerevisiae* (paper II)

To construct a metabolite-specific biosensor, the first step is to identify an appropriate sensing component. In previous work in *S. cerevisiae*, an estradiol-responsive gene expression system based on a chimeric transcriptional activator was established.[214] This transcription activator, termed GEV, consists of a Gal4 DBD, the human estrogen receptor α (ERα) LBD, and a VP16 transactivation domain (TAD). Due to its structural similarity to estradiol (Figure 10A, 8B), it is known that genistein can also bind to ERα.[215] GEV thus presents a potential sensing element for genistein detection. In our initial biosensor design, GEV expression was controlled by the strong constitutive *TDH3* promoter (P_{TDH3}), and the native *GAL1* promoter (P_{GAL1}) harboring several Gal4 DNA binding sites was employed to regulate green fluorescent protein (GFP) expression. In this way, genistein levels were coupled to fluorescence intensity. The hypothesized mechanism is as follows: In the absence of genistein, GEV is localized in the cytosol. Upon addition of genistein, ligand-TF binding leads to the translocation of the sensor protein to the nucleus and activation of *GFP* expression by binding to Gal4 DNA binding sites in P_{GAL1} (Figure 10C).

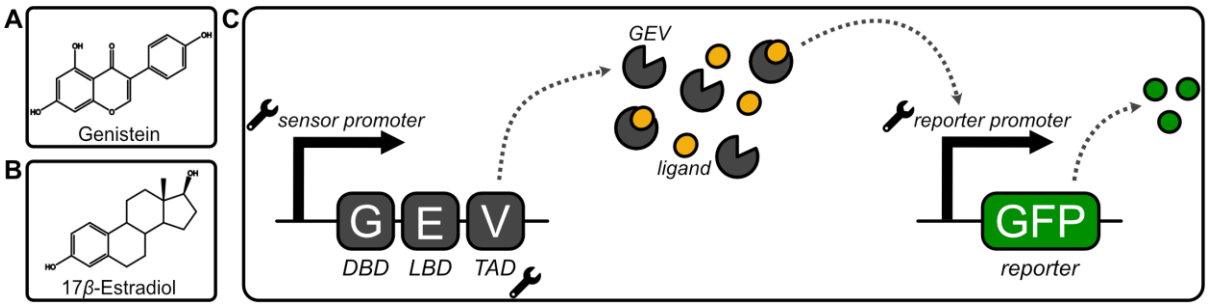


Figure 10. Development of a genistein biosensor. (A, B) Molecular structures of genistein (A) and 17*β*-estradiol (B). **(C)** Biosensor design and function. The sensor element GEV localizes to the nucleus and activates the expression of the *GFP* reporter when bound to its ligand, genistein. The GEV promoter, the GEV TAD, and the reporter promoter sequence can be engineered to improve the biosensor's dynamic range.

The initial design exhibited a dynamic range of ca. 5-fold and rather high leakiness, which means that *GFP* expression was activated even in the absence of the ligand molecule. To further improve its dose-response curve, several sensor components, including the GEV protein itself, its expression level, and the reporter promoter sequence were optimized (Figure 10). First, lowering GEV expression via promoter exchange (P_{REV1} instead of P_{TDH3}) led to reduced background noise. Second, replacing the VP16 transactivation domain for a stronger VPR domain (VP64-p65-Rta),[216] enhanced the fluorescence signal in the presence of the ligand. The combined reduction of sensor protein levels and increase in transcriptional activation strength led to an increase in the dynamic range up to ca. 15-fold. As transcriptional activation necessitates GEV translocation into the nucleus, removal of the additional nuclear localization signal (NLS) within the VPR domain was expected to reduce leaky expression. However, this was only the case at low GEV expression levels. It may be that the ERα NLS was sufficient for nuclear translocation, or that other mechanisms such as protein dimerization also play a role in the response mechanism.

Finally, the reporter-regulating P_{GAL1} sequence was investigated by testing different copy numbers of Gal4 binding sites, removing the native Mig1 (transcription factor involved in glucose repression and inhibition of *GAL* genes) binding sites[217], and adding the bases GTA upstream of Gal4 binding sites, which was previously shown to be important for *GAL4* expression.[218] In this way, the final biosensor design, which consisted of P_{REV1}-driven VPR2 expression (VPR with NLS removed) and a modified reporter promoter lacking Mig1 binding sites and containing 5 Gal4 binding sites, showed a 20-fold dynamic range as well as an improved operational range that covered typical genistein titers expected in current yeast-based production. When employing the sensor in a strain able to convert naringenin to genistein, fluorescence output signals correlated linearly with intracellular genistein concentrations normalized by biomass, but not with total genistein titers.

In summary, we have developed an *in vivo* biosensor for genistein detection in yeast, which can report on intracellular genistein concentrations through fluorescence intensity. Due to its high specificity and broad operational and dynamic range, the sensor may find versatile applications in metabolic engineering of yeast-based genistein production.

Although the genistein biosynthetic pathway and related enzymes have been elucidated, its production has not been explored as exhaustively as naringenin. Figure 11 depicts the reaction steps from naringenin to genistein. First, the aryl migration of ring B from the C2 to C3 position is catalyzed by a cytochrome P450 enzyme, isoflavone synthase (IFS), coupled to a cytochrome P450 reductase (CPR). The resulting intermediate compound is further converted to genistein, which is catalyzed by a 2-hydroxyisoflavanone dehydratase (HID). It has been found that the combination of the three enzymes could have significant effects on resulting titers.[171,219] Furthermore, genistein has been shown to downregulate its own production by inhibiting IFS activity.[171] As our biosensor can differentiate intracellular product concentrations, we contend that it may aid in the

screening of strain or enzyme libraries, for instance, to identify pathway enzymes with higher catalytic activity, beneficial enzyme combinations, or feedback-resistant IFS variants. Additionally, if the sensor is responsive to estradiol (not assessed in this study), it could be used for titrated gene expression by addition of specific estradiol concentrations to the medium, as it is a much cheaper inducer compared to genistein.

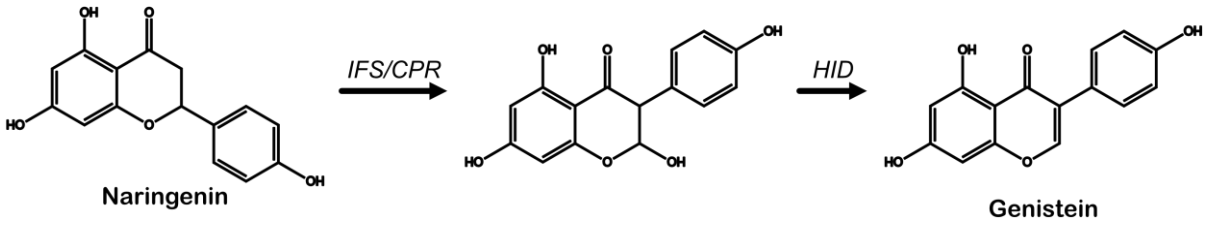


Figure 11. Biosynthetic pathway of naringenin to genistein. The cytochrome P450 enzyme IFS catalyzes the migration of ring B from the C2 to C3 position and requires a cytochrome P450 reductase (CPR) as a redox partner. The dehydration of the resulting intermediate by HID yields genistein.

4.2.2 Biosensor development for *p*-coumaroyl-CoA detection in *S. cerevisiae* (paper I)

The CoA thioester *p*-coumaroyl-CoA is a central precursor for many flavonoids, including naringenin (Figure 12A). Its production is catalyzed by the 4-coumarate:CoA ligase 4CL. While biosensors for end-products are desirable, e.g., to screen for high-producer strains, sensors that detect key intermediates can be useful as well. If a specific step in the pathway is known to be rate-limiting, a biosensor able to detect the corresponding intermediate or its precursor can be used to screen for enzyme variants with higher activity or substrate affinity.

Unlike the genistein biosensor, our *p*-coumaroyl-CoA sensor was based on a bacterial transcriptional repressor, CouR,[220] which responds to *p*-coumaroyl-CoA with high specificity. Its DNA binding sequence has also been elucidated,[221] allowing straightforward implementation in yeast. The CouR DNA binding site was integrated into a yeast native promoter (P_{CCW12}) and used to regulate *GFP* expression. An SV40 NLS was N-terminally fused to CouR to ensure nuclear localization. As CouR expression itself had negative effects on cell growth (paper I, Supplementary Figure S2), its expression had to be adjusted by testing promoters of different strengths.

In the absence of the ligand, *GFP* expression would be repressed by CouR binding. In its presence, CouR-ligand interactions would induce a conformational change in the transcription repressor, releasing it from its DNA binding site and allowing *GFP* to be expressed (Figure 12B). In our experiments, we observed *p*-coumaroyl-CoA accumulation without downstream consumption to inhibit cell growth in yeast. In a *4CL* expressing strain, the addition of 100 mg/L *p*-coumaric acid extended the lag phase significantly (paper I, Supplementary Figure S1). For this reason, the functionality of the sensor design was verified in *de novo* flavonoid-producing strains instead, in which *p*-coumaroyl-CoA was further converted to a non-toxic compound, naringenin. Varying copy numbers of the pathway genes *4CL*, *CHS,* and *CHI* were tested. The idea was to create

strains with different levels of intracellular *p*-coumaroyl-CoA. For instance, if a strain expresses 3x*4CL* and only 1x*CHS* and 1x*CHI*, *p*-coumaroyl-CoA levels should be higher than for a strain carrying 1x*4CL* and 3x*CHS*/*CHI*. As shown in Figure 12C with rising (postulated) intracellular *p*-coumaroyl-CoA levels, GFP signals increased correspondingly. This indicates that our sensor is responsive to the targeted compound, giving a graded output response to different *in vivo* ligand concentrations.

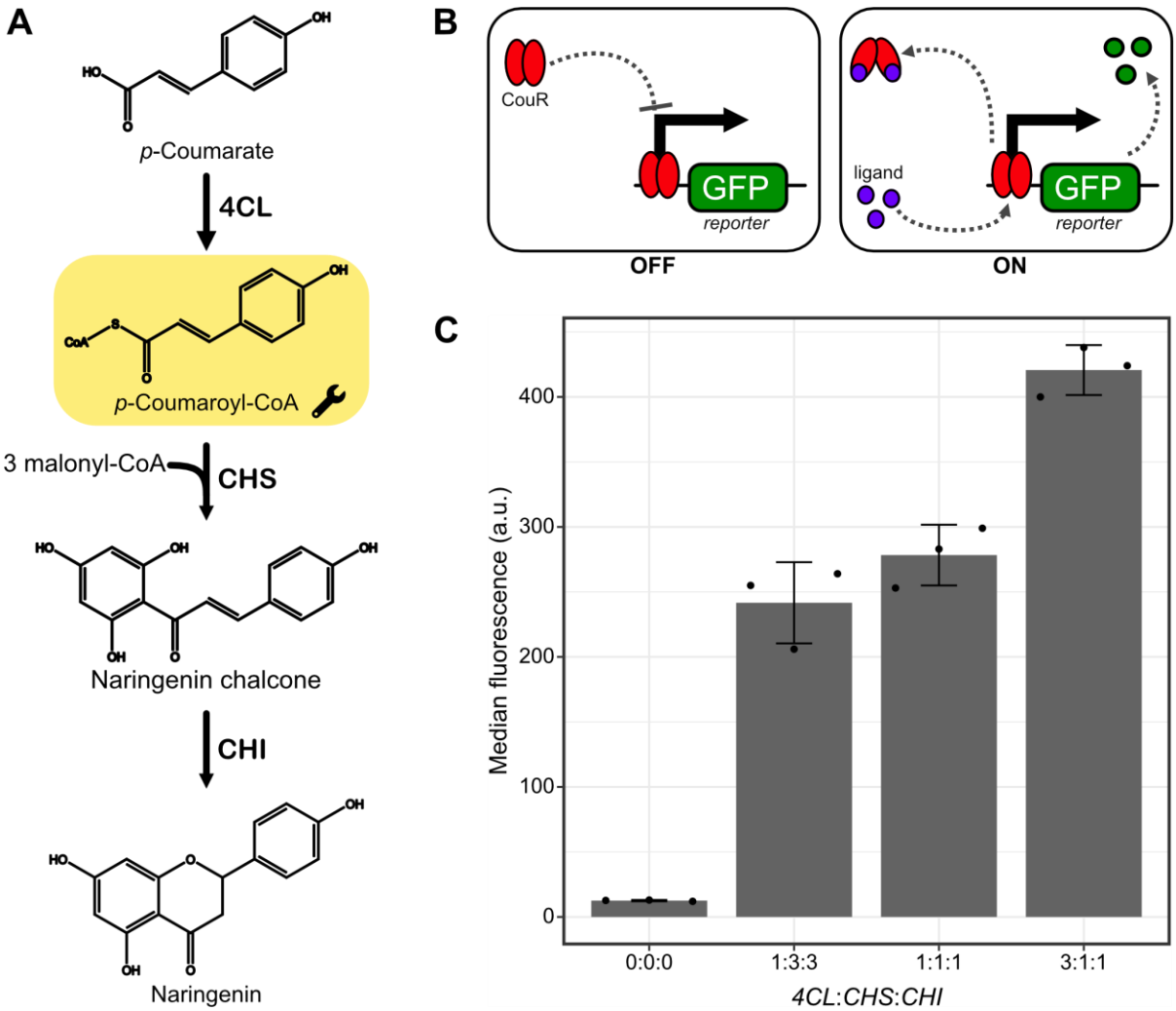


Figure 12. Development of a *p*-coumaroyl-CoA biosensor. (A) *p*-Coumaroyl-CoA as a central intermediate for naringenin biosynthesis. **(B)** Mechanism of action of the CouR-based biosensor. In the OFF state, i.e., in the absence of the ligand *p*-coumaroyl-CoA, CouR can bind to specific DNA binding sites in the reporter promoter, thus repressing *GFP* expression. In the ON state, when the ligand is present in the cell, the ligand-CouR interaction triggers a conformational change in the transcriptional repressor, releasing it from its DNA binding site, and resulting in increased *GFP* expression. **(C)** Median GFP fluorescence (a.u.) in strains carrying the biosensor plasmid and expressing different copy numbers of the naringenin pathway enzymes *4CL*, *CHS*, and *CHI.*

While we demonstrated the functionality of this biosensor, its dynamic and operational range was not extensively optimized. Furthermore, the mechanism behind CouR-associated growth inhibition was not elucidated. Therefore, in the future, one may further expand the potential of this sensor, by reducing CouR toxicity and engineering sensor

components to improve its dose-response curve. It may find applications in both library screening and pathway regulation, the latter of which will be demonstrated in Chapter 4.2.3.

4.2.3 Dynamic pathway regulation (paper I)

The static overexpression of heterologous enzymes and the accumulation of foreign metabolites can exert stress and additional load on the cells. Being able to adjust enzyme expression and regulate toxic intermediate levels autonomously depending on the cell's metabolic state would alleviate such toxicity and allow better allocation of carbon and energy sources at the same time. In nature, dynamic regulation of metabolic pathways is a common strategy for organisms to adapt to changing nutrient availability, environmental stresses, and cell density.[222] While various examples of artificial dynamic control for chemical production exist,[223–225] its application is still constrained by the scarcity of suitable regulatory components.

As pointed out in the previous chapter, *p*-coumaroyl-CoA accumulation appeared to inhibit yeast cell viability. Even with downstream conversion to naringenin, cell growth remained suboptimal due to the low activity of CHS. This in turn led to poor production performance. When feeding *p*-coumaric acid (and malonic acid) to a naringenin-producing strain, titers merely reached a few mg/L. We thus employed the newly developed CouR sensor together with a previously characterized malonyl-CoA sensor (FapR) [193,194] to dynamically control intracellular *p*-coumaroyl-CoA levels. In this way, we sought to reduce its toxic effects, while also avoiding unnecessary pathway gene expression. Collectively, this may improve cell growth and enhance naringenin production.

Like CouR, the malonyl-CoA sensor was based on a bacterial transcription repressor, FapR.[193,194] The dual regulation system, depicted in Figure 13A, was implemented in a SynBio4Flav consortium strain, able to take up and assemble *p*-coumarate and malonate to form naringenin. Besides the naringenin pathway genes *4CL*, *CHS,* and *CHI*, this strain expresses a malonate transporter and malonyl-CoA synthetase for efficient assimilation of malonate. Briefly, malonyl-CoA availability was coupled to *p*-coumaroyl-CoA production by using FapR to control *4CL* expression. In this way, the two precursor levels were balanced, avoiding unnecessary *p*-coumaroyl-CoA synthesis if malonyl-CoA levels were insufficient for conversion to naringenin. In a second step, CouR was employed to regulate *FapR* expression, so that low *p*-coumaroyl-CoA levels would suppress FapR activity and promote *4CL* expression, while high *p*-coumaroyl-CoA levels would lead to *FapR* expression and subsequent inhibition of *p*-coumaroyl-CoA synthesis. Using this circuit, we intended to reduce *p*-coumaroyl-CoA accumulation on the one side, and superfluous *4CL* expression on the other. Taken together, the naringenin titer was increased from 9.95 mg/L (without FapR and CouR) to 47.7 mg/L (with FapR and CouR) (Figure 13B) when feeding strains with *p*-coumarate and malonate. Interestingly, when comparing *p*-coumarate feeding to *p*-coumarate and malonate feeding, additional

malonate only enhanced naringenin titers in the non-regulated strain (Figure 13C), but not in the regulated strains (paper I, Figure 6).

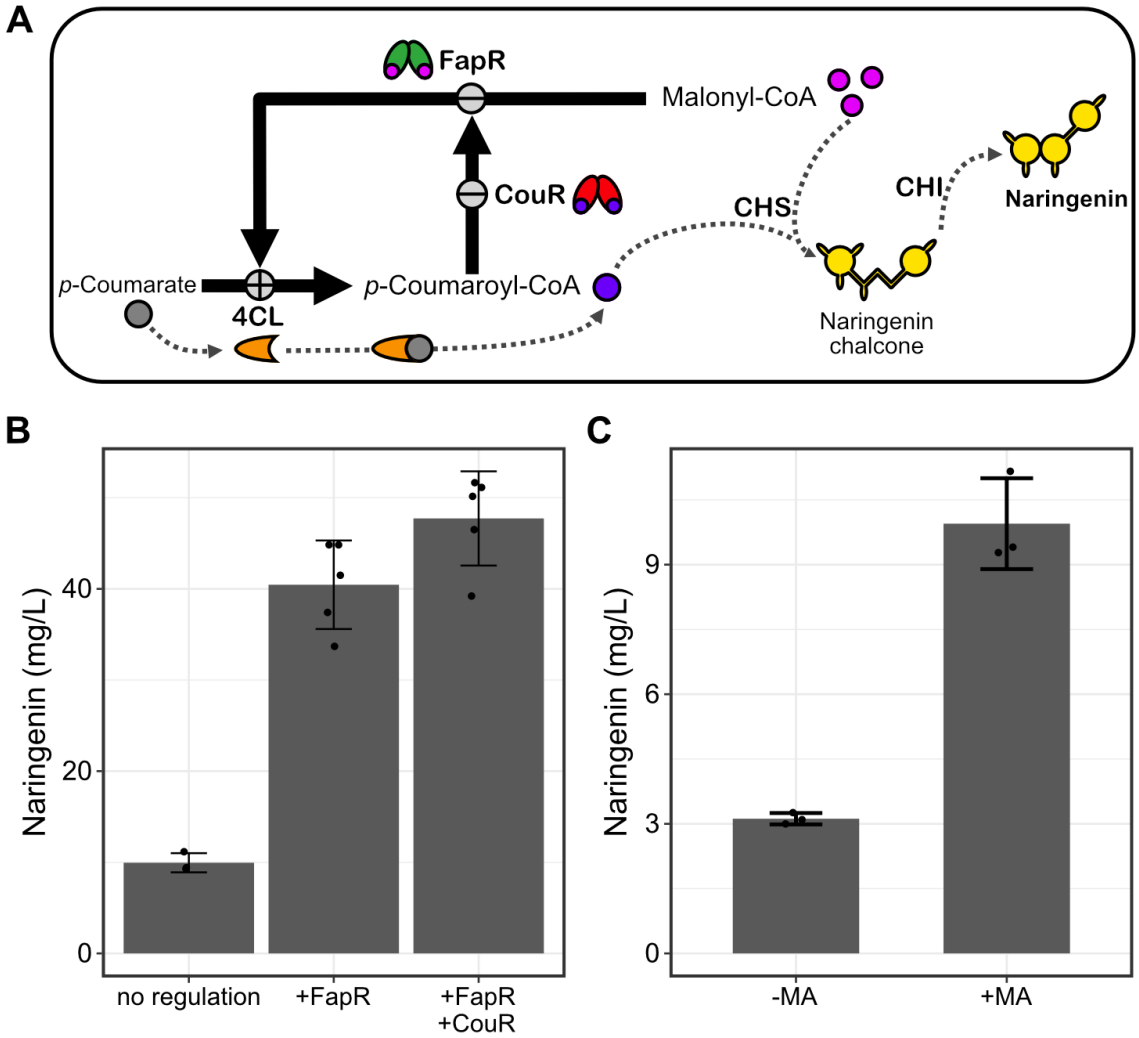


Figure 13. Two-level dynamic regulation of naringenin biosynthesis. (A) Employing the malonyl-CoA responsive FapR and the *p*-coumaroyl-CoA responsive CouR biosensors, *p*-coumaroyl-CoA synthesis was controlled through the transcriptional regulation of *4CL*. **(B)** Naringenin titers in the non-regulated strain compared to the FapR- and FapR-/CouR-regulated strain. All three strains expressed the malonate assimilation pathway, comprising a transporter and a malonyl-CoA synthetase. Precursors malonate and *p*-coumarate were added to the medium and naringenin titers were measured after 72 h. **(C)** The naringenin producer strain lacking FapR/CouR-mediated dynamic regulation was supplemented either with *p*-coumarate only (-MA), or with *p*-coumarate and malonate (+MA). Naringenin titers were measured after 72 h.

As shown here, as well as in many previous studies, dynamic regulation opposed to static overexpression can result in compelling improvements in production. By linking *4CL* expression to the availability of two central naringenin precursors, we obtained dramatic improvements in product titers, without the need for external inducer addition or any manual interference. Admittedly, the final naringenin titers achieved in this work are far below desired values for large-scale production. At the same time, as our system relied on the external supply of *p*-coumaric acid and malonic acid, results are not directly comparable to *de novo* production. Additionally, precursor addition, medium, and growth

conditions could be optimized to potentially increase production. Therefore, the implementation of the same strategy in a high-performance strain could elevate product titers, rates, and yields further, bringing us one step closer to industrial commercialization.

4.3 *In vivo* directed evolution to improve malonate uptake (paper III)

Besides *p*-coumaroyl-CoA, malonyl-CoA represents another central building block for all flavonoid compounds. As it participates in the elongation of fatty acids, it is an essential metabolite. In *S. cerevisiae*, malonyl-CoA is generated from acetyl-CoA, by the acetyl-CoA carboxylase, Acc1. This is a tightly regulated enzyme, both on the transcriptional[226] and post-translational level,[227] resulting in typically low intracellular malonyl-CoA levels and thus limited supply for the synthesis of heterologous compounds.[228] Several strategies have been investigated to increase malonyl-CoA flux for flavonoid production, including *ACC1* overexpression,[229] elimination of protein kinase-mediated Acc1 inactivation,[230] and upregulation of acetyl-CoA production.[231] As an alternative, malonyl-CoA can be synthesized via a heterologous malonate assimilation pathway. This has previously been shown to improve various flavonoid titers in different bacterial hosts.[232–234] Likewise, in our experiments from paper I, we observed that malonate feeding led to a substantial increase in production titers in the non-regulated naringenin production strain (Figure 13C).

However, external supplementation of expensive precursors would increase overall substrate costs, which should be avoided especially in large-scale production. Instead, our goal within the SynBio4Flav concept was to establish a synthetic co-culture, in which both malonate and *p*-coumarate would be supplied by engineered bacterial strains. Rather high levels of *p*-coumarate and malonate production have already been achieved in *S. cerevisiae* and *E. coli* respectively.[163,235] These strains could for instance be employed for co-culturing with our naringenin-producing yeast strain. While *p*-coumarate is readily taken up by *S. cerevisiae*, a native malonate assimilation pathway does not exist. Therefore, the heterologous expression of a malonate transporter and malonyl-CoA synthetase is needed.

To ensure efficient malonate assimilation, we screened different combinations of six dicarboxylic acid transporters and five malonyl-CoA synthetases of diverse origins (bacteria, yeasts, and plants) in a Δ*acc1* strain by feeding malonate (Figure 14). With malonyl-CoA being an essential compound, only strains harboring a functional malonate assimilation pathway could survive, thus allowing growth-based evaluation. From here, the fastest growing strain carrying the Mae1 transporter from *Schizosaccharomyces pombe* (SpMae1) and the malonyl-CoA synthetase MatB from *Rhizobium leguminosarium* bv. *trifolii* (RtMatB) was determined. The superior activity of SpMae1 had been observed in a previous study, where it was shown that SpMae1 acts through an energetically favorable, voltage-dependent mechanism, whereas other transporters rely on proton or sodium ion motive force.[236]

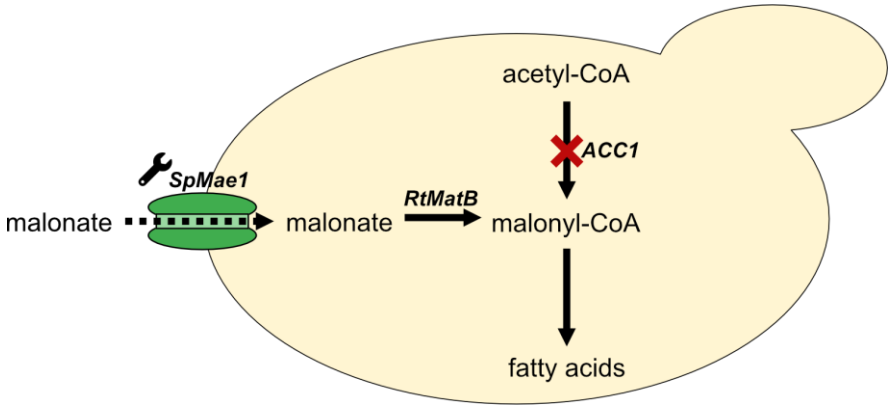


Figure 14. Strategy for evaluating the malonate assimilation pathway. Different combinations of a malonate transporter and malonyl-CoA synthetase were expressed in an Acc1-deficient strain. The best-performing strain, expressing *SpMae1* and *RtMatB*, was identified by comparing growth rates and lag phases under malonate supplementation.

As a dicarboxylic acid, malonate exhibits two pK_a values of 2.83 and 5.69. It had been reported that the actively transported substrate by SpMae1 was the monoprotonated anion (HA^-).[237] This was in line with our observation of malonate uptake being most efficient in low pH environments (pH 4.5, i.e. between the two pK_a values), in which HA^- is the predominant form. This is however in contradiction with the growth requirements of most bacteria, which preferentially grow at a neutral pH. Therefore, we aimed to further improve the transporter protein, not only in terms of generally improved activity but also enhanced functionality at high pH. This can be done by generating an SpMae1 mutant library, followed by enrichment under selective pressure.

Error-prone PCR (epPCR) is a common, straightforward method for *in vitro* mutagenesis. It relies on DNA amplification of the gene of interest (GOI) by a low-fidelity DNA polymerase that lacks any proofreading ability. The diversified PCR product must then be transformed into the selected background strain, making the library size highly dependent on the transformation efficiency of the host organism. Therefore, epPCR-generated libraries are typically plasmid-based, as the efficiency of genomic integrations is often insufficient to achieve desired library sizes. After the identification of improved protein variants, the new GOI sequence must be isolated and sequenced and is often used as template for another round of diversification. This necessitates manual repetition of each step of the evolutionary cycle.[238] Contrarily, *in vivo* directed evolution strategies allow autonomous evolution of the GOI, simply by cell propagation. The process is continuous, and diversification and selection can be carried out with minimal manual interference. *In vivo* continuous hypermutation allows us to explore larger sequence spaces than are generally attainable by epPCR.[238,239] Several approaches inspired by different biomolecular mechanisms (DNA transcription, replication, CRISPR-Cas9 technology) have been developed. OrthoRep,[240] for instance, employs a *Kluyveromyces lactis* linear plasmid system, localized in the cytoplasm, to hypermutate a GOI in *S. cerevisiae*. By using an error-prone DNA polymerase that exclusively replicates the *K. lactis* plasmid, hypermutation does not affect the *S. cerevisiae* genome. MutaT7[241] utilizes

a chimeric protein of a T7 bacteriophage RNA polymerase fused to a nucleotide deaminase. Due to the high promoter specificity of this polymerase, the method enables selective targeting of a GOI localized downstream of a T7 promoter. Originally developed in *E. coli*, MutaT7 has since been expanded to other organisms including *S. cerevisiae*.[242] With the advent of CRISPR/Cas9 technology, Cas9-based hypermutation methods have also been established. EvolvR[243] leverages a nickase Cas9 (nCas9) fused to an error-prone DNA polymerase, which generates point mutations close to the gRNA-targeted nick site. The fusion of a catalytically deactivated Cas9 (dCas9) protein to an activation-induced cytidine deaminase (AID) in CRISPR-X[244] was applied to diversify specific genomic loci in mammalian cells.

To take advantage of the benefits of *in vivo* directed evolution, we employed two dCas9-fused nucleotide deaminases (cytidine deaminase AID*[244] and adenine deaminase TadA8eV106W[245]) for mutagenesis of the *SpMae1* ORF. The complete workflow is depicted in Figure 15. A library of 55 gRNAs, covering most of the SpMae1 ORF, was designed (1). The gRNAs were duplexed for increased mutagenesis efficiency and inserted into a background plasmid which was transformed (2) into the Δ*acc1* P_{TEF1}-*SpMae1* P_{TDH3}-*RtMatB* strain, together with a plasmid carrying a dCas9-fused adenine or cytosine deaminase. The library was evolved (3) in an optimal growth environment and potential beneficial mutants were subsequently enriched under the two selective conditions – high pH and low malonate concentration (4). Promising variants were sequenced and reverse-engineered (5) to verify the positive effects of identified mutations. In total, 3 beneficial mutations, M43V, L76S, and V274A, were found. While the M43V mutant was enriched under high pH conditions, the L76S and V274A mutant were identified at low malonate concentrations. The combination of all three mutations in a triple mutant strain resulted in the best growth performance (Figure 16) and highest overall transport efficiency (paper III, Figure 5). The SpMae1 triple mutant also showed promising results in the SynBio4Flav consortium for naringenin production (unpublished results).

To gain better insight into the effects of the three amino acid substitutions on protein function, AlphaFold2, molecular docking, channel analysis, and molecular dynamics simulations were performed. At higher pH, the fully dissociated form of malonate (A^{2-}) is prevalent. Molecular docking results suggest that the replacement of methionine with valine at position 43 leads to weaker interactions between the residue and the A^{2-} substrate, while also expanding the substrate channel. Both effects may collectively facilitate substrate passage. The L76S mutant exhibited an additional channel compared to the wild-type, which may be beneficial for substrate transport. The V274A mutation, on the other hand, did not show significant differences from the wild-type protein structure. However, molecular docking results indicated increased flexibility of the V274A mutant, which may be beneficial for malonate transport. The combined effects of all three mutations could explain the superior transport activity of the triple mutant.

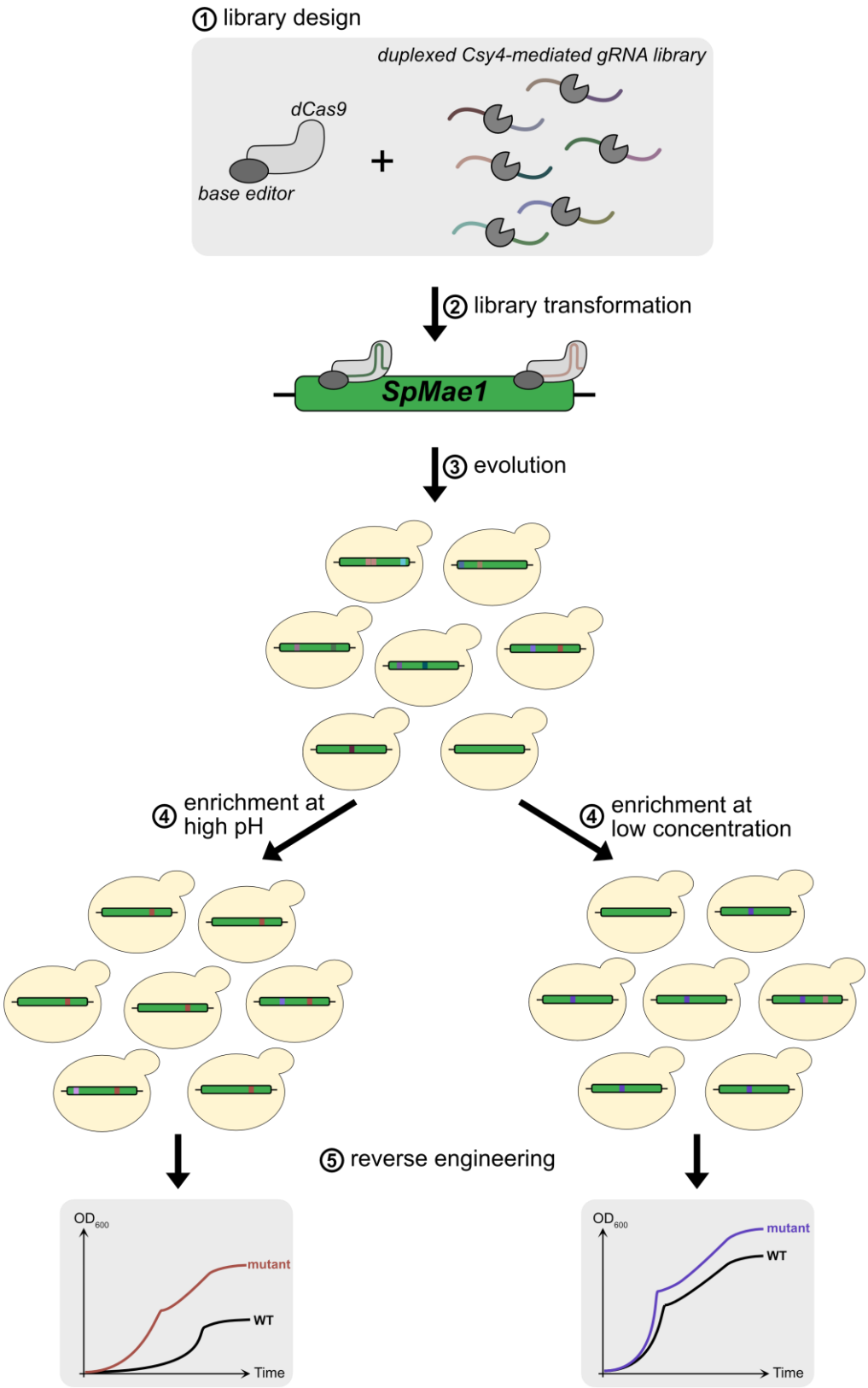


Figure 15. Workflow of SpMae1 evolution and enrichment using an *in vivo* targeted mutagenesis approach. The dCas9-base editor expression plasmid and the library of 55 duplexed gRNAs targeting the *SpMae1* ORF were transformed into the Δ*acc1* P_{TEF1}-*SpMae1* P_{TDH3}-*RtMatB* strain. The *SpMae1* gene was evolved under optimal growth conditions and enriched under selective pressure (high pH or low malonate concentration). Reverse engineering of identified mutations led to the identification of improved SpMae1 variants.

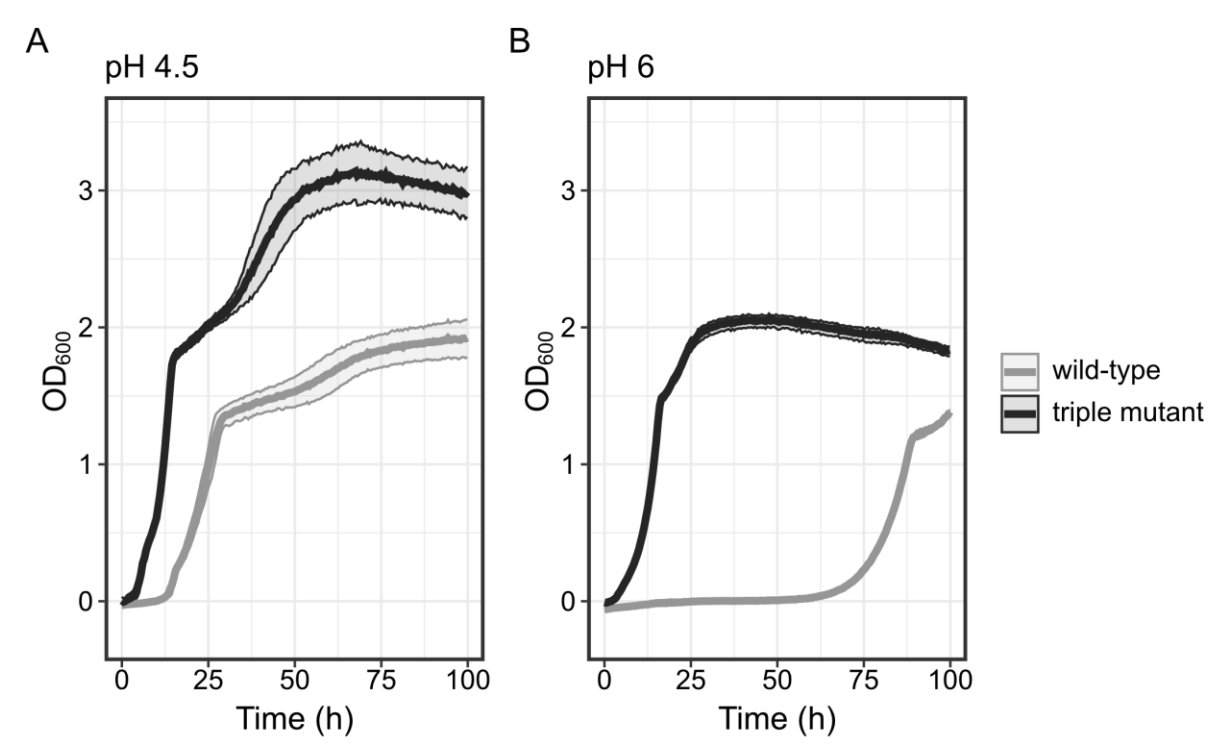


Figure 16. Growth curves of the strain expressing wild-type *SpMae1* and the triple mutant at pH 4.5 (A) and pH 6 (B) respectively. Cells were cultured in defined medium supplemented with 5 mM malonate and OD_{600} was monitored using a growth profiler.

In this application, we demonstrated that *in vivo* targeted evolution presents several benefits over the more commonly used epPCR approach. It poses less stringent requirements on transformation efficiencies, as the number of necessary transformants only needs to cover the size of the gRNA library (in our case 55 duplexed gRNAs, i.e., 2970 combinations) rather than the size of the resulting mutant library. Furthermore, it permits continuous diversification within the defined editing window, in place of sequential rounds of DNA amplification by epPCR. Since mutations are targeted directly at the genomic locus, this eliminates the use of plasmid-based expression, thus reducing cell-to-cell variability. Furthermore, it enables independent mutagenesis of two or more genes. This could for instance be used to co-evolve the RtMatB enzyme and the SpMae1 transporter to improve both steps of the malonate assimilation pathway simultaneously.

Despite the many compelling advantages listed above, several drawbacks of CRISPR base editing technologies should also be addressed, including its reliance on PAM availability, its narrow editing window, the limitation of adenine/cytosine deaminases to transition mutations, and potential off-targeting events. Nonetheless, with continuous advancements, CRISPR-based base editing will undoubtedly become an important tool in the field of directed evolution.

While not investigated in the present study, in the future, we may explore yeast-bacteria co-cultivation to enhance malonyl-CoA supply for improved flavonoid production. Furthermore, this approach could also be transferred to other malonyl-CoA-derived products, such as fatty acids or polyketides. We believe that the use of co-cultures

presents some general advantages over monoculture applications. Instead of engineering malonyl-CoA supply in yeast, which requires extensive metabolic rewiring in addition to the expression of the heterologous pathway itself, malonate production would be outsourced to another host. By doing so, we may optimize the two modules in parallel rather than in sequence. Moreover, this allows us to leverage the unique characteristics of each organism: *S. cerevisiae* provides the necessary cellular environment for the functional expression of eukaryotic (plant) enzymes, while *E. coli* has already been engineered to produce substantial amounts of malonate. Despite these benefits, one should keep in mind that besides pH conditions, yeast and bacteria differ largely in their growth rates and media preferences. Therefore, a strategy for stable co-cultivation is needed. This could be achieved for instance by optimizing the inoculation ratio or establishing a mutualistic dependency.

4.4 Enzyme scaffolding (paper IV)

In paper IV, we explored the biosynthesis of another flavonoid – the dihydrochalcone phloretin. Its biosynthetic pathway is depicted in Figure 17.

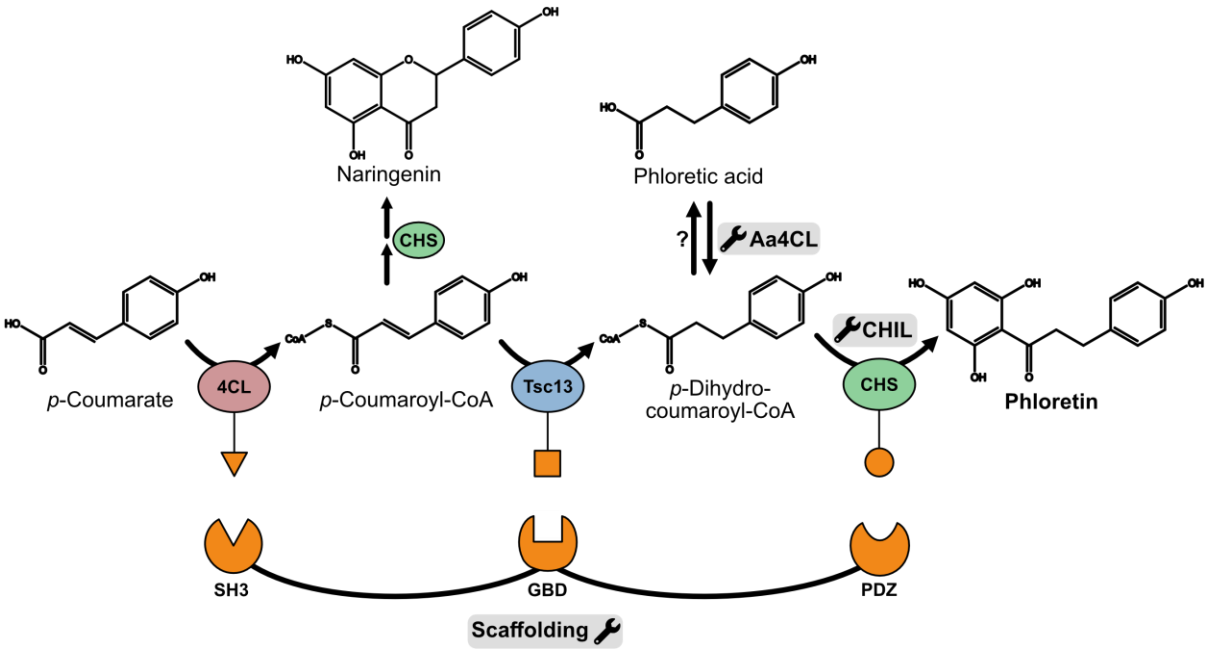


Figure 17. Phloretin biosynthetic pathway and strategies for production improvement investigated in this project. CHI-like enzymes (CHIL) were tested to increase flux towards phloretin. Aa4CL was used to convert phloretic acid to *p*-dihydrocoumaroyl-CoA for reduced byproduct formation. An enzyme scaffolding approach using metazoan protein signaling domains and their cognate ligands was used to spatially organize the three pathway enzymes, 4CL, Tsc13, and CHS, for improved phloretin synthesis.

Like naringenin, phloretin is derived from *p*-coumaric acid, which can be generated from aromatic amino acids via the phenylpropanoid pathway. Upon activation of *p*-coumarate

to *p*-coumaroyl-CoA by 4CL, it is further converted to *p*-dihydrocoumaroyl-CoA by a double bond reductase. In *S. cerevisiae*, this reaction can be catalyzed by the endogenous enoyl reductase Tsc13.[197] CHS enzymes have been reported to exhibit different substrate affinities for *p*-coumaroyl-CoA and *p*-dihydrocoumaryol-CoA, leading to the preferential formation of either naringenin chalcone or phloretin. While the action of CHI can aid in the ring cyclization of naringenin chalcone to naringenin, this step may also occur spontaneously. In previous reports on yeast-based phloretin production, another aromatic compound, phloretic acid, was detected in high amounts.[197] However, how the formation of this compound occurs and whether it is catalyzed by a yeast native enzyme is currently unknown. Consistent with this previous report, we observed phloretic acid and naringenin as two main byproducts in our initial phloretin production strains, expressing *4CL*, *TSC13,* and different variants of *CHS* (Paper IV, Fig. 2). Interestingly, a trade-off between phloretin titers and the phloretin:naringenin ratio was observed (Figure 18). The *EbCHS* expressing strain, for instance, delivered the highest phloretin titer but the lowest phloretin:naringenin ratio. In contrast, the *HvCHS* expressing strain showed the highest phloretin:naringenin ratio, whereas product titers were considerably lower. This is in line with the observation that catalytic activity is often compromised for substrate/product promiscuity in enzymes of secondary metabolism.[80]

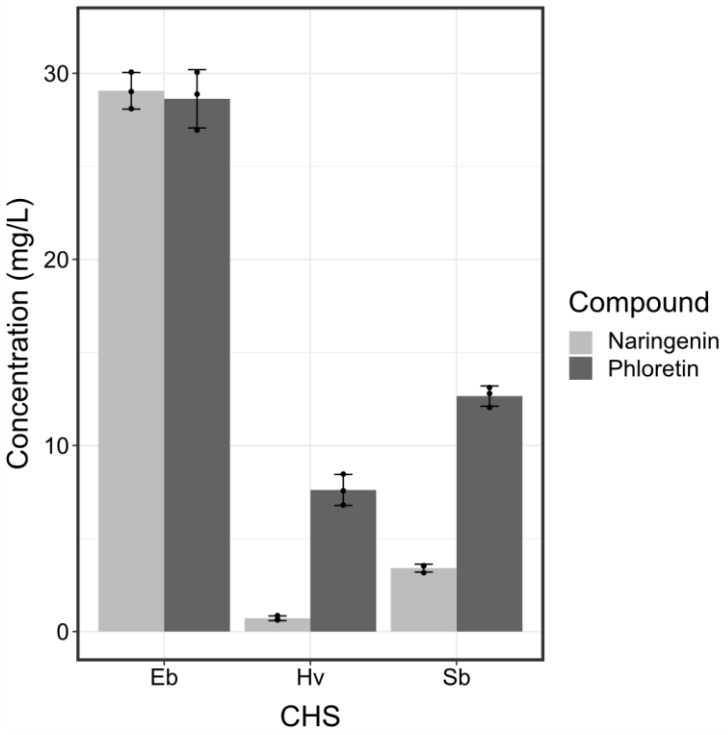


Figure 18. Phloretin and naringenin titers obtained using strains expressing *4CL*, *TSC13*, and different *CHS* variants. EbCHS: CHS from *Erigeron breviscapus*; HvCHS: CHS from *Hordeum vulgare*; PpCHS:CHS from *Pyrus pyrifolia*. Samples were taken after 4 d of shake flask cultivation.

The main objective of this project thus became the reduction of byproduct formation to increase flux towards the desired product. Different strategies were investigated (Figure 17):

1. Converting phloretic acid back to *p*-dihydrocoumaroyl-CoA (Aa4CL)
2. Increasing flux towards phloretin by introducing a CHI-like enzyme (CHIL)
3. Scaffolding of pathway enzymes to improve overall production

While strategies 1 and 2 provided minor improvements in byproduct reduction or product synthesis (detailed in paper IV), I will herein focus on strategy 3, which afforded some promising results.

It is now recognized that cells cannot be simply viewed as homogeneous "bags of enzymes", but that enzymes can physically interact with each other and localize to specific subcellular regions, depending on their function.[246] Protein complexes exist in different forms and take part in many metabolic pathways in all domains of life. Some better-studied examples of multi-enzyme interactions include the citric acid cycle and the glycolytic pathway.[247] Several protein-protein interactions have also been observed between flavonoid biosynthetic enzymes in plants.[248] Such assemblies have been proposed to increase pathway fluxes by channeling substrates between enzymes involved in sequential reaction steps.

This natural phenomenon has inspired metabolic engineers to design and construct synthetic enzyme scaffolds for different applications. The hypothesis is that enhancing the spatial proximity of specific enzymes of sequential reactions could enable substrate channeling and increase local metabolite and enzyme concentrations for increased metabolic efficiency. Moreover, it could reduce the accumulation of toxic compounds and the escape of labile intermediates. Enzyme scaffolding may also allow regulation of metabolic branch points by sequestering metabolites involved in competing reactions. Some scaffolding methods can be used to control pathway enzyme stoichiometries, which could potentially improve flux balances and thus enhance productivity.[249,250]

The simplest way to co-localize two enzymes is by fusing them covalently into a chimeric protein, typically linked by a short flexible peptide linker. This has for instance led to improved production of the polyphenolic compound resveratrol by linker-mediated fusion of 4CL and stilbene synthase (STS).[251,252] However, the direct fusion of proteins is difficult to implement for more than two pathway enzymes and is likely to affect enzyme activity. Additionally, it does not allow for control over enzyme stoichiometry.

Enzyme scaffolds have only been marginally explored for flavonoid production. In a previous paper, Fink et al. (2020)[253] elevated mevalonate production by almost 9-fold in yeast by co-localizing three pathway enzymes via fusion to *de novo* designed coiled-coil forming peptides. However, our attempt to assemble 4CL, Tsc13, and EbCHS using the same peptide proved to be detrimental to phloretin production (paper IV, Figure 8). We speculate that the attachment of this rather long peptide (102 residues) may interfere with enzyme activities and perhaps the fusion of shorter ligands would be less damaging.

Dueber et al. (2009)[254] leveraged the specific interaction of three metazoan signaling protein domains, SH3 (Src homology 3 domain from the adaptor protein CRK), GBD (GTPase binding domain from the actin polymerization switch N-WASP), and PDZ (PSD95/DlgA/Zo-1 domain from the adaptor protein syntrophin), and their respective peptide ligands to develop a synthetic scaffold to enhance mevalonate production. The scaffold domains were connected via 9-residue glycine-serine linkers, and their cognate ligands were fused to three consecutive enzymes of the mevalonate pathway. This method proved to be highly effective, resulting in up to 77-fold titer improvements in *E. coli*. The same protein scaffold was later applied for the recruitment of 4CL and STS in yeast and resulted in an up to 5-fold increase in resveratrol production.[255] We thus sought to investigate whether this approach would be transferable to the phloretin biosynthetic pathway. As these interaction ligands are only 6-32 amino acids long, we expected them to have less impact on enzyme activities. Additionally, as the scaffold and enzymes were expressed separately in this method, it allows modulation of enzyme stoichiometries by adjusting the number of repeats of each scaffold domain. Indeed, with this approach, phloretin titers were increased in two specific stoichiometries of the three scaffold domains, whereas all other configurations resulted in reduced phloretin production. Specifically, the scaffold designs $SH3_1GBD_4PDZ_1$ and $SH3_1GBD_4PDZ_2$ led to 1.6- and 1.7-fold improvements in phloretin titers (Figure 19A). However, byproduct formation was similarly increased (Figure 19B, C). Looking at the product:byproduct ratios, the molar ratio of phloretin:phloretic acid was improved by enzyme scaffolding, while the phloretin:naringenin ratio deteriorated (paper IV, Figure 10 B, C). This overall increase in all three compound concentrations suggests that the metabolic flux through the heterologous pathway was generally enhanced in these two scaffold designs. Interestingly, 4 GBD domains for Tsc13 recruitment seemed to be beneficial, whereas the increase of PDZ domains for EbCHS recruitment from 2 to 4 reduced all compound concentrations considerably, indicating that an excess of Tsc13 enzymes may be beneficial, while the assembly of the same number of EbCHS enzymes was not.

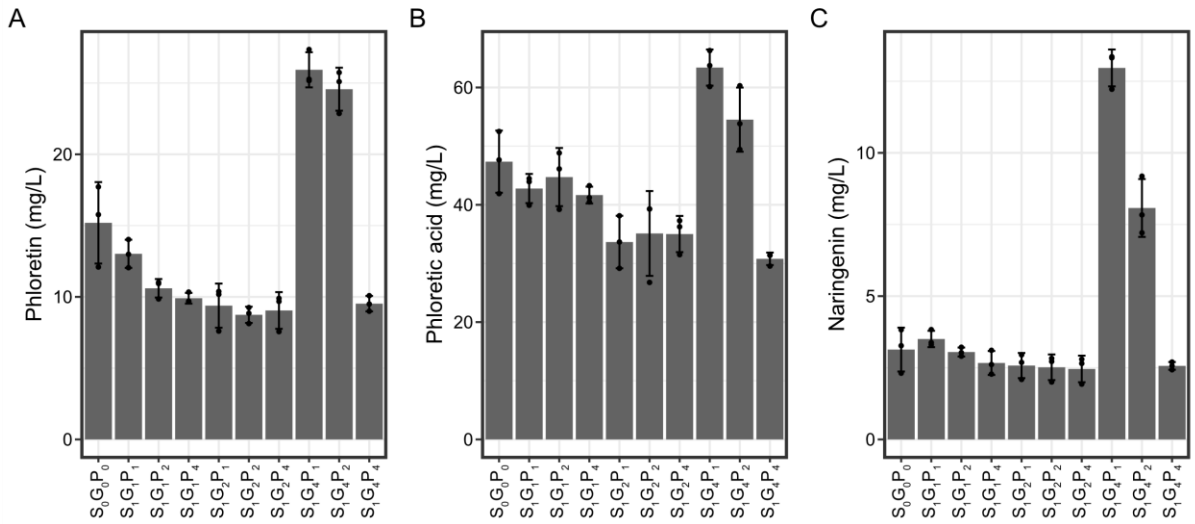


Figure 19. Phloretin (A), phloretic acid (B), and naringenin (C) production using enzyme scaffolding. Scaffold domains SH3, GBD, and PDZ were combined in different numbers of repeats to test the effect of changing pathway enzyme stoichiometry. $S_xG_yP_z$ ($SH3_xGBD_yPDZ_z$) represents the number of each scaffold domain, where x = 1, y = 1, 2, 4, and z = 1, 2, 4.

In summary, enzyme scaffolding presents a promising complementary approach to conventional metabolic engineering. Further optimization of the scaffold design could provide even higher phloretin titers. The SH3-GBD-PDZ-based scaffolding strategy has already been applied to several pathways, demonstrating its versatility. However, due to our limited mechanistic insight, the optimal design (number and order of scaffolding domains, enzyme stoichiometry, linker sequence, etc.) can only be teased out by trial and error for each pathway. This becomes laborious and time-consuming. In the future, a better understanding of the physical interactions between enzymes, for instance through improved modeling techniques, may shift scaffolding applications from trial-and-error-based to become more predictive and rational. With regards to the phloretin biosynthetic pathway, although enhanced enzyme proximity did promote general metabolic fluxes towards phloretin, substantial work on byproduct reduction and productivity, titer, and yield improvement will be needed to permit industrial implementation. Other methods are necessary to avoid concurrent increases in naringenin and phloretic acid formation.

Elucidating the mechanism of phloretic acid formation will be crucial. If an endogenous yeast enzyme is responsible for the conversion of *p*-dihydrocoumaroyl-CoA to phloretic acid, its deletion or downregulation could resolve the issue. If the enzyme is essential for cell fitness, perhaps replacement by a homolog that carries out the essential function without acting on *p*-dihydrocoumaroyl-CoA, could be investigated. Alternatively, switching to a different host organism may also eliminate phloretic acid formation. If, however, the reaction occurs spontaneously, only improving metabolic flux to phloretin can counteract phloretic acid accumulation.

The substrate promiscuity observed in CHS did not come as a surprise. As enzymes of primary metabolism have evolved under stringent conditions, their specificities and

activities are often high. Contrarily, secondary metabolism generates metabolites for ecological interactions rather than cell growth and proliferation. Therefore, enzymes of these pathways require broad substrate and product ranges to create large chemo-diversity among resulting metabolites. This enzyme promiscuity is often accompanied by lower catalytic activity.[80] To enable efficient production of a single natural product, one may screen more CHS variants or use directed evolution approaches to obtain CHS mutants with higher activity and specificity.[256] In this way, naringenin formation as well as other CHS derailment products could potentially be fully erased.

4.5 Product stability (paper IV)

Another major challenge observed in yeast-based phloretin production was the compound instability observed in our cultivation medium compared to water (Figure 20A). Having a stable product is essential for any production process, as degradation will reduce production parameters, such as yield, titer, and productivity. Moreover, degradation products could have unintended adverse effects and must therefore be removed during the purification process. Presuming that this decrease in phloretin concentration in media is caused by oxidation, and potentially facilitated by media components such as transition metal ions, we tested the addition of antioxidants to reduce the rate of degradation. The use of Trolox showed improved compound stability in sterile medium (Figure 20B) but did not have a positive effect on cultivations of a phloretin-producing yeast strain (paper IV, Fig. 4B, C). Another approach is the conversion of the labile compound to a stable product. Phloridzin, the 2'C-glycoside of phloretin for instance, did not show any degradation in the cultivation medium (paper IV, Fig. 3A). Additionally, it has been demonstrated that glycosylation modifications can improve product solubility and enhance extracellular secretion.[165] Moreover, a phloridzin transporter from *Arabidopsis thaliana* has been identified and could be employed to facilitate product export, which may further improve production.[257]

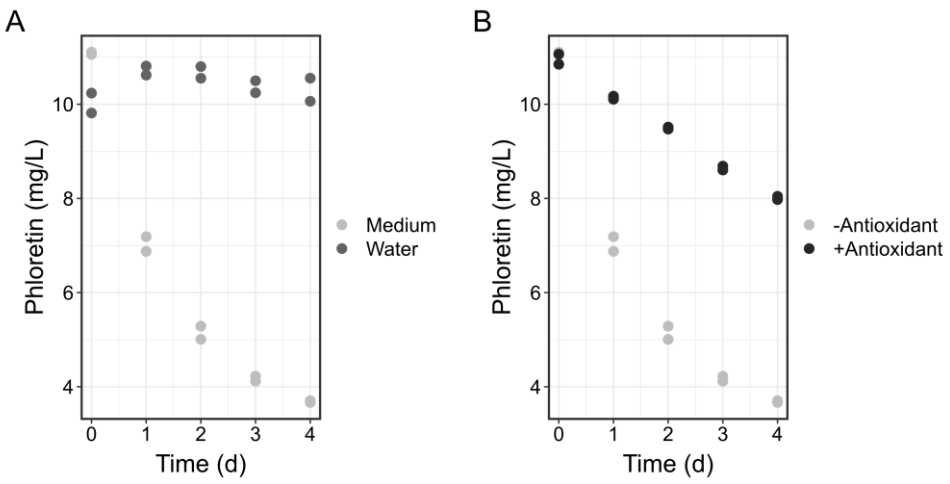


Figure 20. Phloretin degradation in yeast cultivation medium. (A) Change in phloretin concentration in water compared to defined medium. **(B)** Change in phloretin concentration in medium without antioxidant compared to medium with antioxidant addition. The experiment was performed in duplicates.

Our lack of knowledge about the phloretin degradation mechanism presents a serious barrier in yeast-based phloretin production. This exemplifies the importance of considering other aspects of process feasibility (such as product stability), which are outside of conventional metabolic engineering tasks. Since the medium used in our study contains (almost) only essential components for growth, even if we identify the responsible component(s), removing those will likely be detrimental to growth. While the addition of the antioxidant Trolox did reduce the rate of phloretin degradation, the use of expensive supplements is not desirable for industrial processes.

Chapter 5. Conclusions and outlook

Looking at the evolution of metabolic engineering over the last 20-30 years, there is no doubt that much progress has been made. Starting from a handful of chemicals that could be generated by microbial fermentation,[108] we are now at a stage where highly complex[258–260] and even non-natural compounds could be synthesized using engineered or *de novo*-designed enzymes.[261,262] Through the continuous development of novel or improved technologies in DNA synthesis, DNA sequencing, bioinformatics, and systems and synthetic biology, we have broadened our knowledge of metabolic networks significantly and succeeded in the establishment of several impactful industrial processes.[86,87] However, there are still many unknowns and bottlenecks that hinder the rapid development of new cell factories.

In my Ph.D. project, I focused on the development and application of various synthetic biology tools with the overarching goal of speeding up the progress of establishing efficient, sustainable yeast-based flavonoid production. While our major findings have been summarized in Chapter 4, I will conclude this thesis here by giving my perspective on the future of microbial cell factories for flavonoid production. I will also outline current and prospective roles that the previously described engineering tools will play in the construction of microbial production platforms in the next years and decades.

5.1 Addressing challenges and finding solutions for microbial flavonoid production

Many flavonoids have been successfully produced using microbial cell factories. However, titers, rates, and yields so far have not reached sufficient levels for widespread industrial application and many challenges remain.

Enzymes of secondary metabolism typically exhibit low catalytic activities and substrate/product promiscuity, which makes it difficult to produce a specific compound with high titers. In paper IV for instance, we found the low activity of CHS and the formation of side products to be a major hurdle in phloretin biosynthesis in yeast. Biosensors like those developed in papers I and II play a key role in the high-throughput screening of enzymes with higher activity or specificity. Moreover, advanced enzyme design and simulation tools can aid in rational or semi-rational protein engineering approaches.

As secondary metabolites often comprise long pathways, the need for expressing and accommodating many non-native enzymes and metabolites may lead to competition between heterologous reactions and endogenous cellular activities. This may overburden the host organism and result in poor production performance. Furthermore, heterologous products or intermediates, as observed for *p*-coumaroyl-CoA, often exhibit cellular toxicity, which can further hinder cell growth and production. Aside from

screening applications, we showed in paper I that metabolite-responsive biosensors which regulate transcription or translation upon ligand-binding, can be applied for dynamic regulation of biosynthetic pathways to improve flux balances, resource allocation, and overall cell growth and product synthesis.

As discussed in paper III, co-cultures, for example of flavonoid and malonate-producing strains, present several advantages over monoculture-based compound production. By using shorter pathway modules, one can alleviate the metabolic burden and cell stress imposed on a single strain expressing many heterologous genes. The members of the consortium can be engineered in a modular fashion, which reduces the amount of time and the number of genetic modifications required for each host. By spatial segregation of a pathway into different cells, one may avoid autoregulation of a compound on its own production. Finally, when working with a multispecies consortium, one can potentially take advantage of the unique characteristics of each organism. Nonetheless, the co-cultivation of multiple strains can come with distinct challenges. To ensure stable populations over time, compatible growth conditions must be found, so that one strain does not outgrow the other. The efficient transport of metabolites connecting sequential modules is crucial. Sometimes, as in the case of malonate uptake in yeast, additional transporter engineering might be necessary.

Lastly, the mimicking of natural metabolons using synthetic enzyme assemblies (paper IV) presents a promising complementary method to conventional metabolic engineering strategies. Further studies and a better understanding of such scaffolds could lead to significant improvements in microbial flavonoid production.

5.2 Biosensors

The rapid advances in DNA sequencing, synthesis, and assembly, the emergence of increasingly facile genome engineering methods such as CRISPR-Cas9, and the automation of routine laboratory tasks have accelerated strain construction efforts tremendously in recent times. The building phase of the DBTL cycle has thus overtaken our current testing and screening capabilities.[198] As previously described, metabolite-specific biosensors have the potential to alleviate our reliance on low-throughput analytical methods and to expedite the entire cell factory development process. The continuous development of novel biosensors with high specificity, suitable response parameters, and (almost) immediate output signaling enables the rapid evaluation of millions of cells, leading to a massive expansion of previous screening and selection capacities. Additionally, metabolite-responsive transcriptional regulators can be employed to modulate relevant pathway fluxes dependent on the metabolite concentration in a dynamic fashion. This has resulted in considerable improvements in productivity in many cases. For example, in **paper I**, we showed that it is possible to employ dual regulation of the naringenin pathway using two prokaryotic transcription factors in yeast to significantly improve production.

However, the development, characterization, and optimization of any given biosensor still come with many challenges. Although ligand-responsive TFs exist for some metabolites, there is a lack of appropriate sensing elements for many compounds of interest. Existing TF-based biosensors can be repurposed to new ligands through rational approaches or random mutagenesis.[263–265] This, however, is a laborious undertaking. In addition, shifting biosensor selectivity from one compound to another is typically only possible for structurally similar chemicals. Most characterized transcription factors are of prokaryotic origin. Oftentimes they are not directly transferable from one host to another due to differences in the transcriptional machinery. In some cases, as shown in **paper II**, the construction of chimeric proteins, for instance by fusing a ligand binding domain and a native DNA binding domain, presents a viable solution. Nonetheless, continued characterization of new transcriptional regulators, for example from metagenome, transcriptome, and proteome data, is needed to expand our knowledge base and aid the development of new sensors. Moreover, advances in the computational design of ligand-binding proteins may enable the development of novel ligand-responsive regulators from scratch.[266]

Once a suitable sensing element has been found, its operational and dynamic range as well as its ligand specificity are often suboptimal for direct implementation in high-throughput screening or dynamic pathway control. Depending on the application, sensing parameter requirements will differ drastically. We possess limited knowledge of the influence of individual sensor components on the sensor's overall behavior and the interdependency of different parameters.[267] The lack of standardized parts and design principles severely hinders the optimization process, as designs that work for one biosensor will not necessarily apply to another. For this reason, comprehensive engineering and fine-tuning of promoters, TF binding sites, TF expression levels, etc. is commonly needed (see **paper II**) to arrive at a design that exhibits an adequate dose-response curve for the given application. Here, mathematical modeling and computer-aided design automation could play a key role in accelerating the dose-response curve optimization.[268]

With the increase of successful biosensor design studies, a growing repertoire of sensing devices, and a better quantitative understanding of sensor tunability, sophisticated and precise biosensors for a variety of small molecules will become available. This could furthermore extend the complexity of current genetic circuits and screenings to e.g., multiplexed configurations. Overall, I expect biosensors to become increasingly employed in the development of microbial cell factories.

5.3 *In vivo* mutagenesis

Since its development in 2012,[115] CRISPR-Cas9 technology has caused a massive leap in the field of genome engineering for various applications within synthetic biology. The subsequent expansion of this technology into transcriptional regulation (CRISPRi,[269] CRISPRa[270]) and base editing (CRISPR-X,[244] EvolvR[243]) methods has provided further

understanding of biological systems and enabled efficient and precise control over gene expression and genome sequences.

While the development of CRISPR-based targeted *in vivo* mutagenesis tools is still in its infancy, numerous remarkable studies show how these methods may expedite and facilitate sequence diversification of a gene of interest and permit directed evolution objectives that were previously inconceivable.

The conventional workflow of directed evolution generally consists of *in vitro* diversification, typically by epPCR, followed by library transformation, and screening or selection of improved mutants. Each of these steps is both time- and labor-intensive, thus limiting the evolutionary search scale and depth that is typically feasible with this approach.[238,239] Targeted *in vivo* mutagenesis enables the continuous, autonomous evolution of a specified locus without the need for high transformation efficiencies, thereby minimizing manual labor and expanding the sequence space that can be generated and the length of mutational pathways that may be explored.[238,239] This allows us to tackle more ambitious engineering targets than before, for example, the coevolution of two or more genes and even protein complexes or entire pathways. It could also facilitate the search for non-natural enzyme functionalities. With the help of machine learning, such experimental work could be further enhanced through the analysis of large datasets and the creation of more detailed fitness landscapes.[239]

In **paper III**, we employed a dCas9-fused adenine or cytidine deaminase to target the gene encoding the transporter protein SpMae1. While this approach resulted in enriched variants that demonstrated considerable improvements in protein functionality, further optimization of the base editing method is desired. First, the use of deaminases creates a bias towards transition mutations (A>G, G>A, C>T, T>C),[271] which restricts the overall attainable sequence space. It is thus probable that other less biased approaches, such as those based on error-prone DNA polymerases,[243] may be preferred in some cases. Second, off-target events can lead to increased mutation rates outside of the locus of interest,[239] which elevates the risk of enrichment of false positives. As shown for TadA8eV106W,[245] it is possible to engineer mutator enzymes to reduce such off-target effects. Furthermore, in some cases, negative screening or selection campaigns can be designed to eliminate false positives from the population.[211] Third, with increasing mutations in the targeted loci, gRNA-binding may become hindered, reducing the hypermutation efficiency over time. A possible solution would be to employ more promiscuous gRNA sequences or mutators able to edit adjacent regions rather than the gRNA binding site itself. Lastly, in the case of SpMae1, the functionality of the protein of interest was directly coupled to cell fitness, thus enabling a rather straightforward selection and enrichment process. However, that is not always the case. With increasing library sizes, efficient screening or selection methods become increasingly sought after. In the future, the development of more versatile selection approaches and screening platforms could alleviate this bottleneck and enable the directed evolution of a wider range of proteins or cellular properties.

5.4 Enzyme scaffolding

Spatial organization and dynamic assembly of enzymes has been suggested to play a role in pathway regulation and to provide metabolic advantages in nature. The phenomenon has been observed in different metabolic pathways, throughout all domains of life.[272] While synthetic enzyme scaffolds present a relatively new approach in metabolic engineering, some successful examples of artificial assemblies have already led to impressive improvements in product titers.[253,254,273] Enzyme assemblies have been proposed to improve chemical production via several mechanisms of action. By increasing local enzyme and intermediate concentrations, they may enhance metabolic fluxes of the pathway of interest. By sequestering compounds from the bulk aqueous phase of the cell, labile intermediates would be protected from degradation whereas toxic compounds would have a lesser effect on cell fitness. In addition, this sequestration could lower the accessibility of intermediates for competing pathways, resulting in reduced byproduct formation. In this way, enzyme assemblies may exert post-translational control over metabolic branch points. Lastly, some scaffold designs enable control over domain and enzyme stoichiometries. Due to divergent catalytic activities of heterologous enzymes, bottlenecks may arise, leading to the accumulation of intermediates and suboptimal fluxes. Scaffolding allows us to adjust enzyme stoichiometries to better balance biosynthetic pathways.

In **paper IV**, we observed byproduct formation as a major obstacle for yeast-based phloretin production. We then demonstrated that spatial recruitment of three sequential pathway enzymes using previously reported metazoan signaling protein interaction domains led to improved phloretin production in specific scaffold domain stoichiometries. The choice of domain repeats appeared to have a big impact on the production performance, as the use of other domain stoichiometries resulted in reduced titers compared to the control. In this study as well as in previous publications, the design of enzyme scaffolds has largely been based on laborious experimentation, hindered by a lack of mechanistic knowledge or predictive insight.[274] Moreover, many design factors can influence scaffolding performance, such as linker sequence, enzyme stoichiometry, order and positioning of scaffold domains, and the enzyme:scaffold expression ratio.[275] It would take a considerable amount of time and workload to determine the optimal scaffold configuration. Therefore, while this approach presents a promising complementary method to other metabolic engineering strategies, we require a much better understanding of the effects of different parameters. Furthermore, a maximum of only three consecutive pathway enzymes have been assembled in different studies so far. In theory, enzyme scaffolding could be expanded to more enzymes and longer pathways. However, this would be accompanied by higher complexity and even more degrees of freedom. In the future, more predictive models could help us to narrow it down to a feasible design space. Additionally, the possibility of establishing scaffold libraries could accelerate the identification of favorable designs. This could for instance be achieved by combinatorial shuffling of a large variety of genetic parts and subsequent high-throughput screening.

5.5 Concluding remarks

Several engineering strategies were investigated in this thesis, but many unexplored areas and opportunities remain. Protein engineering will, for example, likely be important to obtain better pathway enzymes with higher activities and specificities to improve the production of secondary metabolites. Efficient mutagenesis strategies and high-throughput library screening or selection methods are expected to expedite this process. Furthermore, computational advances in e.g., metabolic modeling and protein design will be crucial to broaden our detailed understanding of metabolic networks and thereby allow accurate prediction of engineering targets. The use of *de novo*-designed enzymes can increase the possibility of producing non-natural flavonoid derivatives and could hence expand our collection of valuable bioactive compounds. The growing standardization of synthetic biology and automation of routine laboratory tasks will, additionally, reduce the time and workload required for building and testing different designs. While most studies are currently conducted in a small number of hosts (mainly *E. coli* and *S. cerevisiae*), other organisms such as *Y. lipolytica* might provide more suitable characteristics for flavonoid production. Even so, the findings made with current model organisms will still contribute importantly as they deepen our fundamental understanding of various aspects of microbial flavonoid production. This will ultimately accelerate the establishment of industrially viable production processes. Besides enabling efficient production, it is also important to obtain better insight into the bioactivities of flavonoids in humans, and where possible, validate these through clinical trials. Increased understanding of flavonoid absorption mechanisms and bioavailability as well as the development of improved drug delivery systems will play a key role in the commercialization of flavonoids as therapeutics.

Flavonoid compounds have already been successfully industrially produced by fermentation. US-based companies such as Conagen (https://conagen.com/) and Blue California (https://bluecal-ingredients.com/) for instance are specialized in the production of natural products, including flavonoids, to be used as flavoring, coloring, and health-promoting agents. I am quite optimistic that with the continuous progress made within the fields of metabolic engineering and synthetic biology, it will be possible to progressively replace plant extraction processes with more sustainable and environment-friendly microbial cell factories.

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Looking back to when I first started at Sysbio in 2019, it almost feels like yesterday. At the same time, a lot has happened, and I wish I could hold on to some of those moments and memories for just a little longer. I have met many wonderful people, I have learned about flavonoids, yeast, and synthetic biology, and I have gone through many ups and downs that have eventually brought me to this point.

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