THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# Combining Metabolic Engineering and Synthetic Biology Approaches for the Production of Abscisic Acid in Yeast

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CHALMERS
UNIVERSITY OF TECHNOLOGY

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CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2023

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ISBN: 978-91-7905-767-1

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Doktorsavhandlingar vid Chalmers tekniska högskola

Ny serie nr 5233

ISSN 0346-718X

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Cover:

“Drawing board sketch” of the contents of this thesis.

Printed by Chalmers Reproservice
Gothenburg, Sweden 2023

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Maximilian Otto, Department of Life Sciences, Chalmers University of Technology

# Abstract

Nature presents us with a myriad of complex and diverse molecules. Many of these molecules prove to be useful to humans and find applications as pharmaceuticals, biofuels, agrochemicals, cosmetic ingredients or food additives. One highly promising natural product with a broad range of potential applications is the terpenoid abscisic acid (ABA). ABA fulfils a pivotal role in higher plants by regulating various developmental processes as well as abiotic stress responses. However, ABA is also produced in many other organisms, including humans. It appears to be a ubiquitous and evolutionary conserved signalling molecule throughout nature.

Genetically engineered microorganisms, referred to as microbial cell factories, can be a sustainable source of natural products. In this thesis, a cell factory for the heterologous production of ABA was established and optimized employing the yeast *Saccharomyces cerevisiae*.

Cell factory development is an inherently time-consuming process. As an enabling technology for subsequent work on the ABA cell factory, we expanded the modular cloning toolkit for yeast and made it more applicable for common genetic engineering tasks (Paper I). The ABA biosynthetic pathway of *Botrytis cinerea* was used to construct an ABA-producing *S. cerevisiae* strain (Paper II). The activity of two *B. cinerea* proteins, BcABA1 and BcABA2, was found to limit ABA titers. Two optimization approaches were devised for the following studies. Firstly, various rational engineering targets were explored, of which the native yeast gene *PAH1* was identified as the most promising candidate (Paper III). Knockdown of *PAH1* benefited ABA production without affecting growth. Secondly, platform strains for screening BcABA1 and BcABA2 enzyme libraries were developed, which utilize an ABA biosensor and enable a high throughput screening approach (Paper IV).

In this work, we combined metabolic engineering and synthetic biology approaches for the heterologous production of ABA, and furthermore provided tools and insights that will be useful beyond the scope of this project.

## Keywords

*Saccharomyces cerevisiae*, *Botrytis cinerea*, abscisic acid, sesquiterpenoid, cell factory, standardization, biosensor, metabolic engineering, synthetic biology

# Preface

This dissertation serves as partial fulfilment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The PhD studies were carried out between April 2018 and February 2023 at the Division of Systems and Synthetic Biology under the supervision of Verena Siewers and co-supervision of Florian David. The thesis was examined by Jens Nielsen. The research was funded by the Swedish Research Council (Vetenskapsrådet) and the Novo Nordisk Fonden.

Maximilian Otto

January 2023

# List of publications

## Papers included in this thesis

I. **Maximilian Otto***, Christos Skrekas*, Michael Gossing, Johan Gustafsson, Verena Siewers, and Florian David.
"Expansion of the Yeast Modular Cloning Toolkit for CRISPR-Based Applications, Genomic Integrations and Combinatorial Libraries."
*ACS Synthetic Biology* 10, no. 12 (2021): 3461–74.

* Authors contributed equally

II. **Maximilian Otto**, Paulo Gonçalves Teixeira, Maria Isabel Vizcaino, Florian David, and Verena Siewers.
"Integration of a Multi-Step Heterologous Pathway in *Saccharomyces cerevisiae* for the Production of Abscisic Acid."
*Microbial Cell Factories* 18, no. 1 (2019): 205.

III. **Maximilian Otto**, Michael Gossing, Florian David, and Verena Siewers.
"Engineering Yeast to Improve Heterologous Abscisic Acid Production."
*Manuscript*

IV. **Maximilian Otto**, Yasaman Dabirian, Florian David, and Verena Siewers.
"Sense and Screen-ability: Development of Tuneable, Biosensor-based Screening Platforms for Abscisic Acid."
*Manuscript*

## Other publications

- Rui Pereira, Olena P. Ishchuk, Xiaowei Li, Quanli Liu, Yi Liu, **Maximilian Otto**, Yun Chen, Verena Siewers, and Jens Nielsen.
"Metabolic Engineering of Yeast."
In *Metabolic Engineering* (editors J. Nielsen, G. Stephanopoulos, S.Y. Lee); John Wiley & Sons, Ltd, 2021; pp 689–733
- **Maximilian Otto**, Dany Liu, and Verena Siewers.
"*Saccharomyces cerevisiae* as a Heterologous Host for Natural Products."
In *Methods in Molecular Biology* (editor E. Skellam), Springer US, 2022; pp 333–367

## Contribution Summary

Paper I:

Conceived part of the research, designed part of the research, performed and analysed part of the experiments, wrote part of the manuscript.

Paper II:

Designed part of the research, performed and analysed most of the experiments, wrote most of the manuscript.

Paper III:

Conceived part of the project, designed most of the research, performed and analysed most of the experiments, wrote the manuscript.

Paper IV:

Conceived part of the project, designed most of the research, performed and analysed most of the experiments, wrote the manuscript.

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# Abbreviations

| | |
|---|---|
| ABA | abscisic acid |
| CBR | cytchrome b5 reductase |
| CPR | cytochrome P450 reductase |
| CYB5 | cytochrome b5 |
| CYP | cytochrome P450 monooxygenase |
| DAG | diacylglycerol |
| DBTL | design-build-test-learn |
| DE | double emulsion |
| DH-α-IE | 1’,4’-trans-dihydroxy-α-ionylideneacetic acid |
| DMAPP | dimethylallyl pyrophosphate |
| DNA | deoxyribonucleic acid |
| epPCR | error-prone PCR |
| ER | endoplasmic reticulum |
| FACS | fluorescence-activated cell sorting |
| FPP | farnesyl pyrophosphate |
| FRET | Förster resonance energy transfer |
| GEM | genome-scale metabolic model |
| GMO | genetically modifed organism |
| GTP | guanosine-5'-triphosphate |
| HFE | hydrofluoroether |
| HPLC-MS | high-performance liquid chromatography – mass spectrometry |
| IPP | isopentenyl pyrophosphate |
| MEP | 2-C-methyl-D-erythritol 4-phosphate |
| MetS | metabolic syndrome |
| MoClo | Modular Cloning |
| MVA | mevalonate |
| ori | origin of replication |
| PA | phosphatidic acid |
| PCR | polymerase chain reaction |
| PL | phospholipids |
| PP2C | protein phosphatase 2C |
| PYR/PYL | pyrabactin resistance / pyrabactin resistance like |
| RNA | ribonucleic acid |
| sdDE-FACS | single droplet double emulsion fluorescence-activated cell sorting |
| SE | single emulsion |
| STC | sesquiterpene cyclase |
| T2D | type-2-diabetes |
| TAG | triacylglyceride |
| TF | transcription factor |
| TRY | titer, rate, yield |
| Y2H | yeast two-hybrid |
| α-IAA | α-ionylideneacetic acid |
| α-IE | α-ionylideneethane |

# About this thesis

Hello there! This is a short note on how this thesis is structured. During my PhD studies I dipped my toes in a variety of topics, ranging from classical metabolic engineering over standardized toolkits to biosensors and microfluidics. In this thesis, I hope to present the different topics in an accessible way, focusing on a broader perspective instead of the details. In-depth analysis can be found in the appended research articles; however, the thesis should be comprehensible without reading them.

Part I of this booklet contains a general introduction, including some historical perspectives. The research papers are presented in Part II, starting with a brief primer on the topic, followed by a summary of the main results as well as a broad discussion of the findings. Part III puts the papers in context and provides future perspectives.

*“Where is my mind? Where is my mind? Where is my mind?”*

– *Where Is My Mind* by *Pixies*

# Part I: Background

# Engineering life

## Biology – An ancient science

Biology is the study of life. The term “Biology”, in the sense that we use it today, was coined in 1800 by the physiologist Karl Friedrich Burdach (Burdach, 1800). Humans have studied life long before Burdach of course. Greek natural philosophers, especially Aristotle (384 - 322 BC), are often credited as the first to have investigated living organisms using a systematic approach resembling modern science (Lennox, 2021). However, written records focused on anatomy, physiology and medicine from Egypt, Mesopotamia, India and China predate ancient Greece by centuries and Greek philosophers likely built on a substantial foundation of knowledge (Magner, 2002). In fact, we have studied biology in one way or another since the very beginning of humanity, and our evolutionary predecessors presumably did too. Knowledge about plants as food sources or understanding their healing properties was essential for hunter and gatherers, and so was studying for example the migration patterns of animals (Magner, 2002). Over time, biological insights led to major advances in agriculture, animal husbandry and medicine.

In the last century, we started to investigate biological processes on a molecular level. This has led to ground-breaking discoveries and paradigm shifts, like the identification of DNA as the carrier of genetic information (Hershey and Chase, 1952), the elucidation of the molecular structure of DNA (Watson and Crick, 1953; Maddox, 2003) and the postulation of the central dogma of molecular biology (Crick, 1970). These insights allowed us to not just observe and understand biology, but to eventually predict and even engineer it. They are the bedrock for numerous new scientific disciplines, including synthetic biology and metabolic engineering, two disciplines central to my research.

Even taking its lengthy history into account, biology appears to have never been more relevant than today. The 21$^{st}$ century has been heralded as “the century of biology” (Venter and Cohen, 2004). Its opportunities have been identified by governments and international organisations alike and there is a global pivot towards a more sustainable “bio-based” economy. Current and future research is predicted to have large implications for human health, nutrition, animal welfare, ecosystems and the global climate.

## Genetic engineering – Copy and paste DNA

Scientists tinkering with biology have long fascinated authors and readers. Early science fiction novels go back to the 19th century, with Mary Shelly's *Frankenstein* (1818) and H. G. Wells' *The Island of Doctor Moreau* (1896) telling the regretful stories of (too) curious scientists. More recent examples include *Brave New World* by Aldous Huxley (1932), *Jurassic Park* by Michael Crichton (1990) and *Never Let Me Go* by Kazuo Ishiguro (2005), which were clearly influenced by concurrent developments in modern genetics and genetic engineering [*]. Genetic engineering has been the basis of all the research included in this thesis. This subchapter will provide a short overview about its concepts and applications.

Genetic engineering can be defined as:

> *" […] the intentional manipulation of an organism's genetic material using tools that cut, move, and reattach (recombine) DNA segments within and across different organisms"*
>
> – Presidential Commission for the Study of Bioethical Issues (2010)

Fundamental discoveries about evolutionary highly conserved biological principles paved the way for genetic engineering to be a feasible and worthwhile endeavour. Nowadays some of these discoveries seem so self-evident that it is difficult to imagine a view on biology without them. Two such discoveries are that the genetic code is close-to universal in living organisms (Crick, 1968; Osawa et al., 1992) and that there is generally a unidirectional flow of information from DNA over RNA to protein, described in the central dogma of molecular biology (Figure 1) [†] (Crick, 1970). The fact that these principles are universal in living organism implies that we can transfer DNA from one organism to another without major alterations, and that basic design principles and technologies are broadly applicable in different species.

Various ways to "cut, move and reattach" DNA have been developed and used since the 1960s, for example in the form of restriction enzymes, transformation/transfection protocols and ligase reactions. Essential verbs for genetic engineering also include "reading", "writing"

[*] The term genetic engineering was coined by scientists in the late 1940s and was popularized in fictional literature by Jack Williamson in his 1951 Sci-Fi novel *Dragon's Island* (Stableford, 2004).

[†] The genetic code is remarkably conserved even between the different kingdoms of life. However, there are slightly variations between organisms and even within organelles (Barrell et al., 1979).

and “copying”, as in sequencing (Maxam and Gilbert, 1977; Sanger et al., 1977), synthesizing DNA (Beaucage and Caruthers, 1981; McBride and Caruthers, 1983) and performing PCRs (Saiki et al., 1985). The most recent technology that has taken the genetic engineering community by storm are CRISPR/Cas systems (Jinek et al., 2012). They have also been essential for my research and are further detailed in Paper I. A timeline of breakthrough discoveries and enabling technologies in the field are illustrated in Figure 2.

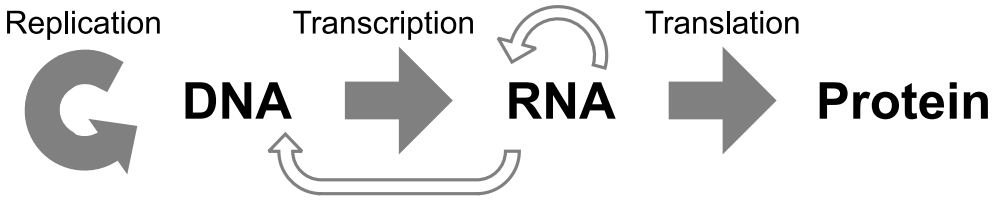



Figure 1: The central dogma of molecular biology (Crick, 1970). Large grey arrows indicate the universal transfer of information in biological systems. Small white arrows indicate exceptions to the universal information flow, e.g. via reverse transcription (RNA to DNA) or RNA replication (RNA to RNA).

A cell’s phenotype is dependent on its genotype and environmental stimuli. Consequently, genetic engineering can be used to change a cell’s behaviour and traits. Historically speaking, manipulation of DNA has been an indispensable tool to deepen our understanding of biological processes, for example in gene knockout studies. Nonetheless, it rapidly became apparent that cells can be reprogrammed with specific goals in mind, for instance for gene therapies (Wirth et al., 2013), resilient crop varieties (Zaidi et al., 2020) or even bioart (Dumitriu et al., 2021). Another major application of genetic engineering is the production of complex biomolecules in modern biotechnology. The next section will specifically address the use of microbial cell factories.

However, before we move forward there is one additional remark about genetic engineering. The reader likely noticed that most of the works of literature mentioned in the introductory sentences of this section are horror or dystopian novels, that deal with the consequences of new technologies. When it comes to genetic engineering of animals, including humans, the ethical implications cannot be overstated (Normile, 2018). This being said, my research concerned single-cell, non-pathogenic organisms in controlled laboratory environments. A more extensive and comprehensive discussion is outside the scope of this thesis, but I can

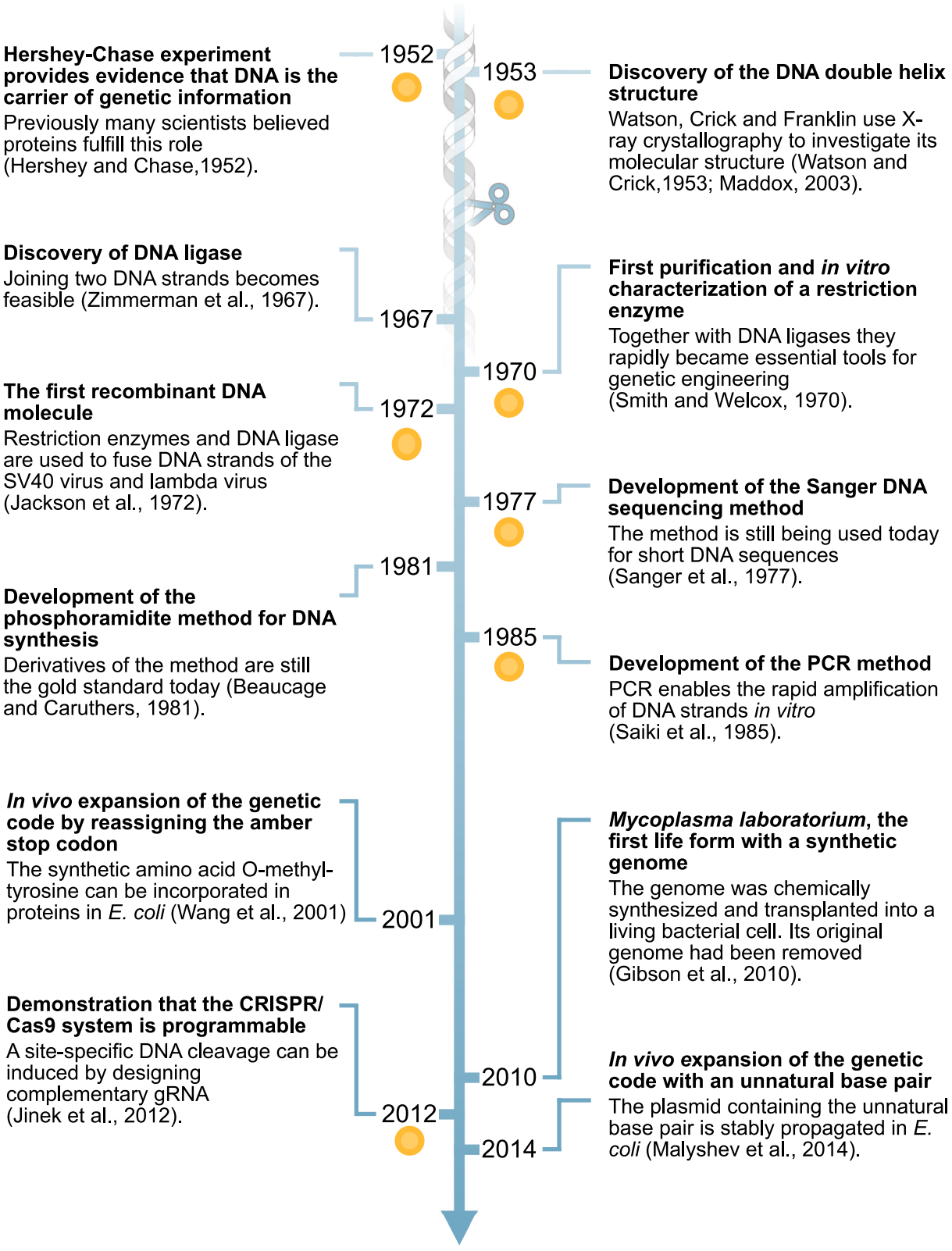



Figure 2: Historical perspective on genetic engineering. The timeline provides an overview of important events and breakthroughs in the field over the last 70 years. "Gold medals" indicate research associated with a Noble prize. The timeline is not comprehensive and represents the subjective opinion of the author.

refer the reader to de Graeff and co-workers (2019) or Niemiec and Howard (2020) for further reading.

## Microbial cell factories – Ferment everything, from beer to explosives

Fermentation of food and drink has been a part of human culture for millennia with evidences going back to 7000 B.C. (McGovern et al., 2004). By providing bacteria, yeasts and moulds with nutrients and favourable growth conditions, they in turn provided and keep providing us with longer-lasting foods containing additional nutrients and flavour, as well as intoxicating beverages. Initially humans utilized fermentations while being oblivious about what was happening on the microscopic scale. Until the mid-1800s it was generally assumed that fermentation is a spontaneous and purely chemical process (Schlenk, 1985). In 1837, three scientist, Theodor Schwann, Friedrich Traugott Kützing and Charles Cagniard de la Tour, independently provided evidence that living yeast cells are essential for alcoholic fermentation (Kützing, 1837; Schwann, 1837; Schlenk, 1985). This research was highly influential for Louis Pasteur's research regarding germ theory and the famous swan neck flask experiment, proving that air-born particles (microbes) caused fermentation and not the air itself (Berche, 2012).

In the first half of the 20$^{th}$ century, the stage for modern biotechnology was set when fermentation was used for the industrial production of chemicals and medicines. The bacterium *Clostridium acetobutylicum* was used during the First World War for the production of acetone, which was required for manufacturing ammunition (Sauer, 2016). During the same time period, the yeast *Saccharomyces cerevisiae* was used for the production of glycerol, an essential ingredient for nitroglycerine explosives (Semkiv et al., 2020). Mass production of penicillin, extracted from moulds of the genus *Penicillium*, started in the early 1940s (Quinn, 2013). Later in the century, it became apparent that microbes could be genetically engineered and thereby optimized as production facilities, so called microbial cell factories. Microbes can be engineered to produce higher amounts of native molecules, to produce molecules that they would not naturally produce, to be able to use different carbon sources or to improve their robustness to industrial environments. This opened seemingly unlimited opportunities for the efficient and sustainable production of complex molecules in

industrial settings. Pharmaceuticals, agrochemicals, cosmetic ingredients, nutritional supplements, biofuels, and commodity chemicals can be produced in cell factories (Pereira et al., 2021). Different host organisms, also called chassis, are being used as microbial cell factories and the choice between different hosts is often product-specific and dependent on the cultivation process (Xu et al., 2020). The yeast *S. cerevisiae* has proven to be an incredibly versatile and competent host organism for cell factories.

## *Saccharomyces cerevisiae* – The case for yeast

A world without *Saccharomyces cerevisiae*, also known as baker's yeast, is hard to imagine. It ferments sugars and in turn produces $CO_2$ and ethanol, making it an essential ingredient in culinary staples such as bread, beer, wine and even chocolate (Parapouli et al., 2020). Its centrality in human life has made *S. cerevisiae* one of the most well studied organisms on the planet and a favourite plaything for scientists. Yeast (in this thesis referring to *S. cerevisiae* unless indicated otherwise) is a eukaryotic model organism in fundamental research (Botstein and Fink, 2011), but is also used in applied science, for example for drug discovery (Hughes, 2002). Furthermore, it is presumably the most commonly used eukaryotic host for cell factories in academic research [*].

Many cellular processes and structures are highly conserved between different eukaryotes. A surprising amount of insights gathered from studying yeast is transferable to humans, e.g. regarding the function of specific genes or regulatory pathways (Smith and Snyder, 2006; Nielsen, 2019). In addition, yeast is simpler to work with than human or mammalian cell lines and the experimental throughput is higher. Multiple Nobel prizes have been awarded to researchers investigating physiological processes in yeast that are relevant for human physiology (Starr, 2016).

[*] Where does *S. cerevisiae* come from? Research of the organism's pangenome suggests that China was *S. cerevisiae*'s cradle (Peter et al., 2018). It appears to have been domesticated afterwards at multiple locals and timepoints independently of each other.

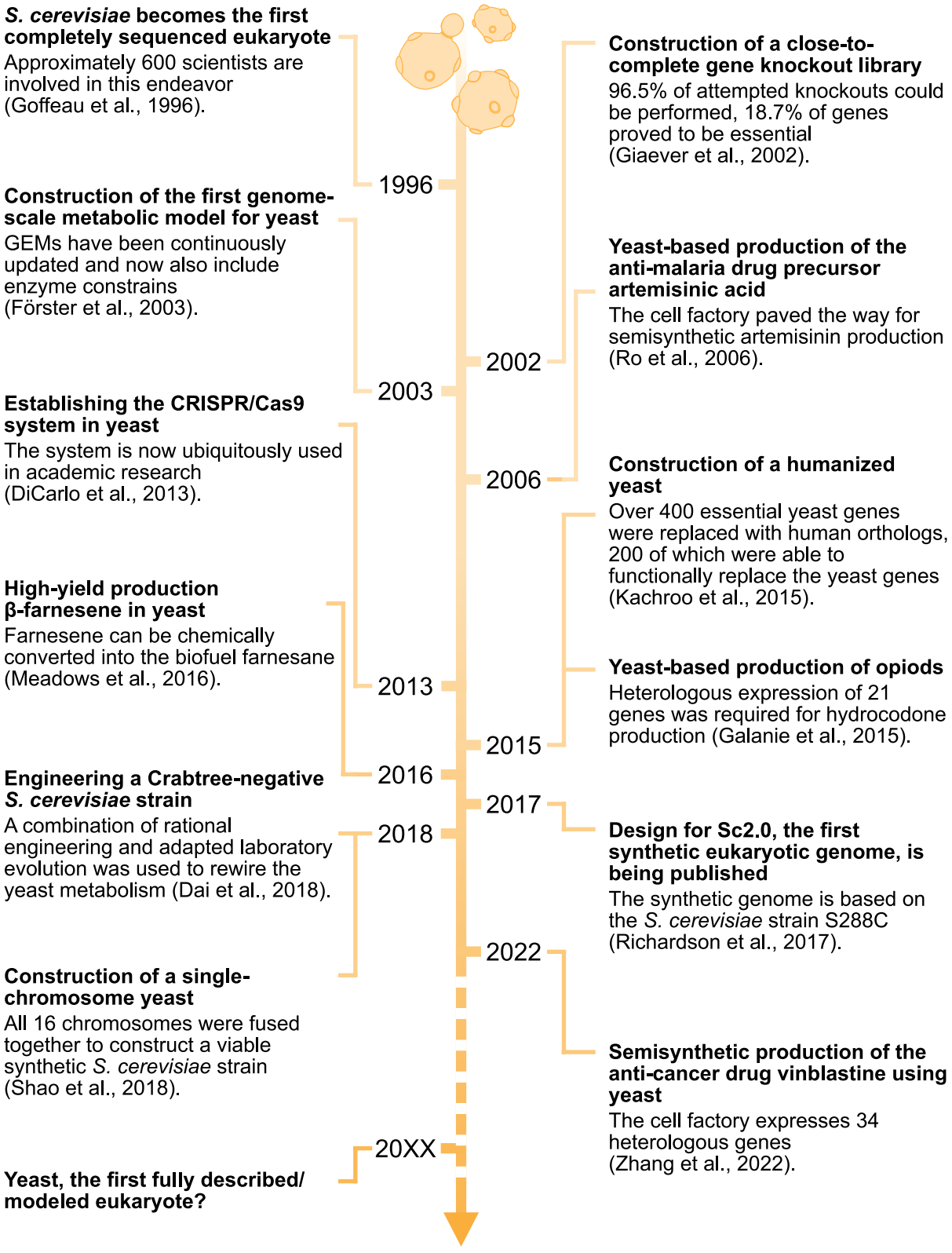



Figure 3: Historical perspective on *Saccharomyces cerevisiae* research. The timeline highlights important studies in basic science as well as applied research focusing on synthetic biology and metabolic engineering applications. The timeline is not comprehensive and represents the subjective opinion of the author.

Besides the mentioned production of glycerol during World War I, yeast has been and is being used for the industrial production of bioethanol [*]. With the advances in genetic engineering technologies, *S. cerevisiae* has been modified to produce non-native products as well. Prominent examples for products sourced from yeast cell factories on an industrial scale include insulin, hepatitis B vaccine ingredients, human serum albumin and the antimalaria drug precursor artemisinic acid [†] (Nielsen, 2013; Kung et al., 2018). The fact that yeast is a eukaryote is also advantageous for its use in biotechnology. Eukaryotes are compartmentalized and enzymes are often bound to the ER membrane or are localized in other organelles. Generally speaking, heterologous genes from other eukaryotes can be functionally expressed in yeast when expression in prokaryotes is challenging (Paddon and Keasling, 2014). Yeast has several other key advantages that make it particular suitable as a cell factory. For once, it is easy to genetically engineer due to its highly efficient homologous recombination machinery. Compared to other eukaryotes it grows fast, requires only simple media composition and is resilient to the harsh physical conditions in industrial bioreactors. *S. cerevisiae* can thrive in acidic conditions, which is especially relevant for the production of organic acids since cultivation at low pH significantly reduces the downstream costs for obtaining the free acid form (Chen and Nielsen, 2016). Furthermore, yeast is non-pathogenic and many products sourced from the organism have the generally-recognized-as-safe (GRAS) designation. Highly sophisticated genome-scale metabolic models (GEMs) have been developed for yeast (Lu et al., 2022). The models illustrate the vast amount of knowledge about the organism that has been gathered over the last century. GEMs have been successfully applied for the computational design of cell factories (Ishchuk et al., 2022). Figure

[*] Bioethanol production became especially relevant during the 1970s energy crisis (Bajpai, 2021). In the recent two decades the bioethanol industry has again seen substantial growth due to increasing concerns about the environmental impacts of fossil fuels. Calling first generation bioethanol a “sustainable” fuel alternative is however largely misleading since it requires intensive agriculture on a large scale, often going hand-in-hand with the destruction of ecosystems (Elshout et al., 2019).

[†] Semi-synthetic artemisinin production (in which artemisinic acid is produced in yeast and then chemically converted to the active drug artemisinin) (Ro et al., 2006; Paddon et al., 2013) has generally been celebrated as a groundbreaking accomplishment and is a poster child for the industrial adoption of cell factories. However, the project has arguably not lived up to the expectations and excessive publicity around it. Previously, artemisinin had been sourced from the plant natural producer *Artemisia annua* and yeast-based production was meant to reduce costs and lead to more consistent yields. Sanofi started the commercial semi-synthetic production of artemisinin in 2014, however it was stopped shortly after due to multiple reasons, including the high costs of the chemical conversion step at scale, reduced costs of agricultural sourced artemisinin and a stagnant demand for the drug (Peplow, 2016; Kung et al., 2018; Czechowski et al., 2020). Nonetheless, the cell factory engineering efforts are an exceptional accomplishment on their own.

3 showcases various milestones in fundamental and applied yeast research. Over recent years, numerous proof-of-concept yeast cell factories have been developed in academic research, many of which have great potential for future adoption in industrial processes. Still, comparatively few yeast cell factories are used commercially. This is largely due to difficulties reaching commercially viable productivities. The next section addresses this inherent challenge in the field of metabolic engineering.

## Metabolic engineering and synthetic biology – same same, but different

The research in this thesis combines metabolic engineering and synthetic biology approaches. The two disciplines are closely related and here I aim to highlight their commonalities and differences in terms of approaches, challenges and goals.

Metabolic engineering can be defined as:

> *"[...] the science of rewiring the metabolism of cells to enhance production of native metabolites or to endow cells with the ability to produce new products"*
>
> – Nielsen and Keasling (2016)

A major goal is to redirect the metabolic flux (the turnover rate of metabolites) towards the cell factory's product and achieve high enough titers, rates and yields (referred to as TRY metrics) for the cell factory to be economically viable and sustainable. Cellular metabolism is organised in a bow-tie structure (Figure 4). Cells can use a wide range of energy and carbon sources. These substrates are subsequently transformed into a very limited number molecules, including energy carriers, redox equivalents and 12 universal precursor metabolites (Nielsen, 2003; Nielsen and Keasling, 2016). The precursor molecules subsequently serve as building blocks to synthesize all primary metabolites, secondary metabolites and macromolecules. Production and consumption of energy and precursor supplies need to be balanced and regulated on multiple levels to ensure cell growth and homeostasis even under changing extrinsic conditions (Nielsen and Keasling, 2016). The highly conserved nature of cellular processes is beneficial for genetic engineering. However, the fact that the central carbon metabolism is conserved to a similar degree and tightly regulated is an immense challenge for metabolic engineering. Rewiring metabolism to produce high quantities of the molecule of interest and to limit the production of biomass or

unwanted by-products is inherently difficult. This is however not the only engineering target. Enabling the utilization of alternative carbon sources or increasing robustness of the cell factory are also essential for industrial application (Gong et al., 2017; Šuchová et al., 2022).

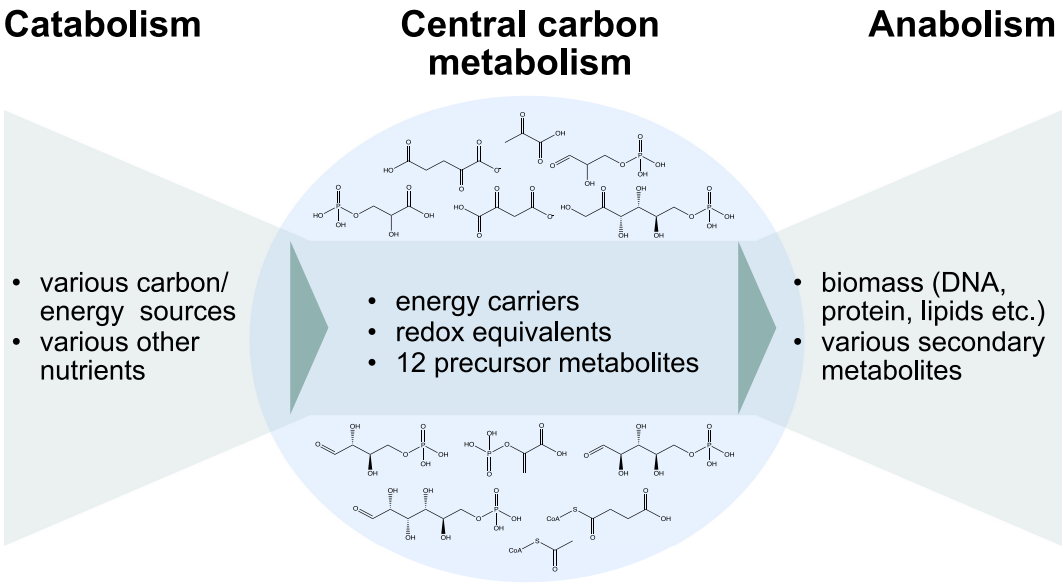



Figure 4: The bow-tie structure of metabolism. Structurally diverse energy and carbon sources are transformed into a very limited number of energy carriers, redox equivalents and 12 precursor metabolites, which are subsequently transformed into various forms of biomass, primary metabolites and secondary metabolites. The 12 precursor metabolites are: glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, erythrose-4-phosphate, glyceraldehyde-3-phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, oxaloacetate, 2-oxoglutarate, acetyl-CoA and succinyl-CoA.

Even with the sophisticated GEMs available for *S. cerevisiae*, we are still not able to sufficiently predict the outcomes of specific genetic modifications due to the mentioned complex regulation and interconnectivity. As a result of this, metabolic engineering is a time-consuming, iterative process following the so-called Design-Build-Test-Learn (DBTL) cycle (Nielsen and Keasling, 2016). The Design phase initially encompasses the choice of host chassis and afterwards the choice of genes to introduce, knockout or overexpress. In the Build phase genetic engineering methods are used to modify the organisms accordingly. The cell factories are then tested using low- or high-throughput technologies, like chromatography or fluorescence-activated cell sorting (FACS). After the analysis the newly learned insights inspire the next design choices. The first DBTL cycle could for example include literature research on a heterologous pathway (Design), transfer of the suggested pathway enzymes to the host chassis (Build), determining if the molecule-of-interest is being produced (Test) and

elucidating which enzymes are essential or beneficial for production (Learn). The goals of the next cycles could be to find the production bottleneck and to alleviate it.

Synthetic biology is closely related to metabolic engineering with many overlapping aspects (Nielsen and Keasling, 2011). Both are based on genetic engineering and (for now) have to rely on the DBTL cycle due to the complexity of biological systems. In both disciplines, biological machines are designed and constructed to fulfil specific purposes. A consensus definition of synthetic biology is arguably missing, likely because the research field is younger and its boundaries are less well defined than metabolic engineering. Common motives when synthetic biology is described in the literature include:

- applying traditional engineering principles, such as standardisation, to biology
- characterising genetic parts and assembly of the parts into genetic circuits
- producing and utilizing molecules that do not occur in nature
- developing biological machines on a molecular or cellular level
- designing and constructing synthetic genomes and synthetic minimal cells

(Benner and Sismour, 2005; Keasling, 2008; Purnick and Weiss, 2009; Schwille, 2011; Nielsen and Keasling, 2011; Meng and Ellis, 2020). Overall, the aim of synthetic biology is to construct useful and programmable biological machines in similar ways that electrical engineers are building electronic devices. These biological machines arguably include cell factories [*], but their applications reach beyond the sole production of molecules. They can be used as biological sensing devices (Hicks et al., 2020), drug delivery systems (Anderson et al., 2006), organoids (Hofer and Lutolf, 2021), biological computers (Pandi et al., 2019) or for bioremediation purposes [†] (Cases and de Lorenzo, 2005).

Naturally, there are synergies between metabolic engineering and synthetic biology (Nielsen and Keasling, 2011; Stephanopoulos, 2012). The research in this thesis illustrates some of these synergies, using a cell factory for the production of abscisic acid (ABA) as an example.

[*] Guo and co-workers provided an interesting perspective of how synthetic biology and metabolic engineering can be combined in future cell factories (Guo et al., 2018). They constructed a hybrid system in which inorganic chemistry is used to harvest light. The resulting photoexcited electrons are used by a genetically engineered *S. cerevisiae* strain to produce NADPH.

[†] Bioremediation is sometimes also included as a metabolic engineering discipline (Sharma and Shukla, 2022), further illustrating the blurry line of the disciplines

The border between the disciplines is blurry at best. Both have been utilized to different degrees in the research papers of this thesis. Figure 5 aims to categorize the four papers on a subjective scale from metabolic engineering to synthetic biology.

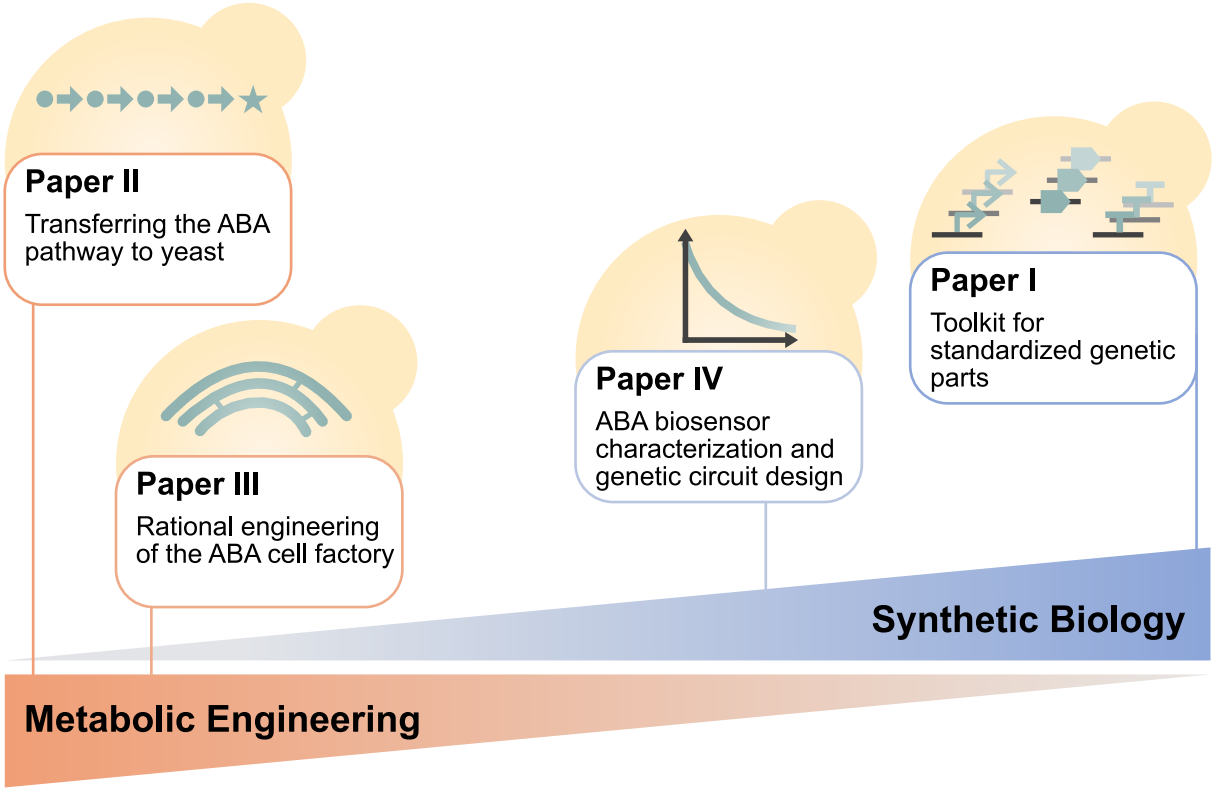
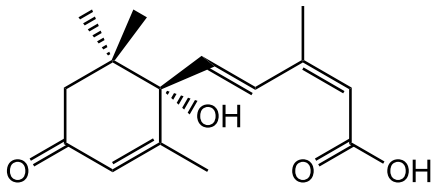



Figure 5: Is this Synbio yet? In this thesis, approaches of two closely related disciplines, metabolic engineering and synthetic biology, were combined for the heterologous production of ABA. This schematic illustrates to what degree the research papers utilized one or the other discipline. The toolkit developed in Paper I is an example for a synthetic biology project, whereas Paper II was a "classical" metabolic engineering project. Similarly, Paper III followed a metabolic engineering approach; nonetheless, standardized genetic parts were used in this project. Synthetic biology tools like biosensors and a genetic circuit were used in Paper IV; however, the main objective was to improve productivity of a cell factory. This figure was inspired by Figure 1 from Nielsen and Keasling (2011).

# Abscisic acid

When I started my PhD studies, I was excited to have a project at the intersection of metabolic engineering and synthetic biology. I did not know much about abscisic acid (ABA) but it sounded like an interesting molecule to work on. The more I read about ABA the more intrigued I became by its ubiquity and its applications. The following chapter aims to summarize ABA's unique features without being overly exhaustive.




Abscisic acid (ABA)

Figure 6: Chemical structure of (*S*)-(+)-2-cis,4-trans ABA

## ABA the phytohormone – Plants get stressed too

In 1963, a growth-inhibiting compound was isolated from birch tree buds, which was subsequently called dormin (Eagles and Wareing, 1963). In the same year, a molecule with the formula $C_{15}H_{20}O_4$, extracted from cotton fruit, was shown to cause the abscission of leaves, and consequently was named abscisin II (Ohkuma et al., 1963). Later it was shown that dormin and abscisin II were actually the same molecule and its name was changed to abscisic acid (ABA) to better represent its chemical structure [*] (Figure 6) (Addicott et al., 1968). Since ABA is derived from isoprene it is part of the terpene family of natural products, specifically sesquiterpenoids since it has 15 carbon atoms (the -oid suffix indicates that it contains functional groups such as hydroxyl groups). The naturally occurring enantiomer is (*S*)-(+)-ABA, predominantly found in the 2-cis, 4-trans configuration.

[*] ABA does likely not directly induce the abscission of leaves, but rather controls senescence and stress responses that subsequently lead to abscission (Finkelstein, 2013). The name dormic acid had been used for ABA before and, in retrospect, might have been a better choice.

In the decades following its discovery, ABA was shown to fulfil crucial roles in plant growth and developmental processes (reviewed in Cutler et al., 2010) and it is now presumed that ABA is present in all vascular plants [*]. The molecule joined the ranks of other phytohormones such as auxins, cytokinins, ethylene, gibberellins, salicylic acid and jasmonic acid. Besides seed dormancy it is involved in germination, flowering, fruit ripening, closure of stomata and senescence to name a few (Finkelstein, 2013; Leng et al., 2014; Martignago et al., 2020). ABA is also a central regulator of abiotic stress responses in plants, such as drought, cold, UV, salt and heavy metal-induced stress (reviewed in Finkelstein, 2013 and Hu et al., 2020). More recent studies demonstrate that ABA also increases resilience to certain biotic stresses (Lee and Luan, 2012; Olds et al., 2018); however, its impact appears to be more nuanced and potentially dependant on the pathogen (Stevens et al., 2022). Olds and co-workers (2018) hypothesized that in some cases ABA itself could act as a plant defence molecule, since herbivore attacks trigger ABA production in affected leaves (Schäfer et al., 2011) and ABA in turn affects the reproductive ability of some insects (Visscher, 1980).

The mentioned processes are a testimony of how central ABA is in plant biology. ABA is also produced in cyanobacteria and most of the investigated species of algae, however much less is known about the physiological role of the molecule in these organisms (Hartung, 2010). It has been hypothesized that ABA biosynthesis originally occurred in algae and cyanobacteria, but ABA signalling only became crucial after plants colonized land (Hartung, 2010). This would be in line with ABA's essential role in the response to water scarcity. Nonetheless, it is noteworthy that the ABA signalling network is highly complex with effects depending on plant tissue and varying between species. ABA works agonistically with or antagonistically against other plant hormones and the cross-talk between them further complicates investigations (Verma et al., 2016; Berens et al., 2017; Lievens et al., 2017; Emenecker and Strader, 2020). Even after roughly 60 years of research open questions remain about ABA signalling in plants.

[*] Refresher on botany: vascular plants, historically referred to as "higher plants", are plants that have xylem and phloem as dedicated transport tissues for water and nutrients, respectively. It includes seed-bearing plants but also plants that reproduce via spores, such as ferns for example. Over 300.000 species of vascular plants have been identified so far (Christenhusz and Byng, 2016).

## ABA in other organisms – Not a phytohormone after all?

A remarkable number of organisms were found to produce ABA and the list seems to grow longer every year. ABA-producing organisms have so far been found in six of the seven kingdoms of life [*], with the exception of Archaea (Hauser et al., 2011). Compared to plants, the role of ABA is less central in other kingdoms, but it still fulfils important functions and shows remarkable physiological effects.

Plant-associated microorganisms produce ABA, likely either to communicate in symbiotic relationships or to hijack the ABA-mediated response in pathogenic infections. Examples of such microorganisms include nitrogen-fixing rhizobacteria (Cohen et al., 2009) and plant pathogenic fungi such as *Magnaporthe oryzae* (Spence et al., 2015). ABA is also produced in multicellular animals such as marine sponges, which are evolutionary speaking among the oldest animal lineages (Olds et al., 2018; Gross, 2021). Further examples for ABA-producing organisms include the unicellular parasite *Toxoplasma gondii* (Nagamune et al., 2008), the European honey bee *Apis mellifera* (Negri et al., 2015), and mammals, including *Homo sapiens* (Bruzzone et al., 2007).

From what we know so far, ABA seems have maintained a stress-related role in many microbes and animals, to the point where it has been referred to as an "alarmone" (Scarfì et al., 2009; Sakthivel et al., 2016). For instance, supplementation of ABA made honey bee larvae more resilient to cold temperatures (Ramirez et al., 2017). Heat-stress increased ABA biosynthesis in marine sponges (Zocchi et al., 2001). Accordingly, increased ABA production was observed in human granulocytes after heat-stress (Bruzzone et al., 2007). Furthermore, it has been suggested that there is a conserved ABA-mediated response to UV-B radiation in plants and animals (Tossi et al., 2012).

In mammals, ABA is produced in various cell types, including but not limited to granulocytes (Bruzzone et al., 2007), macrophages (Magnone et al., 2012), pancreatic β-cells (Bruzzone et al., 2008) and mesenchymal stem cells (Scarfì et al., 2008). ABA appears to be ubiquitously present in mammalian organs and has been detected in the heart, lungs, liver, kidney and blood plasma, with the highest ABA concentration found in the brain (Le Page-Degivry et al.,

[*] Since Carl Linnaeus the kingdoms of life have been redefined ever so often. The seven kingdoms of life according to Ruggiero et al. (2015) are Bacteria, Archaea, Protozoa, Chromista, Plantae, Fungi and Animalia.

1986; Bruzzone et al., 2012). Research in murine animals and humans has shown that ABA is tightly interlinked with inflammation and glucose metabolism but is also involved in cell proliferation.

Interestingly enough, ABA can act as both, a pro- and anti-inflammatory agent. *In vitro* studies report activation of immune cells while anti-inflammatory effects are generally observed in animal models (reviewed in Sakthivel et al. (2016) and Lievens et al. (2017)). Regarding glucose metabolism, Bruzonne and co-workers (2008) describe a signalling cascade in pancreatic β-cells in which glucose stimulated ABA secretion, and ABA subsequently stimulated insulin secretion. *In vitro* assays furthermore showed that ABA induces GLUT4-mediated glucose uptake in myoblasts and adipocytes (Bruzzone et al., 2012). The blood glucose stabilizing effect of ABA has been confirmed in human subjects (Magnone et al., 2015). Reports also show that ABA can positively affect cell proliferation of mesenchymal stem cells and hematopoietic progenitors (Scarfì et al., 2008, 2009; Malara et al., 2017).

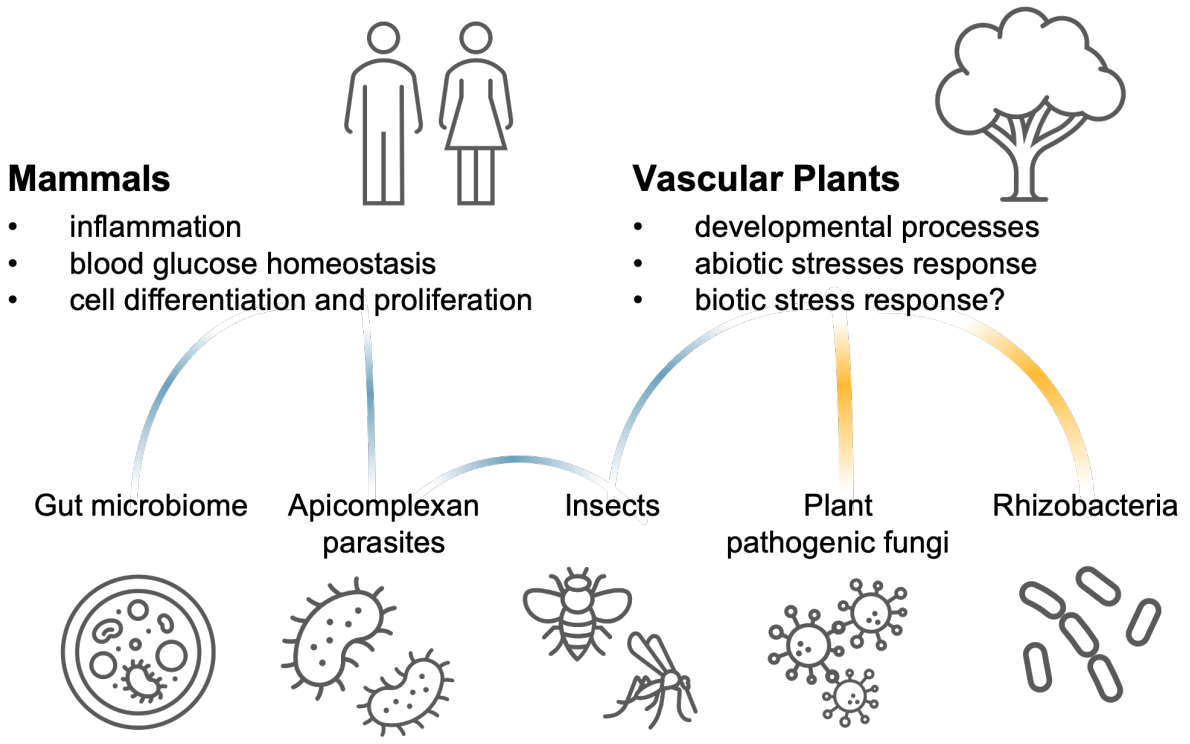



Figure 7: Processes regulated by ABA and its use in inter-species interactions. ABA-regulated processes in mammals and vascular plants are summarized. ABA-mediated interactions between organisms are shown as blue or orange lines. Orange lines indicate proven interactions, blue lines indicate hypothesized interactions. All listed groups of organisms have species that produce ABA, with the exception of the gut microbiome for which ABA production has not been proven yet. To our current knowledge all mammals and vascular plants produce ABA.

Its biological ubiquity and conserved nature make ABA a particularly suitable candidate for inter-species communication. Besides endogenously produced ABA and ABA absorbed from fruit and vegetables, the gut microbiome could be a potential third source of the molecule (Kim et al., 2020). ABA was shown to block bitter-taste receptors in the human gut (Pydi et al., 2015) and it has been suggested that ABA might mediate host-microbiome interactions (Kim et al., 2020). However, so far it is not known if gut microbes produce significant amounts of ABA.

With the on-going research in this field, it is arguably misleading to call abscisic acid a phytohormone. The molecule might not be as essential in animals as it is in plants; however, many important processes on a cellular and systems level are facilitated by it. Figure 7 summarizes the effects of ABA in mammals and vascular plants, and shows examples of (potential) ABA-mediated interactions between species.

## ABA applications – A molecule to save the world

As outlined above, ABA shows a large repertoire of physiological effects in many different organisms. Its potential applications are hence similarly broad and include medicine, nutrition and agriculture (Figure 8). ABA could play a role in solving current societal challenges such as food security, changes in diets and the surge of lifestyle-related diseases.

In the coming century climate change will severely affect agricultural yields (Devereux and Edwards, 2004). More severe and longer-lasting periods of abiotic stress, such as drought and heat stress, can acutely endanger food supplies. Exogenous application of ABA was shown to improve abiotic stress resistance in various crop plants (reviewed in Dar et al., 2017). The molecule could be a valuable natural agrochemical that can be deployed according to weather predictions. ABA could also be used to inhibit early seed germination potentially resulting in crop loss or reduced yields. Applications of ABA that are more plant-specific include the regulation of fruit colouration, potentially also affecting the nutritional value by increasing anthocyanin production in the fruit (Ban et al., 2003; Karppinen et al., 2018; Oh et al., 2018). Two products validate the commercial interest in ABA as a plant growth modulator (BioNik™ and ProTone®, both from Valent BioSciences).

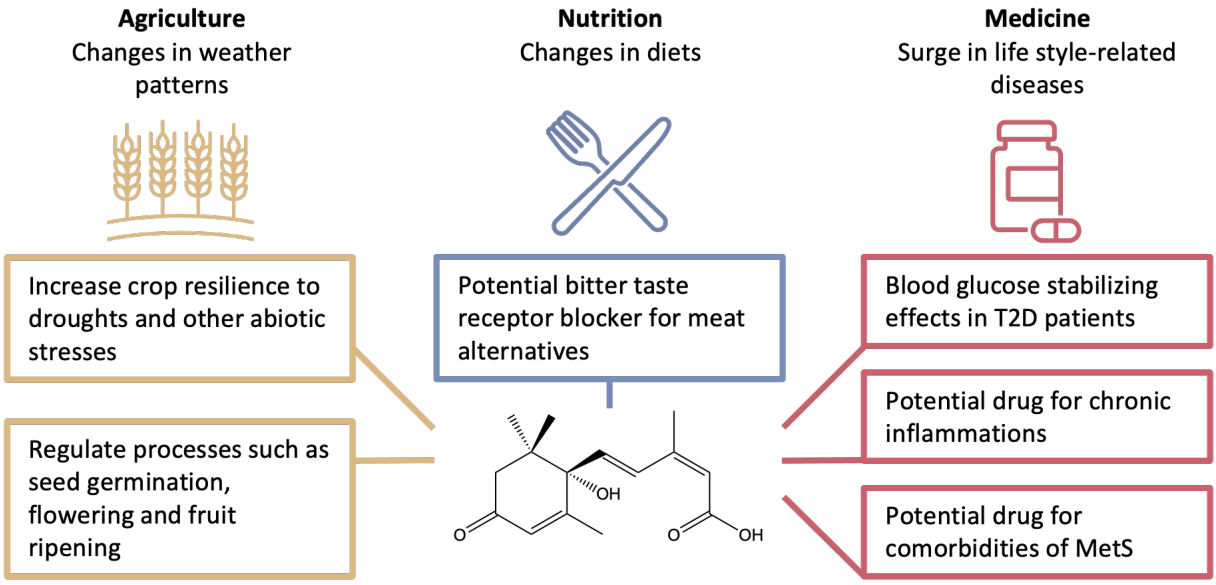



Figure 8: Applications of ABA with respect to current societal challenges. Global warming will lead to changes in weather patterns with detrimental effects on agriculture. ABA could be applied to crops according to weather predictions to prevent extensive crop loss, e.g. due to droughts or early seed germination. Diets are changing due to increased concerns about livestock-based carbon emissions and animal welfare. ABA could be used as a bitter taste receptor blocker in meat alternatives. Life style-related diseases, like type-2-diabetes (T2D) and metabolic syndrome (MetS), have surged in recent decades. ABA could become a drug for such diseases or their comorbidities.

As a bitter-taste receptor blocker, ABA could potentially be used as a food additive, e.g. in meat alternatives (Pydi et al., 2015; Singh et al., 2022). ABA might be a particularly appealing choice compared to other bitter blockers since it can provide additional nutraceutical benefits to the product as outlined below.

Phytohormones have a long history in medicinal applications. The most prominent example being salicylic acid and its synthetic ester acetylsalicylic acid, commonly known as Aspirin [*]. ABA's anti-inflammatory properties were confirmed in animal models for chronic inflammatory diseases or inflammation-associated diseases. Noteworthy examples include obesity-related inflammation (Guri et al., 2007), arteriosclerosis (Guri et al., 2010) and inflammatory bowel disease (IBD) (Guri et al., 2011). In a murine influenza model, ABA supplementation reduced lung tissue damage, apparently not by affecting the virus directly but by inhibiting the host immune response and preventing a "cytokine storm" (Hontecillas et al., 2013). *Plasmodium* infections, such as malaria, can cause serious damage to the liver

[*] Plants rich in salicylic acid seemingly have been used for medical purposes already 30.000 years ago (Hardy et al., 2012; Weyrich et al., 2017; Kim et al., 2020).

and spleen, which are also largely caused by an exacerbated host immune response. In *Plasmodium yoelii* infected mice, ABA reduced liver and spleen pathologies, likely in a similar manner as in the influenza model (Hicks et al., 2016). ABA is a highly promising pharmakon for type-2-diabetes (T2D) since it improves blood glucose homeostasis at comparatively low concentrations (≈ 1 µg ABA per kg body weight) (Magnone et al., 2015). In fact, a supplement for T2D patients made from ABA-containing fig extract [*] has recently come to market (Atkinson et al., 2019) and a phase-2 human trial is currently being conducted (AdventHealth Translational Research Institute, 2022). Animal studies suggest that ABA might reduce pathology-related memory loss as well as depression (Qi et al., 2015, 2016; Ribes-Navarro et al., 2019; Espinosa-Fernández et al., 2019). The brain contains high concentration of ABA and there is a recuring theme since neuroinflammation can lead to memory loss or depression. However, further research is required to validate the findings in humans. A recent review by Kim and co-workers (2020) comprehensively examines the multi-layered effects of ABA (and other plant hormones) on metabolic syndrome (MetS), a complex disorder related to a combination of obesity, cardiovascular issues, type-2-diabetes and chronic inflammations. ABA is a highly promising drug candidate for metabolic syndrome since it affects various aspects of the disease.

ABA's role in cell proliferation and differentiation could be employed in regenerative medicine or cancer therapy. For example, *in vitro* application of ABA induced apoptosis and cell differentiation in glioma tissue (Zhou et al., 2016). Furthermore, ABA appears to downregulate angiogenesis (Chaqour et al., 2018), a phenotype of various tumours. Fundamental research also indicates that ABA has anti-mutagenic effects *in vitro* (Saxena et al., 2017).

Many of the cited reports have only been published recently and describe initial findings that need to be confirmed and evaluated further. Historically speaking, insights gathered from *in vitro* experiments or animal models are not directly transferable to human physiology.

[*] Eat your figs! The bioavailability of ABA from fresh fruit appears to be high and figs naturally contain high amounts, about ≈ 75 µg ABA per 100 g wet weight (Magnone et al., 2015). Compared to other fruit and vegetables apricots also contain high amounts, ≈ 32 µg ABA per 100 g wet weight (Magnone et al., 2015). This is obviously not medical advice!

Nonetheless, the sheer number of studies with promising results in various pathologies demonstrate the potential of ABA for therapeutic or prophylactic applications.

## ABA biosynthesis and signalling – So conserved and yet so not

ABA is a highly conserved signalling molecule throughout living beings. What makes ABA truly intriguing from a scientific point of view is the fact that its biosynthesis and its receptors are not conserved in the same manner. The terpene and terpenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) can be produced via two distinct pathways. Animals and fungi exclusively use the mevalonate pathway (MVA) located in the cytoplasm. Plants can produce IPP and DMAPP either via the MVA pathway or via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway located in plastids. Interestingly enough, plants produce ABA via an "indirect" biosynthetic pathway in which IPP and DMAPP (produced via the MEP pathway) are used to synthesize a C40 carotenoid, which is subsequently broken down to from the C15 molecule ABA (Figure 9) (Nambara and Marion-Poll, 2005). Plant-pathogenic fungi such as *Botrytis cinerea* in contrast use a "direct" pathway in which ABA is synthesized from the C15 precursor farnesyl pyrophosphate (FPP) (Figure 9) [*]. Nonetheless, this pathway is not strictly conserved and different intermediates are being used in fungi (Tudzynski and Sharon, 2002; Takino et al., 2018, 2019). ABA production in *B. cinerea* will be described in more detail in the chapter about Paper II. ABA biosynthesis in humans has not been elucidated yet. Humans neither have the MEP pathway, nor do they produce carotenoids. Homologues for three of the four *B. cinerea* ABA pathway genes have been found in mammalian genomes (Lievens et al., 2017). It seems likely that mammals produce ABA via a similar, but not necessarily the same, "direct" pathway as fungi. ABA biosynthesis in prokaryotes is equally elusive at the moment. Some ABA-producing species also produce carotenoids, indicating biosynthesis via an "indirect" pathway (Lievens et al., 2017), however biosynthesis might vary depending on the microbe.

[*] Due to the different biosynthetic pathways ABA is referred to as an apocarotenoid (compounds derived from carotenoids) or a sesquiterpenoid (a C15 terpenoid) in the literature.

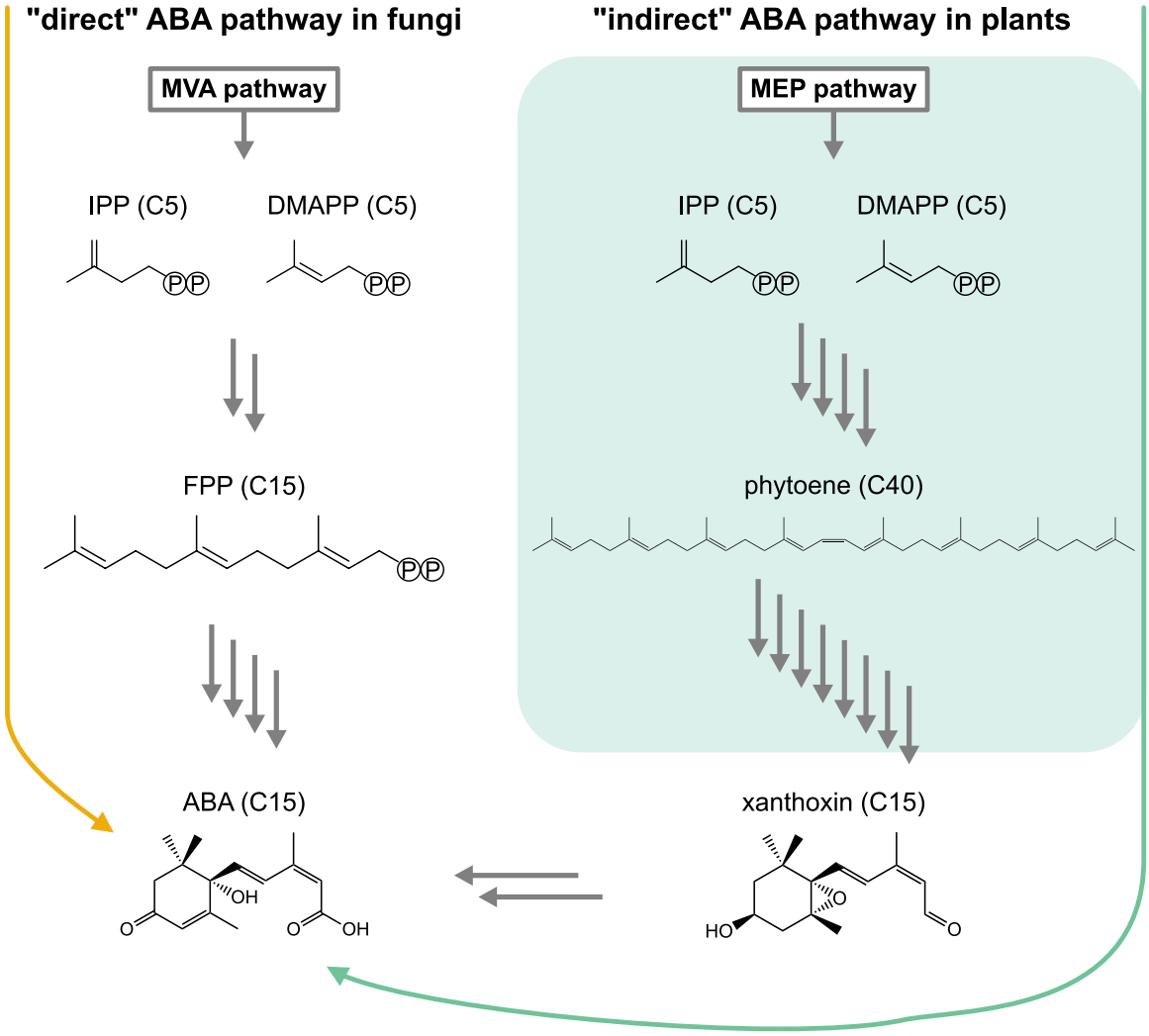



Figure 9: Comparison of the ABA biosynthetic pathways in fungi and vascular plants (Finkelstein, 2013). Fungi produce ABA via "direct" pathways localized in the cytosol using the C15 precursor FPP. Vascular plants produce ABA via an "indirect" pathway. In plants, a C40 carotenoid is synthesized in plastids, which is broken down to form ABA. The last steps of this pathway are localized in the cytosol. The terpene precursors IPP and DMAPP are produced via the MVA pathway or the MEP pathway, respectively in fungi and plants. Abbreviations: MVA = mevalonate, MEP = 2-C-methyl-D-erythritol 4-phosphate, IPP = isopentenyl diphosphate, DMAPP = dimethylallyl diphosphate, FPP = farnesyl pyrophosphate.

ABA signalling in plants is highly complex (reviewed in Cutler et al., 2010). It includes various independent receptors localized in different cellular compartments. A family of proteins called pyrabactin-resistance (PYR) or pyrabactin-resistance-like (PYL) receptors, localized in the cytosol, appear to be the most impactful ABA signalling components. In *Arabidopsis thaliana*, 14 such receptors have been found (Fujii et al., 2009). Upon ABA binding, they inhibit class-2C protein phosphatases (PP2Cs), resulting in a signalling cascade and transcriptional response. PYR/PYL receptors are further discussed in Paper IV. Besides the previously

mentioned bitter taste receptor (Pydi et al., 2015), a membrane-bound protein called LANCL2 seems to be the main ABA receptor in animals. A plant homologue of LANCL2 exists, but its involvement in ABA signalling in plants remains questionable (Cutler et al., 2010). In animals, binding of ABA to LANCL2 results in the activation of the transcription factor PPARγ (Lievens et al., 2017), which has been associated with energy metabolism, inflammation and cell differentiation (Houseknecht et al., 2002).

## ABA from an evolutionary perspective – Simple chemistry?

How can ABA be present in that many organisms and yet be produced and sensed in different ways? Out of all the possible chemical structures, why did evolution "pick" this molecule as a virtually universal signalling molecule? At this point in time, definitive answers for these questions are lacking and we can only speculate. One theory postulates that ABA biosynthesis was not present in the last universal common ancestor but was first established in prokaryotes, some of which eventually became chloroplasts in photosynthetic eukaryotes (Hartung, 2010). The "direct" ABA biosynthetic pathways likely occurred via convergent evolution since it allowed inter-species communication (Lievens et al., 2017), initially mostly with photosynthetic organisms (for example the plant-rhizobacteria interaction mentioned above), but after "re-invention" of the ABA pathway in other organisms potentially also more broadly. Continuous exposure to ABA via plant-based foods might also have played a role in this evolutionary development.

From a chemical perspective, ABA appears to be sufficiently water soluble while retaining the ability to permeate membranes. ABA conforms with Lipinski's Rule of Five, a rule of thumb to predict the suitability of a given molecule as an orally administered drug in terms of solubility and permeability [*] (Lipinski et al., 1997). ABA also conforms with other drug-likeness rules, e.g. regarding the number of rotatable bonds and polar surface area of the molecule (PubChem accessed 16.12.2022; Veber et al., 2002). ABA's acid group ($pK_a$ = 4.75) changes its

[*] Lipinski's Rule of Five actually has four criteria, but each criterion is a multiple of five hence the name. Generally, three out of the four criteria should be true. The criteria are: no more than five hydrogen-bond donors, no more than ten hydrogen-bond acceptors, a molecular weight of less than 500 Da, and a partition coefficient (logP) of less than five. ABA fulfils all four criteria. The rule does not apply to substrates for of biological transporters (Lipinski et al., 1997).

ability to diffuse through membranes in a pH dependent manner, with the anion not being able to penetrate membranes (Slovik et al., 1992). Taken together, ABA's molecular structure appears to be highly favourable for a signalling molecule and might be one of the reasons why the ability to synthesize and sense ABA is so conserved throughout life. This is not to say that ABA is a superior signalling molecule compared to other possible molecules. Presumably ABA's chemical structure was sufficient to fulfil its evolutionary role and appeared early enough in our evolutionary history to eventually become as conserved as it is.

# Part II: Research

## Connecting the dots

Part II of this thesis accommodates my original research. The four following chapters review the individual papers, presenting a general introduction, a short summary of the main findings and a broader discussion. The goal of this part was to, whenever possible, not repeat the contents of the papers, but to provide an entry and wider perspective on the topics covered. Detailed method descriptions, data and discussion of specific results are included in the papers.

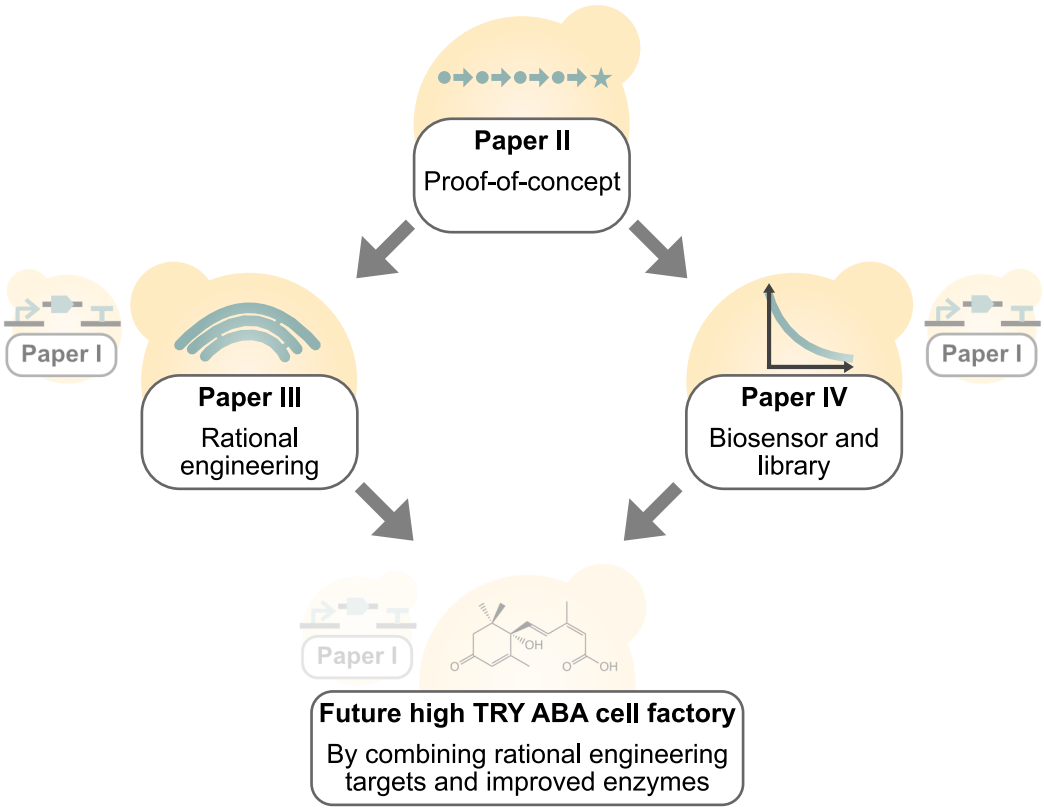



Figure 10: Overview of the research papers. In Paper II a proof-of-concept ABA cell factory was developed. Two different optimisation strategies were adopted for Paper III and Paper IV. The toolkit developed in Paper I was used to construct the strains of Paper III and Paper IV. In the future, the findings of Paper III and Paper IV can be combined to construct an ABA cell factory with high titer, rate and yield (TRY).

This thesis is about the construction and optimization of an ABA yeast cell factory. Figure 10 illustrates how the papers relate to each other. Paper I describes a toolkit that has been used as a general enabling technology for the subsequent research. In Paper II a proof-of-concept yeast strain for heterologous ABA production was established. Paper III and Paper IV use different approaches to optimize ABA production. The findings of these two papers can be combined in the future to construct ABA cell factories with high titers, rates and yields.

# Paper I: Expanding the yeast MoClo toolkit

## Standardization of DNA parts – Putting the "engineering" in genetics

Standards are indispensable in all engineering disciplines, from mechanical to software engineering. Standardization has been a major facilitator of the industrial revolution and standards are ubiquitous in our everyday life. Standardized parts, like screws and nuts or diodes and transistors, enable the construction of complex systems out of simple parts (Figure 11). Standardized parts bring numerous advantages, including any or all of the following: compatibility, interoperability, reproducibility, predictability and reusability. Overall, standards reduce time investments and costs, and substantially increase scalability.

As was highlighted in Part I, biology is highly standardized on a fundamental level. This allows us to transfer basic engineering strategies from other disciplines to biological systems [*]. Standardization has been proposed from the early days as one of synthetic biology's central pillars (the others being decoupling and abstraction) (Endy, 2005). Early on, it was recognized that the assembly of recombinant DNA constructs is time-consuming, tedious and potentially limiting advances in the field. Gene expression cassettes can be thought of as modular, fundamentally consisting of separate genetic parts assigned to specific functions, such as the promoter, coding sequence and terminator. Standardization of these genetic parts would simplify and accelerate the construction of recombinant DNA fragments, reduce costs and ensure reproducibility. Pioneering work by Rebatchouk et al. (1996) and Knight (2003) proved that genetic parts can be standardized and assembled in a modular fashion. Today the benefits of genetic assembly standards are arguably universally acknowledged and various standards have been developed and refined over the years, most notably the BioBrick (Knight, 2003), Goldenbraid (Sarrion-Perdigones et al., 2011) and Modular Cloning (MoClo) standard (Weber et al., 2011).

[*] Biological engineering is in a unique position since we are largely restricted to a top-down engineering approach in which we are taking an existing system, in this case a cell, and add and/or remove parts. Relying on systems that are a product of evolution is however problematic. In contrast to an engineer who follows a systematic, goal-oriented approach, evolution has been compared to a tinkerer who adapts whatever parts and tools are available at a given moment (Jacob, 1977). This results in a large amount of complexity, redundancy, pleiotropy and low regulatory hierarchy, all of which are generally not desirable in engineering projects. Research in bottom-up engineering approaches is on its way (Mutschler et al., 2019), but arguably still in its infancy.

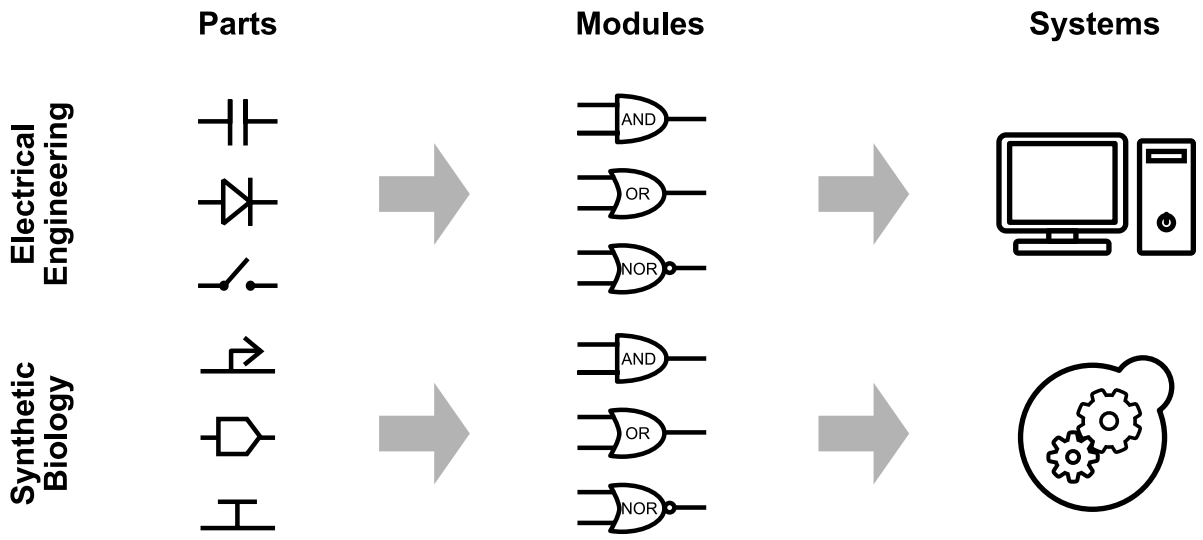



Figure 11: From parts to systems. Engineering disciplines use standardized parts to construct modules, e.g. logic gates in electrical engineering. Subsequently the modules can be combined to construct systems, e.g. electronic devices. Synthetic biology aspires to a similar modularity and abstraction. Characterized and standardized genetic parts can be used to construct logic modules inspired by electrical engineering, which can then be used to develop biological devices.

## Modular Cloning – One-pot assembly magic

In Paper I, we expanded the capabilities of the existing yeast MoClo standard developed by Lee and co-workers (2015). The MoClo workflow is briefly described in the following text and is illustrated in Figure 12. In MoClo, genetic parts are categorized by "type", e.g. type 2 parts are promoters, type 3 parts are coding sequences, type 4 parts are terminators *et cetera*. The assembly procedure is based on Golden Gate cloning (Engler et al., 2008, 2009). Type IIS restriction enzymes, BsaI and BsmBI, are used to generate type-specific DNA overhangs, e.g all parts that are of the promoter type have the same overhang. The overhangs align with the subsequent part and therefore can be ligated in a directional manner, e.g. overhangs of type-2 parts containing promoters align with type 3 parts containing coding sequences. This enables the assembly of expression cassettes or whole plasmids in an efficient one-pot restriction-ligation reaction. MoClo uses a hierarchical assembly architecture with dedicated "levels". Individual genetic parts are stored in level-0 part plasmids, e.g. containing a single type 2 promoter part or a single type 3 coding sequence (in a bacterial backbone for amplification purposes). Multiple level-0 part plasmids are combined to construct level-1 plasmids that contain a complete expression cassette (and a yeast-compatible backbone).

Lastly, multiple level-1 plasmids can be combined to form level-2 plasmids with up to six expression cassettes. For the assembly of level-2 plasmids, specific connector elements (type 1 and type 5 parts) were designed (Lee et al., 2015).

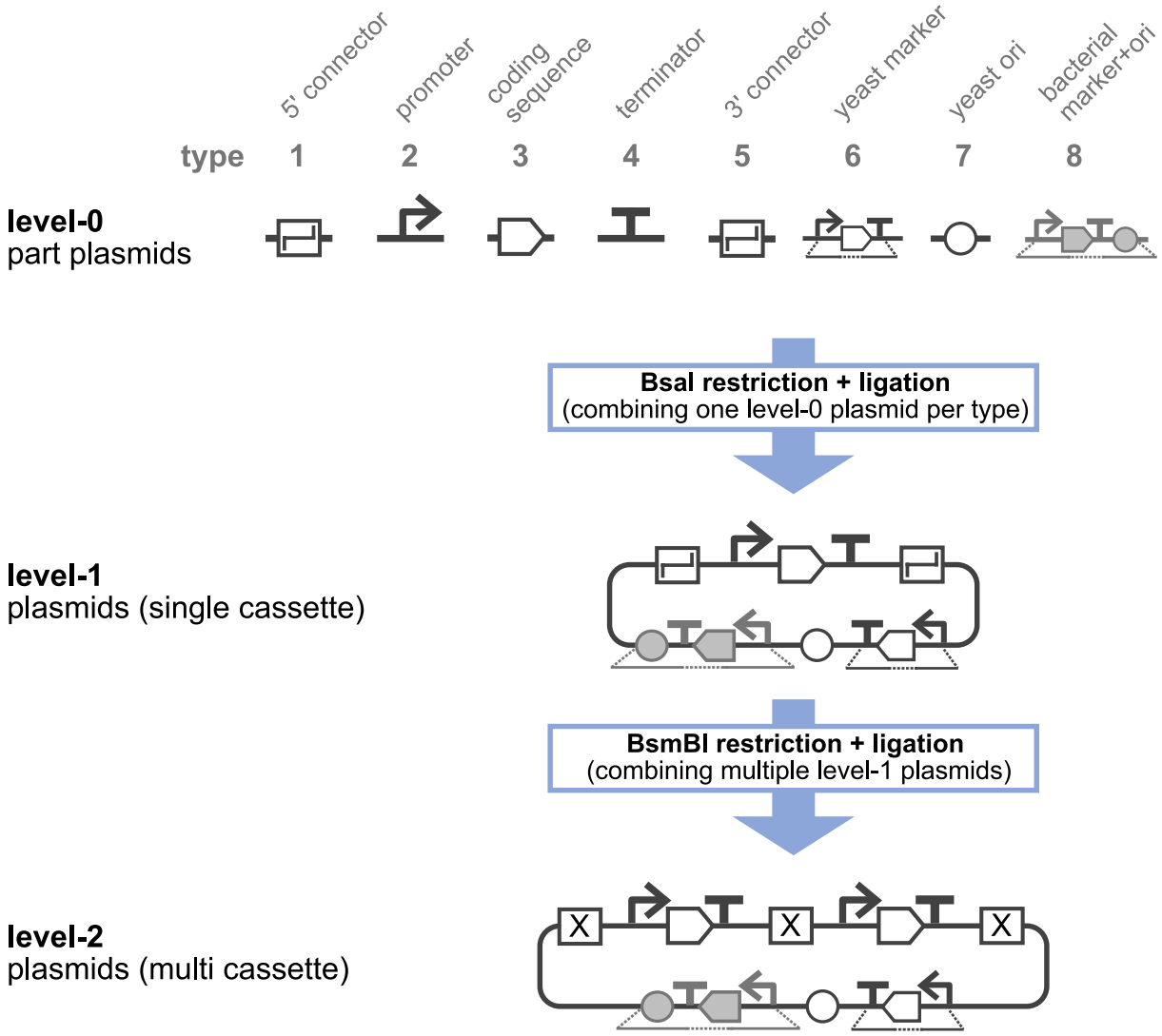



Figure 12: Modular Cloning (MoClo) workflow. The illustration shows how single cassette and multi cassette plasmids are constructed from standardized parts. Symbols filled in grey represent bacterial parts. For the construction of level-2 plasmids the restriction sites in the type 1 and type 5 connector parts are used and destroyed (indicated by "X").

Any type 0 part plasmids in the MoClo library can be combined directly and interchangeably in restriction-ligation reactions without the need for specific "one-time-use" PCR primers that are for example required in Gibson or uracil excision assemblies. The yeast MoClo toolkit developed by Lee and co-workers (2015) provided the basic assembly framework as well as a library of 96 MoClo plasmids that include commonly used promoters, coding sequences, terminators, yeast origins-of-replications, yeast selection cassettes, bacterial backbones and

connector elements. We expanded the existing toolkit to make it more applicable for common metabolic engineering and synthetic biology applications. Paper I is summarized in Box 1.

Box 1: Summary of the main goals and findings of Paper I.

**Aims and results of Paper I:**

- Aim 1 - Expand the yeast MoClo toolkit for common genetic engineering tasks
  - Ten plasmid backbones for chromosomal integrations were added to the MoClo library
  - A workflow for Csy4-mediated gRNA multiplexing was developed
  - A dual counter-selection marker for yeast and bacteria useful for the construction of combinatorial libraries was added to the MoClo library
- Aim 2 - Further characterize existing parts:
  - Inclusion of a BglII site in promoter parts was shown to negatively affect protein production
- Aim 3 - Simplify the adoption of the standard and improve its usability:
  - An online tool to streamline the design of new MoClo parts was developed
  - An online tool for the design of gRNA arrays was developed

## Adoption of standards – Of toothbrushes and scientist

The MoClo standard and its expansion has been an enabling technology for the research in this thesis, specifically for Paper III and Paper IV (Paper II had been published before). Nonetheless, standards are especially useful at scale. One of the main motivations for this project was to establish a standard at our research group that is applicable for everyday engineering tasks. Parts shared with co-workers can be used immediately and new plasmids can be purified the next day. This nicely illustrates how a standard can accelerate the Build phase of the DBTL cycle. In contrast, ordering primers for Gibson assemblies would take two to five days, not to speak of the additional costs. Other institutions that utilize the same standard benefit similarly and can rapidly utilize shared DNA constructs and reliably

reproduce results. Beyond academia, standardization in biological engineering (including but not limited to standardized genetic parts) is seen as crucial for a bio-based economy (de Lorenzo and Schmidt, 2018).

Standardization attempts have been around for nearly two decades and their benefits are widely accepted in the synthetic biology community. Why are genetic part standards not adopted more widely in academia yet? In this context the Physics Nobel Laureate Murray Gell-Mann has been quoted before saying:

> *"A scientist would rather use someone else's toothbrush than another scientist's nomenclature"*
>
> – Gell-Mann (1995) and Beal et al. (2020)

There might be some truth to the particular stubbornness of scientists. Nevertheless, for the adoption of genetic standards, there might be two other impactful factors, namely the restrictive nature of standardization and the initial investments of resources required for their adoption (Beal et al., 2020). Standards are by definition restrictive which is particularly an issue for innovative research performed in academia. However, depending on the research, the impact of these restrictions might often be overestimated and the additional throughput achieved by standardization is likely worth the trade-off, especially since innovation often requires rapid prototyping (Beal et al., 2020). From my own experience, the initial adoption of a standard is time-consuming, requires establishing an administrative system (e.g. how is the MoClo library stored, distributed and expanded) as well as financial resources (e.g. for acquiring a basic part library and providing sufficient freezer storage space). Further investment is required from the researchers who need to learn a new assembly procedure. This specific issue was partly addressed in Paper I by developing tools that streamline the design of new genetic parts, a process that is tedious and error-prone when done manually. Standards will only be widely adopted if they provide substantial value to engineers and their advantages outweigh the disadvantages. On a small scale this was illustrated by the adoption of the MoClo standard at our research division. While many group members were initially hesitant to switch to another assembly standard, over the course of a year, more and more colleagues tested the MoClo approach and now use it regularly or exclusively. Different reasons lead to individuals adopting the assembly standard, including reoccurring difficulties with PCRs for Gibson assemblies due to the long primer sequences, wanting to adapt

promoter or coding sequences from co-workers that were using MoClo already, getting DNA constructs from other institutions that were using MoClo parts, and the overall faster prototyping e.g. when fine-tuning expression levels using various promoters. From a practical perspective, important features of the MoClo standard are the efficient and reliable Golden Gate assembly protocol, as well as the user-friendly screening method for correctly assembled plasmids that makes verification less tedious and time-consuming [*].

This being said, the MoClo standard comes with some disadvantages. Firstly, it is not possible to directly assemble level-2 plasmids with multiple expression cassettes from level-0 part plasmids. Instead, level-1 plasmids with a single expression cassette need to be constructed first and then combined to form level-2 plasmids. In addition, the choice of connector elements predefines the assembly of level-2 plasmid, therefore level-1 plasmids cannot be combined universally in any number or order.

MoClo promoter parts include a BglII restriction site at the 5' end of the coding sequence, enabling compatibility with the BglBrick standard for genetic parts (Anderson et al., 2010). Generally, cross-compatibility is highly desirable for standardized parts. Nonetheless, in Paper I, we demonstrated that including the restriction site can reduce protein production levels. Decreased protein production either was deemed to be less important than cross-compatibility or might not have been anticipated when the standard was designed. This highlights an issue with biological standards that is not present in other engineering disciplines to the same degree. At the moment, a holistic understanding of cells and their regulation is still missing. How genetic parts or modules behave when perturbed, e.g. by extrinsic stimuli or other genetic modifications, is often unpredictable. This could potentially require fundamental design changes in a given standard and require re-characterisation of genetic parts. However, standards have been iterated on in other disciplines as well, adapting to new insights or new applications.

[*] The screening method is based on different antibiotic markers, as well as GFP or RFP dropout cassettes which are replaced if the plasmid is assembled correctly. Colonies that carry correctly assembled plasmids appear white on the plate. More details can be found in the supplementary material of Lee et al. (2015).

## The future of standards – Beyond parts

The standardization of genetic parts is one piece of the puzzle towards biology as a genuine engineering discipline. Various other standardization efforts have been established already and are adopted by the community to different degrees. For example, by using the Synthetic Biology Open Language (SBOL, https://sbolstandard.org) biological designs can be represented in a circuit-like manner. OpenWetWare (https://openwetware.org/) provides standardized operating procedures for laboratory techniques. Furthermore, Standards for Reporting Enzymology Data (STRENDA, https://www.beilstein-institut.de/en/projects/strenda/) have also been established.

Standards will impact biological engineering beyond just accelerating the Build phase of the DBTL cycle. Instead, it is likely that standardization will substantially reduce the required number of DBTL iterations for a given engineering project. Reported results are often not reproducible (Baker, 2016). More standardization could play a significant role in alleviating this reproducibility crisis. Furthermore, standardized testing and reporting procedures, as well as comprehensive data repositories can facilitate the Learn phase, leading to more promising designs in the following DBTL cycle. Having data repositories that are suitable for training and evaluating machine learning algorithms will be key for many metabolic engineering and synthetic biology applications.

To utilize standards to their full potential wide-spread adoption by the community is required. This is as much a cultural issue as it is a scientific one. Establishment of a single genetic part standard for biological engineering agreed upon by experts appears unlikely. This is arguably also undesirable due to different needs and expectations in the research community. Nonetheless, scientific journals and grant agencies could encourage the use of standards regarding the reporting of data and the use of standardized genetic parts. Importantly, this needs to be done in a non-restrictive way, not forcing the use of a single standard but promoting the general use of any.

# Paper II: A yeast cell factory for heterologous ABA production

## *Botrytis cinerea* – A noble pathogen

Most of us have likely encountered *Botrytis cinerea* in the supermarket or at home (Figure 13A). The plant pathogenic fungus infects an extensive range of host plants, 586 genera of plants have been reported so far (Elad et al., 2016). It can often be found on grapes, strawberries and tomatoes, and is typical referred to as "grey mould" [*]. *Botrytis cinerea* causes major financial damage in agriculture and in particular viticulture. Damages due to rotten products are difficult to estimate, but global expenses to prevent or mitigate *B. cinerea* infections likely surpass $1 billion/year (Dean et al., 2012). Due to its negative impacts in agriculture, *B. cinerea* is the most widely studied necrotrophic fungus (Dean et al., 2012). Sometimes however, *B. cinerea* infection of grapes, known as noble rot, can be desirable and is actively pursued at some vineyards with suitable climatic conditions (Magyar, 2011). Botrytized wines are said to be naturally sweet and complex.

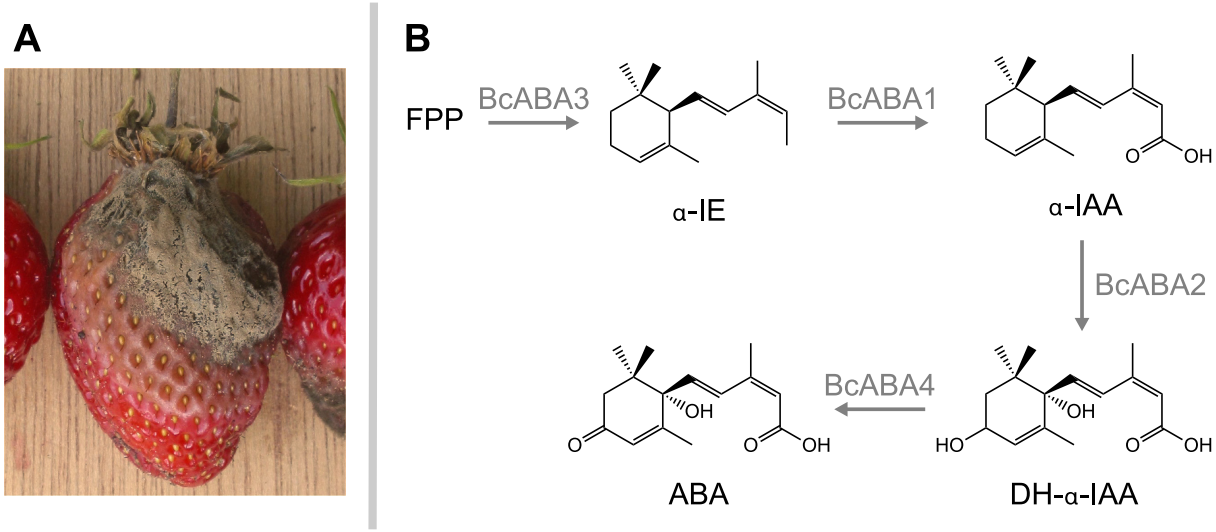



Figure 13: *Botrytis cinerea* and its ABA biosynthetic pathway. A: Picture of a strawberry infected with *B. cinerea* (picture by Rasbak, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=192867). B: ABA biosynthetic pathway of *B. cinerea* (Takino et al., 2019). Enzyme names are shown in grey above the arrows. Abbreviations: FPP = farnesyl pyrophosphate, α-IE = α-ionylideneethane, α-IAA = α-ionylideneacetic acid, DH-α-IE = 1',4'-trans-dihydroxy-α-ionylideneacetic acid, ABA = abscisic acid.

ABA is generally regarded as a virulence factor for *B. cinerea*, interfering with plant defence signalling (Kettner and Dörffling, 1995; Audenaert et al., 2002; Curvers et al., 2010).

[*] The name *Botrytis cinerea* stems from the Latin words for "grape" and "ash", describing the structure of the fungal spores and their greyish color respectively.

Specifically, it appears to interfere with the salicylic acid signalling network that is associated with biotic stress resistances, increasing the plant's susceptibility to the fungus. However, there is also evidence that ABA can reduce the susceptibility to *B. cinerea* (Korolev et al., 2008). Additionally, *B. cinerea* strains that, in axenic culture, do not produce ABA are still highly pathogenic (Siewers et al., 2006). More research will be required to understand ABA's role in this pathogen-host interaction fully.

## ABA biosynthesis in *B. cinerea* – Unveiled after 15 years

In Paper II we developed a proof-of-concept strain for the heterologous production of ABA in *S. cerevisiae*, in which the biosynthetic pathway of *B. cinerea* was used (Figure 13B). Using this ABA pathway instead of the pathway from plants seemed promising for multiple reasons. In plants, most steps of the ABA pathway are localized in plastids, an organelle that is not present in yeast. *S. cerevisiae* and *B. cinerea* are evolutionary more closely related (both belong to the phylum Ascomycota), increasing the likelihood that heterologous enzymes can be functionally expressed. Furthermore, and arguably the most relevant reason for metabolic engineering applications, the *B. cinerea* ABA biosynthesis requires fewer enzymatic reactions and is more efficient in its carbon and energy usage. Of the ABA-producing fungi, ABA biosynthesis in *B. cinerea* is best described. Nonetheless, at the start of this project in 2018, its ABA biosynthetic pathway had not been elucidated completely and there was still uncertainty about the number of enzymes involved and their specific roles.

A cluster of four genes, *bcaba1* to *bcaba4*, had been described before (Siewers et al., 2006). Three of the genes, *bcaba1*, *bcaba2* and *bcaba3*, had been shown to be essential for ABA production (Siewers et al., 2004, 2006). While not essential, *bcaba4* still positively affected ABA titers in *B. cinerea*. ABA was thought to be produced in at least four steps, starting with the cyclisation of the sesquiterpene precursor FPP to form the ABA carbon backbone, which consecutively would be decorated with oxygen-containing functional groups. None of the clustered genes however showed typical motives of a sesquiterpene cyclase (STC) that could catalyse the first reaction. In addition, it was not certain which of the oxidation reactions were catalysed by which enzyme and in what order they were performed.

One study found that a gene named *bcaba5* encoding an STC was essential for ABA production in the native host, supposedly catalysing the initial cyclisation of FPP (Izquierdo-Bueno et al., 2018). This was however rebutted by a study that closely investigated BcABA3, a protein with no homology to previously characterized enzymes (Takino et al., 2018). Takino and co-workers (2018) found that BcABA3 is a hitherto undescribed type of STC. Shortly after, the same group investigated the other genes of the *bcaba* cluster, elucidating the ABA pathway and its intermediates in more detail (Takino et al., 2019). Interestingly, Takino and co-workers reported that BcABA2 also exhibits an unusual functionality. BcABA2, a cytochrome P450 monooxygenase (CYP), appears to catalyse two oxidation steps at different positions and on different faces of the molecule (Takino et al., 2019).

Box 2: Summary of the main goals and findings of Paper II.

**Aims and results of Paper II:**

- Aim 1 - Establish ABA production in *S. cerevisiae* and confirm conflicting studies about ABA pathway genes
    - Expression of *bcaba1* to *bcaba4* were sufficient for heterologous production
    - Expression of *bcaba5* did not benefit ABA production
    - ABA is predominantly present in the culture supernatant and is mostly produced during growth in glucose
- Aim 2 - Pinpoint production bottlenecks in the ABA cell factory
    - Neither FPP supply nor NADPH supply is limiting ABA production
    - (Over)expression of a cytochrome P450 reductase gene, either the native yeast *NCP1* or the *B. cinerea BcCPR1,* increased ABA titers
    - An as yet undescribed pathway intermediate or side-product with the mass-to-charge ratio 233 accumulated in ABA producing strains
    - Activity of the ABA pathway enzymes, specifically the CYPs BcABA1 and BcABA2, are limiting ABA titers in the current strain
    - The ABA titer was increased 4-fold by expressing additional copies of *bcaba1* and *bcaba2*, reaching > 10 mg/L ABA

In Paper II, we confirmed that the 4 genes of the *bcaba* cluster are sufficient to produce ABA in *S. cerevisiae*. The proof-of-concept strain produces ABA in a mg/L range. Besides, we were able to pinpoint production bottlenecks in the strain which became the basis for further investigations in Paper III and Paper IV. Box 2 briefly summarizes the findings of Paper II.

## Heterologous ABA production – Worth the trouble?

Besides its use in speciality wines, *B. cinerea* can fulfil another useful role for humans, namely the biotechnological production of ABA (Kimura et al., 1994). In Paper II, the BcABA amino acid sequences of the ABA-overproducing *B. cinerea* strain ATCC 58025 were used. This strain produces close to 100 mg/L ABA in complex media after 17 days of cultivation in shake flasks (Izquierdo-Bueno et al., 2018). On the one hand, this is substantially higher than in the ABA titer reported in Paper II. On the other hand, mineral media was used in our study and the production rate was higher. Other studies report ABA titers of > 1 g/L in fed-batch cultivations for a *B. cinerea* strain that has been mutagenized using UV radiation (Gong et al., 2014).

This leads to the question if it is worthwhile to construct and optimize an *S. cerevisiae* cell factory for the production of ABA. Calculating and comparing the ABA yields is difficult since not enough data is provided (the media feed rate for the fed-batch cultivations is not stated). However, obvious advantages of *S. cerevisiae* are its faster growth rate and no hyphal growth. Moreover, the following two studies of this thesis, Paper III and Paper IV, illustrate the potential of *S. cerevisiae* in terms of genetic engineering possibilities and experimental throughput. Performing the same genetic modifications in *B. cinerea* would have been substantially more time-consuming and difficult. Compared to yeast (admittedly a lofty standard), *B. cinerea* lacks genetic tools. To my knowledge, no dedicated framework for standardized genetic parts exists for *B. cinerea*. Only recently has a CRISPR-Cas system been developed for use in the fungus (Leisen et al., 2020). The use of genetic circuits in *B. cinerea* is still in its early stages (Olivares-Yañez et al., 2021). No small-molecule biosensors, comparable to ones described in Paper IV, has been developed in *B. cinerea* to the best of my knowledge [*]. Another advantage of *S. cerevisiae* is the existing expertise and infrastructure

[*] Details about how the previously mentioned, UV-mutagenized *B. cinerea* strain was constructed and screened for are not accessible. Presumably individual mutants have been screened "by hand" using low throughput methods.

for yeast cell factories in the industry, e.g. regarding large-scale fermenters and down-stream processing. Biotechnological ABA production and potential cell factory chassis are further discussed in Part III of this thesis.

# Paper III: Improving ABA production by rational engineering

## Cytochrome P450 monooxygenases – Needy enzymes

Depending on how much is known about the pathway-of-interest, establishing a proof-of-concept cell factory is often not trivial. However, as outlined in Part I already, improving titer, rate and yield of a cell factory has proven to be all the more challenging and is one of the major reason why few cell factories are currently used in industrial settings (Nielsen and Keasling, 2016; Nielsen et al., 2022). The goal of Paper III and Paper IV was to improve ABA production in the proof-of-concept strain. The activity of the CYPs BcABA1 and BcABA2 were shown to be production bottlenecks. In Paper III we improved CYP performance without engineering the enzymes themselves.

CYPs are complex enzymes that catalyse oxidation reactions using heme as a co-factor. They rely on co-enzymes called cytochrome P450 reductases (CPRs) to supply electrons using NADPH. Cytochrome b5 (CYB5) and its cognate cytochrome b5 reductase (CBR) can provide an alternative source of electrons. CYPs and their various co-enzymes are often anchored in the ER membrane [*]. Any of these factors could limit the activity of BcABA1 and BcABA2 in the ABA cell factory.

To investigate this further, rational engineering targets were chosen from the published literature (Michener et al., 2012; Paddon et al., 2013; Arendt et al., 2017; Kim et al., 2019), which included *B. cinerea* as well as the native yeast genes. We analysed the effects of expressing the *B. cinerea* CYB5 and CBR, overexpressing the native *HEM13* gene to increase heme supply and investigated effects of genetic modifications affecting ER morphology, namely overexpression of *INO2* or deletion of *OPI1* and *PAH1*. We furthermore investigated if ABA production can be improved by preventing the export of pathway intermediates via native yeast transporters. After probing the cell factory using these diverse engineering targets, we homed in on *PAH1* for further experiments since it appeared to be a highly promising target. A more detailed summary of the results can be found in Box 3.

[*] CYPs are a large superfamily of very diverse enzymes. Their sequence identity can be less than 20% (Werck-Reichhart and Feyereisen, 2000) and general statements can be misleading. For example, prokaryotic CYPs are not membrane-bound. For some CYPs their CPR is fused to the enzyme, while other CYPs do not require a CPR at all, instead they use NAD(P)H directly as an electron donor. Most CYPs, but not all, use molecular oxygen. The text above refers to class II CYPs, the most common class in eukaryotes.

Box 3: Summary of the main goals and findings of Paper III.

**Aims and results of Paper III:**

- Aim 1 - Investigate if the CYB5 of *B. cinerea* improves the activity of BcABA1 and BcABA2
    - Expression of BcCYB5 and its cognate reductase did not improve ABA titers
- Aim 2 - Investigate if modifications in the native yeast metabolism can improve ABA production
    - Overexpression or knockdown of the transcription factors *PDR1* and *YRR1*, involved in transporter gene expression, did not increase ABA titers
    - *HEM13* overexpression, with the goal of increasing heme supply, did not increase ABA titers
    - Three genes involved in ER homeostasis were modified. *INO2* overexpression and Δ*opi1* did not affect ABA production, whereas Δ*pah1* caused a growth defect but improved ABA titers when normalized to $OD_{600}$
- Aim 3 - Avoid the growth defect caused by Δ*pah1*
    - A *PAH1* knockdown (exchanging the native promoter with a minimal synthetic promoter) mediated the growth defect while still improving ABA production

## ER proliferation – A detour into lipid metabolism

*PAH1*, *INO2* and *OPI1* are genes involved in yeast lipid metabolism. The former encodes an enzyme whereas the latter two encode transcriptional regulators. Generally, cells use lipids in three ways: as energy and carbon source, as energy and carbon storage and as building blocks for cellular compartments, such as the ER. *PAH1* encodes a phosphatidate phosphatase, catalysing the conversion of phosphatidic acids (PAs) to diacylglycerols (DAGs) (Figure 14). The reverse reaction from DAGs to PAs is catalysed by the enzyme Dgk1 (diacylglycerol kinase). PAs are precursors for membrane phospholipids (PLs) via the cytidine diphosphate diacylglycerol (CDP-DAG) pathway (Fakas, 2017). DAGs on the other hand are precursors for triacylglyceride (TAG) storage lipids. The reactions catalysed by Pah1 and Dgk1

are decisive in terms of how lipids are being used in yeast cells, either for membrane homeostasis and expansion or for energy and carbon storage.

Not surprisingly, PL and TAG biosynthesis are tightly regulated in yeast cells (Henry et al., 2012). A key regulator is the Ino2/Ino4 complex, activating various genes involved in the CDP-DAG pathway (Figure 14). Opi1 acts in opposition to Ino2/Ino4 by binding Ino2 and preventing gene activation. Knockout of *PAH1*, overexpressing *INO2* or knockout of *OPI1* all lead to the PA/DAG balance being shifted towards PAs (Carman and Han, 2009). They result in similar phenotypes in which an expansion of the ER has been observed, referred to as ER proliferation (Arendt et al., 2017; Kim et al., 2019).

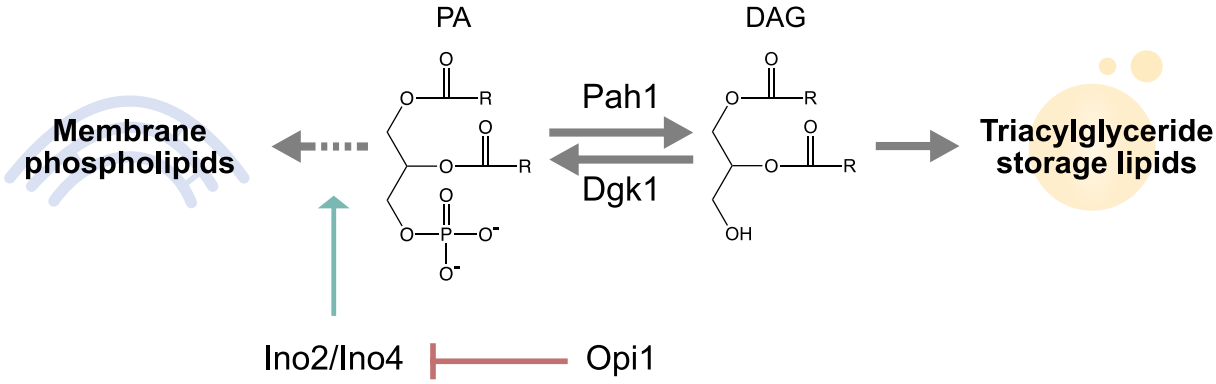



Figure 14: Regulation of membrane phospholipid (PL) and triacylglycerid (TAG) production in yeast. The enzyme Pah1 catalyses the conversion of phosphatidic acid (PA) to diacylglycerol (DAG). The reverse reaction is catalysed by Dgk1. DAG is used for the production of TAG storage lipids, whereas PA is a precursor for PLs. The Ino2/Ino4 transcription factor complex activates various genes involved in PL metabolism. Opi1 inhibits the formation of the Ino2/Ino4 complex, thereby acting as a negative regulator of PL metabolism genes. Overproduction of PLs can result in phenotypes with an expanded ER. In the chemical structures, "R" represents the aliphatic moiety of a fatty acid.

ER proliferation is a tried-and-tested engineering strategy for improving the activity of heterologous CYPs (Hu et al., 2022; Jiang et al., 2022). An enlarged ER possibly enhances folding of membrane-anchored enzymes and/or increases the physical space available for said enzymes to function. All three described modifications have been applied in yeast metabolic engineering studies before, however beneficial effects seem to vary depending on the product (Arendt et al., 2017; Kim et al., 2019; Liu et al., 2021). Growth defects caused by Δ*pah1* are not always observed (Liu et al., 2021). Paper III illustrates that a *PAH1* knockdown is another valid engineering option to improve heterologous CYP activity besides the ones previously described in other studies.

## Further experiments – Less applied, more basic

The ubiquity of CYPs in biosynthetic pathways of secondary metabolites makes them exceedingly important for metabolic engineering projects. In comparison to engineering the enzymes themselves, manipulating the native yeast metabolism is advantageous since insights are potentially transferable to other cell factories expressing different CYPs. Additionally, in biosynthetic pathways with multiple CYPs, as is the case with ABA production, the enzymes do not need to be engineered individually but likely benefit similarly.

Nonetheless, in the case of ER proliferation more basic research is necessary to make insights useful and transferable. Specifically comparing the phenotypes of *PAH1*, *INO2* and *OPI1* modifications in detail would be useful and describing how they affect different membrane-anchored proteins. Paper III would for example benefit from confocal or transmission electron microscopy images that would confirm ER proliferation and make the study more comprehensive. Quantitative and qualitative parameters (e.g. clustered or disorganized ER expansion) could be measured (Papagiannidis et al., 2021). Basic research regarding ER proliferation is of particular interest for metabolic engineering applications since current GEMs do not capture ER membrane space.

There are likely many more engineering targets in the native yeast metabolism that would benefit ABA production, be it other genes involved in phospholipid metabolism or genes fulfilling completely different cellular functions, such as chaperones (Kim et al., 2019). Knock-out or overexpression libraries could be analysed to find promising engineering targets (Giaever et al., 2002; Jones et al., 2008). Alternatively, GEMs could be used to predict relevant engineering targets, while keeping the number of samples to a minimum compared to the previously mentioned libraries (Ishchuk et al., 2022). Nevertheless, a high-throughput screening approach would be required for comprehensive analysis.

# Paper IV: Platform strains for high-throughput screenings

## Biosensors – A very short introduction

Cells continuously measure their intracellular and extracellular environment, adjusting their metabolism accordingly to ensure homeostasis and growth. In our daily lives, electronic sensors are used to control our environment, for example to turn on lights upon sensing motion, or to understand and predict our environment, as in the case of meteorological sensors for weather forecasting. Could cells be engineered to fulfil similar functions, in particular functions that are difficult to perform using electronic parts?

Biosensors are devices that can translate an input signal, e.g. the concentration of a molecule, into an easily detectable output signal, such as a fluorescent signal. For this purpose, naturally occurring sensor molecules and their regulatory networks can be adapted and engineered. Biosensors can consist of individual biomolecules, such as receptor proteins, or of whole cells resulting in self-replicating devices. Various whole-cell biosensor designs have been developed over the years, including biosensors based on riboswitches, Förster resonance energy transfer (FRET), transcription factors (TFs) or multi-component systems. Biosensors have been constructed for a multitude of applications ranging from the detection of toxins and explosives in the environment (Shemer et al., 2015; Saltepe et al., 2022), to biological computing (Rondon et al., 2019) and medical diagnostics (Chang et al., 2017). In metabolic engineering, biosensors can be used for the selection or screening of large mutant libraries, e.g. enzyme libraries, with the goal of finding improved variants [*].

Biosensors need to fulfil various criteria to be of use. They need to (selectively) respond to the molecule of interest, referred to as a biosensor's ligand specificity. They need to respond at a relevant concentration range, called the operational range. The signal intensity of an activated biosensor needs to be clearly distinguishable from a biosensor that has not been activated. The maximum difference in signal intensity between a biosensor's "ON" state and its "OFF" state is referred to as the dynamic range. The operational and dynamic range of a biosensor are visible in its dose-response curve (Figure 15A). A biosensor's sensitivity is the slope of the dose-response curve. Lastly, a biosensor's orthogonality describes the cross-talk

[*] A maybe obvious but important point is that biosensors do not directly measure the enzyme's activity but report the product (or substrate) concentration, which is used as a proxy for activity.

between the biosensor components and the native metabolism (Rantasalo et al., 2018). High orthogonality is generally desired, meaning that the biosensor has little to no effects on the native metabolism [*]. All the mentioned criteria are highly application-dependent and biosensor characteristics usually need to be optimized for the specific application at hand.

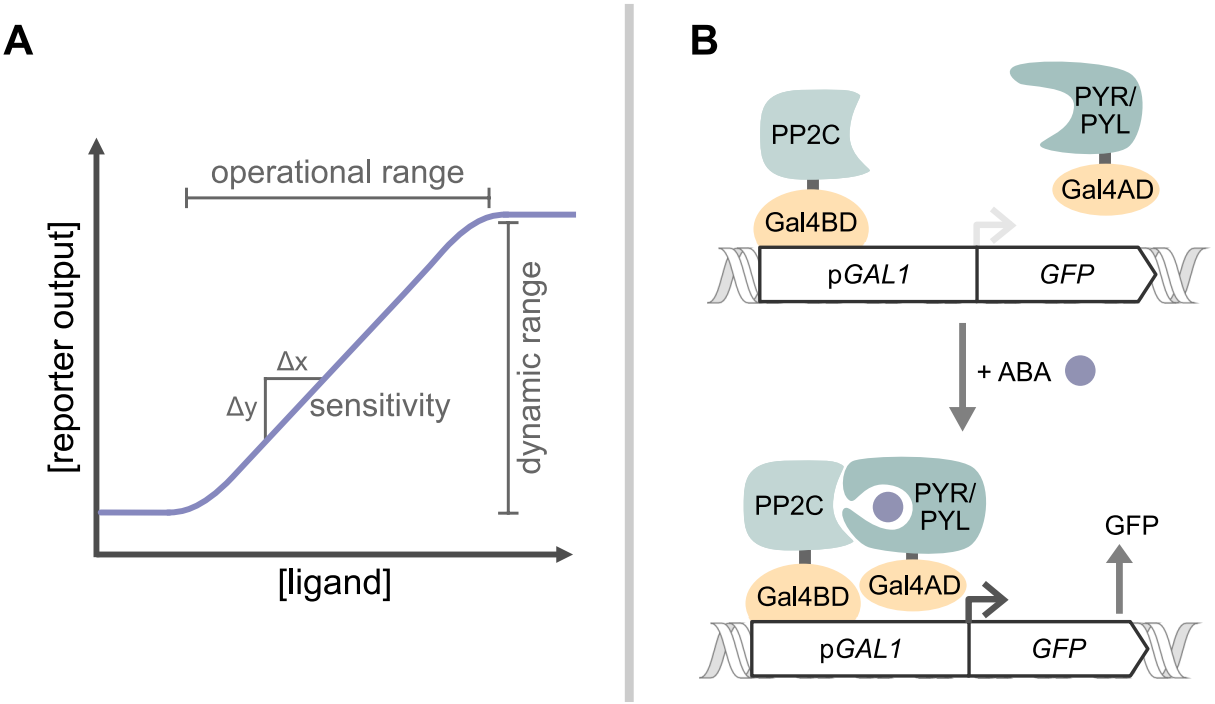



Figure 15: Biosensor schematics. A: Illustration of a biosensor dose-response curve highlighting important biosensor characteristics. B: Design schematic of the yeast-2 hybrid ABA biosensors characterised in Paper IV. The biosensor consists of two fusion proteins, one containing the Gal4 DNA binding domain (Gal4BD), the other containing the Gal4 activation domain (Gal4AD). Upon ABA binding the plant PYR/PYL receptor interacts with the plant protein phosphatase 2C (PP2C), the fusion proteins co-localize at the *GAL1* promoter and the GFP reporter is expressed.

In Paper IV, multiple candidates for whole-cell ABA biosensors were characterized. The biosensor candidates are based on engineered transcriptional activators, specifically utilizing the yeast 2-hybrid (Y2H) system (Fields and Song, 1989). The Y2H system was originally developed to identify protein-protein interactions and is still widely used across biological disciplines. The ABA biosensors use the split Gal4 activation domain (Gal4AD) and Gal4 DNA

[*] Orthogonality is sometimes defined slightly different in this context. It can also describe how transferable the biosensor design is to other organisms (Mahr and Frunzke, 2016). Interactions with the native metabolism are relevant for both definitions.

binding domain (Gal4BD) fused to a plant PYR/PYL receptor and a plant PP2C respectively [*] (Figure 15B). The plant proteins interact if ABA is present, which leads to co-localisation of the activation and DNA binding domain resulting in GFP expression. The most promising biosensor candidate was used to construct platform strains that are specifically engineered to screen BcABA1 and BcABA2 enzyme libraries. The ABA production in the platform strains is tuneable, allowing optimization of the biosensor output. Paper IV is summarized in Box 4.

Box 4: Summary of the main goals and findings of Paper IV.

**Aims and results of Paper IV:**

- Aim 1 - Characterize different ABA biosensor candidates
    - Genomic integration of the biosensor proteins reduced population heterogeneity
    - The time of measurement is a critical parameter and changes the biosensor response curve
    - The PYLcs-ABIcs$^{D413L}$ candidate showed the most suitable operational range and was used for constructing the screening platform strains
- Aim 2 - Develop platform strains optimized for BcABA1 or BcABA2 mutant screening
    - ABA production, and in extension the biosensor output, can be tuned by using the thiamine-repressible promoter p*THI4* for *bcaba1* or *bcaba2* expression
    - The time of measurement is again a critical parameter
- Aim 3 - Construct error-prone PCR libraries for *bcaba1* and *bcaba2*
    - Using a high-efficiency transformation protocol, large enzyme libraries were created, estimated to have $10^7$ and $10^6$ distinct variants of BcABA1 and BcABA2 respectively

[*] The interaction of PYR/PYL receptors and PP2Cs has historically been a "hot topic" in the research community. In 2009, two studies simultaneously described the interaction in plants (Ma et al., 2009; Park et al., 2009). In 2014, two reports independently described FRET-based ABA biosensors in plants that make use of the PYR/PYL-PP2C interaction (Jones et al., 2014; Waadt et al., 2014). In 2022, two groups changed the ligand specificity of PYR/PYL receptors to construct novel biosensors (Beltrán et al., 2022; Zimran et al., 2022).

## Enzyme libraries – Let's be irrational

In Paper II and Paper III, we demonstrated that additional gene copies of BcABA1 and BcABA2 lead to multi-fold increased ABA titers. However, expression of additional copies also resulted in a growth defect. Cellular resources are constraint, meaning that reallocation of precursor molecules and energy, e.g. amino acids and GTP for expressing a heterologous protein, ultimately comes at the expense of other processes (Björkeroth et al., 2020). Brute-force overexpression of enzymes is obviously not a sustainable engineering strategy to reach relevant titers, rates and yields. Engineering the native metabolism to improve enzyme activity, as was done in Paper III, is a more promising approach. However, eventually enzyme kinetics, for example its turnover number, impose a "hard" boundary for such optimization approaches. This is especially relevant for enzymes of the secondary metabolism, such as the BcABA enzymes, since they generally exhibit low catalytic efficiencies (Bar-Even et al., 2011).

Rational engineering of enzymes remains exceptionally difficult. Random mutagenesis, e.g. via error-prone PCR (epPCR), is a validated and more accessible approach to optimize proteins. In Paper IV, epPCR was used to construct mutant libraries for *bcaba1* and *bcaba2*, which were subsequently transformed into yeast. The resulting yeast libraries are estimated to contain $10^7$ and $10^6$ distinct enzyme variants, for BcABA1 and BcABA2 respectively. Libraries of this size are not screenable with conventional HPLC-MS (high performance liquid chromatography-mass spectrometry), which was used for ABA quantification in the previous papers. Instead, biosensors coupled with fluorescence activated cell sorting (FACS) are commonly used to rapidly screen millions of cells and isolate promising mutants within a few hours [*].

ABA can exit and enter the cell, which provides an additional challenge for high-throughput screening approaches. Even in non-producing cells (e.g. cells that do not express a functional *bcaba1/2* gene) the biosensor will be triggered since ABA produced by other cells can enter the non-producer. Therefore, mutants need to be cultivated separate from each other.

[*] The used HPLC-MS setup requires ≈ 10 min per ABA sample. However, a significant amount of time is spent on sample extraction, about 100 samples can be reasonably extracted per day and per person. FACS on the other hand requires minimal sample preparation and, depending on the instrument and desired sorting accuracy, the sample throughput is $10^6$ to $10^7$ per hour. However, FACS requires a functional biosensor and comes with its own challenges, such as high rates of false positives due to cellular noise (Raser and O'Shea, 2004; Liang et al., 2012) .

Droplet microfluidics provide a high throughput approach for encapsulating and cultivating cells in picolitre-scale compartments.

## Droplet microfluidics – Another very short introduction

Integrated circuits, and microelectronics in general, emerged in the 1960s (Kilby, 1964) as a promising technology that became the foundation of the digital revolution. The premise that miniaturization would not just reduce costs but also result in more efficient devices held true. Early work on microfluidic systems was interlinked with the developments in microelectronics, trying to achieve the same main goals (Convery and Gadegaard, 2019). Microfluidic devices use channels in the µm-scale or smaller and equally small volumes. An early application of microfluidics was the inkjet printer head (Sweet, 1965). A more mature version of the technology later permeated other disciplines such as analytical chemistry (Terry et al., 1979) and biochemistry (Woolley and Mathies, 1994). An obvious advantage of microfluidic devices is that they require less volume of precious samples or expensive reagents. Additionally, on micro-scale devices, physical phenomena such as laminar flow, shorter diffusion times, and dominance of capillary forces can be harnessed to increase efficiencies and automate processes (Manz et al., 1990; Convery and Gadegaard, 2019). So called “lab-on-a-chip” devices aim to miniaturize and parallelize virtually every liquid handling workflow, from PCR or -omics analysis to DNA assembly and transformations [*] (Mark et al., 2010; Szita et al., 2010; Linshiz et al., 2016).

Droplet microfluidics refers to the encapsulation of reactions or cells in droplets. Usually this involves combining two immiscible liquids, an aqueous phase and an oil phase, in a microfluidic chip. Different chip designs have been developed for droplet generation. In the so-called flow focusing chip design, the oil phase enters a perpendicular intersection from opposing sides “pinching” the aqueous stream to generate water-in-oil droplets. Pumps are being used to ensure consistent flow of the two phases. The aqueous phase can be water, buffer or media, while the oil phase often consists of hydrofluoroethers (HFEs). Compared to

[*] Unfortunately, we have not quite arrived in the miniaturized future yet. In a recent review, Convery and Gadegaard (2019) argue that lab-on-a-chip devices at this point remain an academic endeavor more accurately described as “chip-in-a-lab”. Echoing the discussion about standardized genetic parts earlier in this thesis, Convery and Gadegaard state that a lack of standardization is hindering lab-on-a-chip technologies.

conventional oils, HFEs allow for better gas transfer and exhibit lipophobic as well as hydrophobic properties, thereby preventing the exchange of organic molecules between droplets (C. Zhang et al., 2022). To stabilize the droplets, surfactants are used to reduce the droplet surface tension. Droplets can subsequently be captured, incubated and analysed. Isolating individual cells in droplets allows detailed analysis of nutrient consumption or of the production of extracellular metabolites. Furthermore, different organisms can be co-encapsulated to study or exploit their interactions (Siedler et al., 2017; Bowman et al., 2021).

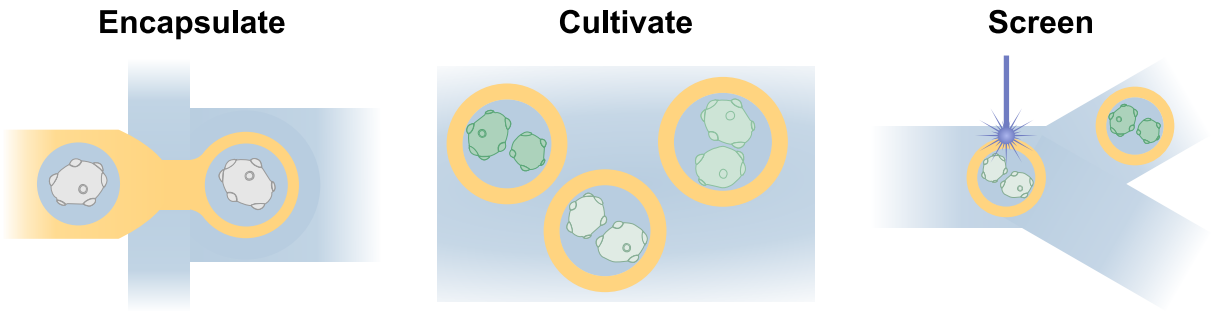



Figure 16: Droplet screening workflow. Cells of the enzyme library are encapsulated in droplets using a microfluidic device. The cells are cultivated separate from each other in the generated droplets. During the cultivation ABA is produced and the biosensor is activated. The biosensor output is used to screen the cell-containing droplets for high ABA producers.

The in Paper IV constructed BcABA1 and BcABA2 libraries could be screened using droplet microfluidics. Individual clones would be encapsulated in droplets, cultivated until sufficient ABA is produced to activate the biosensor and subsequently screened (Figure 16). Fluorescence-activated droplet sorting (FADS) could be used to screen droplets. This however would require specialised droplet sorting equipment. As an alternative, Brower and co-workers (2020a, 2020b) developed a method in which conventional FACS machines can be used to analyse and sort double-emulsion (DE) droplets. The method is called single droplet double emulsion FACS (sdDE-FACS). DE droplets consists of an inner aqueous core (media and cells) surrounded by an oil shell (HFE) that are suspended in an aqueous outer sheath (media or buffer). The outer aqueous solution is required for FACS analysis. DE droplets can be generated using a single-chip design in which two flow focusing intersections are set in series (Figure 17). At the first intersection, single-emulsion droplets (SE) are generated as described in the previous paragraph; at the second intersection, DE droplets are generated. The DE droplets generated by this method can be screened in a FACS machine, they are highly

uniform in volume and droplets without cells are stable for months according to the authors (Brower et al., 2020a; Brower et al., 2020b).

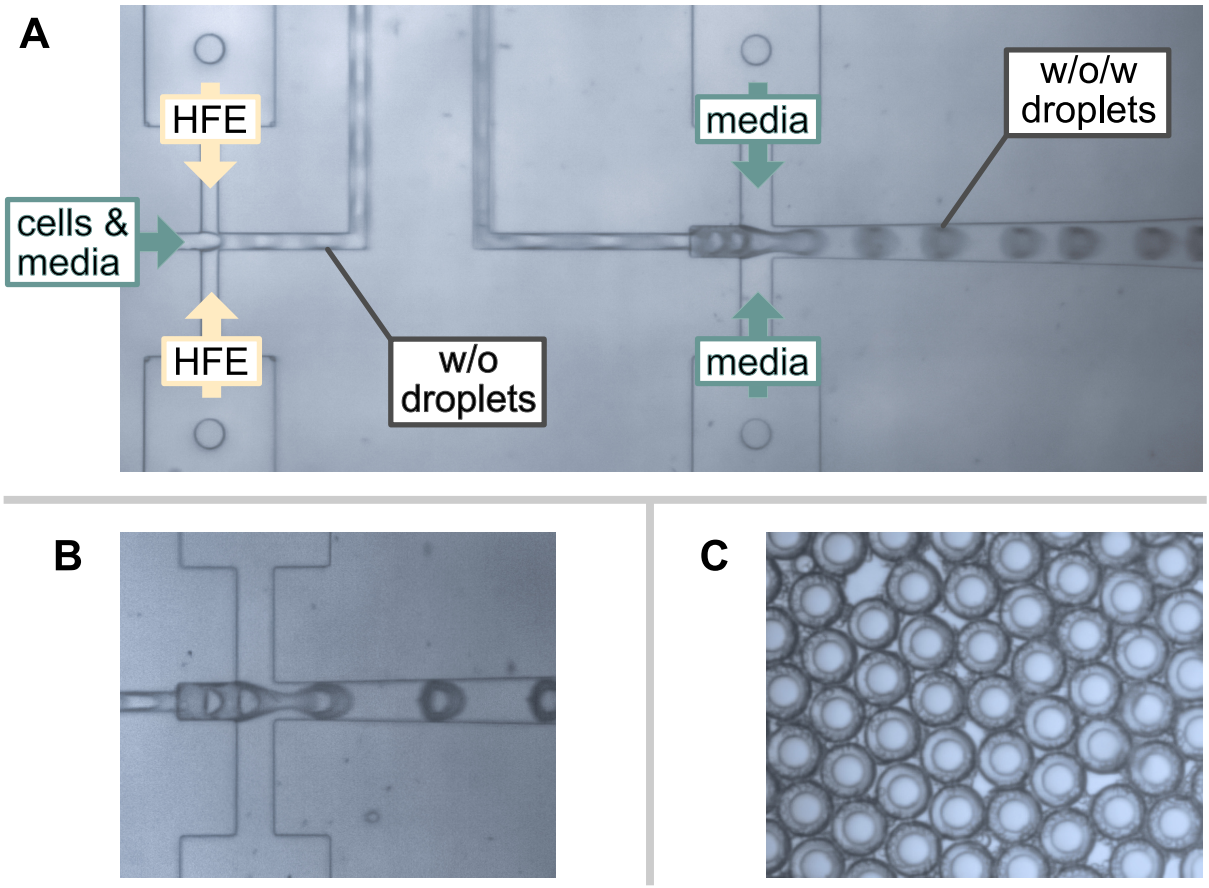



Figure 17: Double-emulsion droplet generation. A: Picture of the microfluidic chip. A dual flow focusing design (Brower et al., 2020b) is used to generate double-emulsion droplets. At the first intersection cells are encapsulated in water-in-oil (w/o) droplets, at the second intersection water-in-oil-in-water (w/o/w) droplets are generated. B: Close-up picture of the second intersection. C: Picture of the generated double emulsion droplets (without cells).

## Screening the BcABA libraries – It's all about the throughput

During my PhD studies, co-workers and I established the DE droplet workflow developed by Brower and co-workers at our division. Nonetheless, experiment-specific parameters, such as the time of measurement and oxygen availability, still require optimization before the libraries can be screened.

In Paper IV, we demonstrated how the biosensor output differs if ABA is added extrinsically or if ABA is produced in the cell. We furthermore showed that the biosensor response differs

at different measurement times. Nonetheless, the data gathered are not transferable to cultivation in DE droplets. Cultivation in DE droplets reduces growth rates and in extension affects ABA production rates and the biosensor output. Determining a suitable time point for the library sorting will be essential.

Oxygen transfer is limited in droplet cultivations even when fluorinated oils are being used (Mahler et al., 2015). Besides affecting growth, hypoxic conditions might also hamper ABA biosynthesis since BcABA1 and BcABA2 use molecular oxygen as substrate (Inomata et al., 2004). Additionally, GFP, which is being used as the biosensor's reporter, is only fluorescent after autooxidation (Heim et al., 1994). Oxygen limitations have been shown to affect fluorescence in SE droplets (Siedler et al., 2017). Initial experiments with control strains that constitutively express GFP showed that fluorescence is reduced in DE droplets (data not shown). Oxygen availability could be improved via agitation; however, it is unclear how resilient the droplets are to shear forces. Droplet incubator setups could also be explored (Mahler et al., 2015).

Can $10^7$ cells be encapsulated and screened in a reasonable timeframe using the sdDE-FACS workflow? Droplets can be generated at a rate of 1 to 10 kHz (Brower et al., 2020a), for this back-of-the-envelope calculation a 5 kHz generation rate is assumed. However, not all the droplets will contain cells. Droplet occupancy, the percentage of generated droplets that contain cells, follows a Poisson distribution (Collins et al., 2015). The higher the cell concentration is, the higher is the droplet occupancy, but the more droplets will contain multiple cells. Assuming a single-cell droplet occupancy of 5% (resulting in ≈ 0.1% droplets that contain more than one cell), it would take > 10 h of continuous droplet generation to encapsulate $10^7$ cells. This calculation disregards the need to oversample to increase the likelihood that all library variants are included in the screening.

Besides droplet generation, the FACS analysis speed also limits throughput. Brower and co-workers (2020a) suggest restricting the event rate to 1000 events per second to avoid droplet breakage and ensure sort purity (regular FACS analysis can be performed at 12,000 events per second). In an average droplet FACS run ≈ 50% of the detected events are droplets, the remaining half being debris (Brower et al., 2020b). Taking this and the previous calculation regarding droplet occupancy into account, 25 cells can be screened per second, meaning that

> 100 h of continuous FACS analysis is required to screen $10^7$ cells (again neglecting oversampling).

Overall, the throughput limitations regarding droplet generation and analysis will likely not be detrimental for discovering improved BcABA1 and BcABA2 variants. While, screening the libraries in their entirety would yield the highest chance for success, improved enzymes were isolated from 100-fold smaller libraries before (Schendzielorz et al., 2014; Xiong et al., 2017) and screening a fraction of the BcABA libraries could suffice.

# Part III: Perspectives

## Engineering future ABA cell factories

There is a multitude of possible approaches to improve titer, rate and yield of the ABA cell factory. Screening of the *bcaba1* and *bcaba2* epPCR libraries will hopefully lead to the discovery of improved enzyme variants. At this stage, various aspects could impede BcABA enzyme activity in yeast. Enzymatic parameters like $k_{cat}$ and $K_M$ could limit ABA production, but translation efficiency, folding or degradation of the heterologous proteins could also play a role. It was demonstrated that even synonymous mutations can affect mRNA levels, translation rate and folding (Zalucki et al., 2009; Zhou et al., 2015; Chaney et al., 2017; Shen et al., 2022). Random mutagenesis libraries are naïve and likely contain variants that are altered in any of the mentioned traits. Discovered variants might not necessarily exhibit improved catalytic efficiency but instead be optimized for functional expression in yeast specifically. Promising mutations from different mutants can be combined to determine potential synergies. As shown in Figure 10, the discovered enzymes could subsequently be transferred to the *PAH1* knockdown strain constructed in Paper III for further performance analysis.

Incorrect processing of heterologous CYPs can result in them being oriented towards the ER lumen instead of towards the cytosol (Galanie et al., 2015). This can severely affect their activity (Galanie et al., 2015). So far, the localization of BcABA1 and BcABA2 has not been investigated but would be worthwhile. In addition, the CYPs could be improved by modifying their N-terminus, to render them water-soluble (Schoch et al., 2003; Chen et al., 2017). Another validated strategy to increase catalytic efficiency is the construction of artificial CYP-CPR fusions (Yabusaki, 1995; Zhao et al., 2016). These strategies could be investigated in small-scale rational engineering approaches. For the analysis, the newly characterized ABA biosensor could be utilized in a microtiter plate format, instead of relying on HPLC-MS measurements.

Nonetheless, the ABA biosensor combined with microfluidic droplet sorting opens up a range of possibilities for large-scale library screenings. As an alternative to constructing CYP-CPR fusions, a combinatorial library with promoters of different strength can be used to fine-tune the expression levels of the CYPs and the CPR. In Paper III and Paper IV, a growth defect was observed in strains with additional copies of *bcaba1* and *bcaba2*. Fine-tuning the expression levels could improve the electron transfer efficiencies between CYP and CPR, and prevent

formation of reactive oxygen species (Paddon et al., 2013; Zangar, 2004). With a similar approach the expression levels of the four ABA pathway genes could be fine-tuned and optimized. In this context, pathway scaffolding could also be explored. In this approach the enzymes of a biosynthetic pathway are co-localized to increase local concentrations of intermediates and prevent the formation of side-products (Siu et al., 2015). Furthermore, various libraries focusing on the native yeast metabolism could be screened, such as overexpression, knockout, CRISPR activation or CRISPR interference libraries. GEMs could be used to design "smart" libraries including only the most promising engineering targets. For some of these libraries it might be preferable to separate the ABA sensing and ABA production tasks. This could reduce the metabolic burden and metabolic imbalances present in extensively engineered cells. Droplet microfluidics can be used to co-encapsulate sensing and producing strains, and studies confirm the feasibility of this approach (Siedler et al., 2017; Bowman et al., 2021).

Besides screening applications, the biosensor can be used to build genetic circuits, such as feed-back or feed-forward loops. Genetic circuits can be employed to dynamically regulate metabolic fluxes in cell factories (David et al., 2016; Tan and Prather, 2017). Once the productivity of the current ABA cell factory has been improved, dynamic regulation could be a promising approach for bioreactor cultivations, e.g. to separate growth and production phases.

## ABA cell factory hosts

In a recent study, *Yarrowia lipolytica* was engineered for the heterologous production of ABA (Arnesen et al., 2022). The oleaginous yeast *Y. lipolytica* is seen as a promising host for terpenoid cell factories due to a presumed high acetyl-CoA flux (Arnesen and Borodina, 2022). This poses the question which microbial host is the most promising for the production of ABA: the natural producer *B. cinerea*, the non-conventional yeast *Y. lipolytica* or the model organism *S. cerevisiae* [*].

[*] *Escherichia coli* has also been used for terpene production before (Wang et al., 2018). However, *E. coli* is an unsuitable host for expressing eukaryotic CYPs, as was demonstrated for heterologous artemisinic acid production (Paddon and Keasling, 2014). Takino and co-workers (2019) used *Aspergillus oryzae* as a heterologous chassis to elucidate the ABA pathway, making the organism another potential ABA cell factory

So far, *B. cinerea* has been used for the biotechnological production of ABA and high titers of over 1 g/L in 10 day fed-batch cultivations have been reported (Gong et al., 2014), but important details for calculating carbon yields are missing. In small-scale cultivations with mineral media, *S. cerevisiae* and *Y. lipolytica* appear to produce very similar ABA/glucose yields of ≈ 0.6 mg/g (Paper II and Arnesen et al., 2022). In terms of production rate, it seems likely that *S. cerevisiae* and *Y. lipolytica* will outperform *B. cinerea* in industrial fermentations since the yeast species grow faster.

As in *S. cerevisiae*, BcABA enzyme activity is currently limiting ABA production in *Y. lipolytica* (to my knowledge no bottlenecks are described for *B. cinerea*). Consequently, it is essential to improve these enzymes, before considering the metabolic flux of the precursor pathway. For this purpose, *S. cerevisiae* is the most promising candidate due to its ease of engineering and its vast knowledge base. *S. cerevisiae* excels at rapid prototyping, and numerous examples of successful high-throughput screenings using biosensors are published. Performing similar approaches in *Y. lipolytica* is of course possible but would likely require more time and resources. A recent review identifies various challenges for *Y. lipolytica* terpenoid cell factories, including fewer engineering tools, limited understanding of its physiology and a lack of characterized regulatory elements like metabolite-responsive transcription factors (Zhang et al., 2022). Improved BcABA enzymes discovered in *S. cerevisiae* could subsequently be transferred and tested in *Y. lipolytica* or *B. cinerea* ABA-overproducers used in industrial setups. In this case, *S. cerevisiae* would serve more as a screening platform to improve other cell factories, since high throughput screening capabilities are lacking for *B. cinerea*. Similarly, rational engineering targets tested in *S. cerevisiae*, like the ones described in Paper III, could be investigated in *B. cinerea* to improve existing processes.

Redirecting carbon flux towards cytosolic acetyl-CoA and the MVA pathway is challenging in both yeast species. By nature, *S. cerevisiae* exhibits a high metabolic flux towards ethanol production [*] (Gambacorta et al., 2020), whereas in *Y. lipolytica*, flux is directed towards lipid biosynthesis (Zhang et al., 2022). A similar engineering challenge might be posed by the

candidate. Compared to *S. cerevisiae*, an extensive knowledge base about *A. oryzae* is missing and the organism is more difficult to engineer.

[*] Eliminating ethanol production in *S. cerevisiae* with the goal of redirecting metabolic flux has been achieved (Dai et al., 2018), but it remains to be seen if this is a suitable approach for industrial cell factories.

regulation of ABA biosynthesis in *B. cinerea*. Since ABA appears to fulfil important physiological functions, metabolic flux through its biosynthetic pathway is likely highly regulated. However, this regulation might have already been altered in the UV-mutagenized ABA-overproducer.

Another important factor to consider in the choice of chassis is the feedstock that can be used in biotechnological processes. Glucose is expensive and its production requires the use of arable land that could otherwise be used for food production. Ideally, biorefineries utilize abundant waste products, such as xylose from lignocellulosic biomass, to produce chemicals. *S. cerevisiae* is not able to utilize xylose natively; however, heterologous xylose utilization has been a major engineering focus in recent years (Kwak and Jin, 2017; Li et al., 2019). *Y. lipolytica* can metabolize xylose but the activities of involved enzymes are suboptimal at best and more research is required to utilize the carbon source efficiently (Lee et al., 2021). *B. cinerea* can also utilize xylose natively (Gentile, 1954). Nevertheless, for the fed-batch cultivations media optimized for ABA production was used (containing glucose, sucrose, yeast extract and soybean meal) (Gong et al., 2014). It is questionable how readily the fungal cell factory could switch to other carbon sources and still produce similar ABA titers [*].

All the hosts have potential, and, at this stage, it would be ill-advised to dismiss one of them over another. Research in any of them is likely beneficial for the other ABA cell factories. The required TRY metrics to make a process economically viable are highly dependent on the use of the product. Lower titer, rate and yield can be acceptable for high-value products such as pharmaceuticals, whereas agrochemicals that are deployed on large scales need to be produced at low cost in high amounts. ABA could embody both, a high- and a low-value product.

## A critical perspective on ABA applications

ABA could find applications in agriculture, medicine or nutrition. Food security is a major concern today and its relevance will increase in the future (Anderson et al., 2020). ABA could be used to prevent crop loss (e.g. due to droughts) or as a growth modulator (resulting in

[*] In the context of carbon and energy sources it is worth noting that ABA-producing microalgae could provide an autotrophic cell factory chassis. Microalgae biotechnology comes with its own challenges however (Khan et al., 2018).

improved yields). For its wide-spread use in agriculture, ABA would need to be produced cheaply and in large quantities, posing a major engineering challenge in terms of TRY metrics. Since ABA is a conserved signalling molecule in various organisms, its wide-spread use could affect ecosystems in unexpected ways. More research is required on this topic, but extensive use of ABA could make crop plants more susceptible to certain pathogens (Edwards, 1983; Gietler et al., 2020; Li and Heath, 1990). Another issue with agricultural applications is the light sensitivity of the molecule. The half-life of ABA under mild UV exposure is under 30 min (Cao et al., 2013), impeding its utility in agriculture. One way to address this issue is adding photo protectants to ABA-containing products (Gao et al., 2016).

Instead of the naturally occurring molecule, synthetic ABA agonists could be used in agriculture. ABA agonists can exhibit better half-life and can trigger ABA-mediated effects more selectively by binding only a subset of PYR/PYL receptors (Dejonghe and Cutler, 2019). To the best of my knowledge no ABA agonists have been commercialized yet. Their industrial production would however depend on fossil carbon-derived chemicals. Many ABA agonists differ substantially from ABA with regard to their structure. Nonetheless, there are structural ABA analogues that are more stable towards UV radiation while retaining biological activity (Wenjian et al., 2013). In theory, synthetic ABA analogues could be produced in yeast by engineering enzymes and/or following a retrobiosynthesis approach (Coelho et al., 2013; Heel et al., 2014; Hadadi and Hatzimanikatis, 2015).

It remains to be seen which medical applications of ABA hold up in human trials, but the variety and number of potential uses encourage optimism. Pharmaceuticals are generally low-volume high-value products allowing lower titers, rates and yields to still be profitable. The fact that ABA shows pharmaceutical effects at low doses possibly decreases these requirements (Magnone et al., 2015). In contrast to *B. cinerea*, which can cause allergies (Jurgensen and Madsen, 2009), *S. cerevisiae* is an established and save host for drug production (Nielsen, 2013). Similar to the use of analogues in agriculture, (yet-to-be-found) ABA analogues with increased potency or other desirable medicinal characteristics could be produced in yeast. In fact, yeast could potentially be used to screen for biologically active ABA analogues (Pausch, 1997; Hughes, 2002). Furthermore, ABA could find applications as a dietary supplement if prophylactic properties are confirmed, and no unwanted side-effects

are observed [*]. Further analysis is required to assess the performance of ABA as a bitter taste receptor blocker in comparison to other products that are already on the market (Singh et al., 2022). Its potential nutritional value could make ABA a preferable choice if produced in a cost-competitive manner.

## Metabolic engineering and the bioeconomy

To prevent the most severe effects of climate change, we need to drastically reduce our carbon emissions as soon as possible. In part, this could be achieved by replacing petroleum-based production processes with more sustainable biotechnological processes. High expectations have been put on metabolic engineering in this regard. However, the discrepancy between the numerous cell factories that are developed in academic laboratories and the limited number of cell factories that are currently used in industrial settings is striking. Metabolic engineering, and to some extend synthetic biology, have been criticized to mainly focus on individual proof-of-concept studies (Yadav et al., 2012), which arguably generate little transferable knowledge. My research started as a very application-oriented project (chronologically Paper II was the first project I worked on), but I nevertheless hope that some of the gathered insights and tools are of use beyond the ABA cell factory.

The toolkit developed in Paper I will hopefully be useful to the yeast genetic engineering community in general and simplify common engineering tasks. Combinatorial libraries, if coupled with a high-throughput screening strategy, could be particularly useful to generate new insights since a high degree of diversity can be created rapidly. The selection cassette provided in the toolkit will facilitate their construction. Paper II is a proof-of-concept study. Nonetheless, the fact that the *S. cerevisiae* CPR appears to be compatible with *B. cinerea* CYPs could be of interest to other studies. At this stage, Paper III is lacking evidence about how the ER proliferation targets affect membrane structure and benefit ABA production. Detailed analysis of electron microscopy pictures and/or analysis of lipid profiles could help elucidate

[*] ABA-producing *S. cerevisiae* strains might be used to fortify fermentation products like nutritional yeast, or even bread or beer. Even the low titers produced in the current strains could suffice for this purpose. Open questions regarding the thermostability of ABA would need to be considered in some cases. *Aspergillus oryzae* is used for the production of soy sauce and miso paste. An ABA-producing strain, like the one constructed by Takino and co-workers (2019), could be utilized in these fermentations. This would, however, require a shift in attitude towards GMOs in food products, specifically in the European Union.

the phenotypes. Ideally, the targets would be investigated in a side-by-side comparison in various cell factories expressing membrane-bound proteins to facilitate our understanding of ER proliferation for metabolic engineering purposes. The main goal of Paper IV was to explore the concept of screening platform strains with tuneable production. While the results look promising, the constructed libraries will need to be screened to validate the usefulness of this approach. Gathered insights could be transferable to other Y2H-based biosensors (Beltrán et al., 2022; Scott et al., 2022; Zimran et al., 2022).

Metabolic engineering would benefit from a more systematic approach and, in my opinion, key elements of such an approach are the use of standards (including but not limited to genetic parts) and more emphasis on understanding the phenotypes. In other words, putting a greater focus on the Learn phase as well as basic research. A central issue in metabolic engineering studies is that underperforming strains are usually discarded without further analysis and even improved strains are often not analysed in terms of why they perform better. Investigating the causalities of phenotypes would facilitate the rational design of cell factories, result in valuable information for developing GEMs and other models and, in the long term, enable a bottom-up engineering approach. However, this shift away from proof-of-concept and optimization studies would require a change of incentives. To publish in high-impact journals, metabolic engineers are incentivised to chase TRY metrics, whereas the time and resources invested in understanding phenotypes or investigating transferability is not rewarded to a similar degree [*]. A shift back to more primary research has also been advocated for in other disciplines (Thorp and Yaffe, 2023). Thorp and Yaffe (2023) argue that neglecting basic science will come at the expense of future applications and should be seen as a long-term investment instead.

Nevertheless, there are other hurdles to the bioeconomy that cannot be solved by either applied or basic academic research. One of which is the, often subsidised, low price of fossil carbon, masking its true costs in terms of environmental damage (Parry et al., 2021) and resulting in a lack of monetary incentives for companies to switch to more sustainable production processes. In addition, companies would be required to make large initial

[*] Another argument for reconsidering the objectives of academic research is the fact that robots appear to be much better suited for chasing TRY metrics than humans (Singh et al., 2023).

investments in R&D (e.g. for scaling up fermentations) and infrastructure (e.g. manufacturing plants). These challenges require a political solution instead of a scientific one.

## Science fiction

Cell factories have immense potential for the sustainable production of virtually any molecule, from structurally simple commodity chemicals to highly complex speciality chemicals. The choice of product is not restricted to naturally occurring molecules (Agostini et al., 2017; Jung et al., 2010; Zhang et al., 2010). Indeed, engineered enzymes can catalyse reactions *in vivo* that were never observed in nature before and that hitherto were only accessible through synthetic chemistry (such as the formation of carbon-silicon bonds for example) (Kan et al., 2016; Yang and Arnold, 2021). Recent advances in metabolic engineering and synthetic biology have enabled novel ways to design, engineer and understand biological systems. In the (hopefully not-too-distant) future, synthetic minimal cells could provide the foundation of a bottom-up engineering approach. *De nov*o designed pathways could then be used to adapt these chassis for specific purposes. Early research indicates that even highly complex traits, like the ability to perform photosynthesis, could become a transferable "engineering module" (Cournoyer et al., 2022). Furthermore, inorganic-biological hybrid systems (Guo et al., 2018) might require us to recontextualize the basic building blocks of cell factories.

# Acknowledgments

Looking back on the last 4+ years is a quite a surreal experience. To me it feels like my first day was yesterday and at the same time it feels like a lifetime has passed. There are numerous people that have contributed to this thesis in so many ways, many of which had nothing to do with science.

Verena, I am glad you did *Take a Chance on Me* with the ABA project [*]. Thank you for giving me the freedom to explore my scientific interests and for putting the trust in me that had to go along with it. Your openness and genuine interest in your students' success will always be greatly appreciated. Florian, I suppose I am a bit of a sceptic at heart and your positivity and optimism was indispensable at times. It has been a pleasure to work with both of you and I truly think I could not have found a better fitting supervisor duo.

Of course, none of the projects in this thesis would have been possible without the help of my big-brained collaborators. Yassi, I learned a lot from you, but more importantly working with you has always been great fun. I was lucky to have had you as a lab buddy and to still have you as a friend. Christos, thank you for initiating the MoClo project; it would not have happened without you and I really needed a break from ABA. Michi and Johan, thank you for joining the project without hesitations and bringing good vibes as well as new perspectives with you. Thank you, Xin, Jerry, Jing and Xiaozhi for joining me on the microfluidic adventure. Also, special thanks to Håkan for his expertise and hands-on support in this matter. Paulo and Maria, your help with my first-ever paper is much appreciated. Dany, thank you for writing the book chapter with me; it was a very welcome distraction during the early COVID days. Angelica, Emelie, Marie, Yili, Abder, Gunilla, Erica and Martina, I am very grateful for all your help and support over the years.

My, Mauri and Olha, the three of you have been with me since the very beginning of my stay at Sysbio. You are not just incredibly kind and fun to be around but spending time with you also made me feel at home somehow, thank you! A big thank you to my long-term office mates and friends John, Veronica, Oliver, Dany and Xiaowei. Having you around to discuss

[*] ba dum tss

science, have fika, vent frustrations, celebrate successes or just hang out has been a great pleasure.

I feel very lucky to have stumbled into Sysbio. It is the people that make this place truly special and I have many fond memories of afterworks, conferences, game nights, quizzes, mushrooming, parties and dinners. Thank you all for making my stay here so memorable and rewarding (and forgive me for not mentioning you by names).

All PhD studies have their ups and downs, and mine were not an exception. I could, however, always rely on you to bring me back on my feet, Tereza. I am incredibly thankful for your unwavering encouragement, patience and understanding. I believe I owe you my sanity for keeping my mind off work at times. Last but certainly not least, I would like to thank my mum, dad and brother. No matter what I set my mind to, I could always count on your unconditional and never-ending support for which I am forever grateful. None of this would have been possible without you.

# References


Addicott, F.T., Lyon, J.L., Ohkuma, K., Thiessen, W.E., Carns, H.R., Smith, O.E., Cornforth, J.W., Milborrow, B.V., Ryback, G., Wareing, P.F., 1968. Abscisic Acid: A New Name for Abscisin II (Dormin). Science 159, 1493–1493. https://doi.org/10.1126/science.159.3822.1493.b

AdventHealth Translational Research Institute, 2022. Abscisic Acid Effects on Glucose Homeostasis and Insulin Sensitivity (Clinical trial registration No. NCT04722354). clinicaltrials.gov.

Agostini, F., Völler, J.-S., Koksch, B., Acevedo-Rocha, C.G., Kubyshkin, V., Budisa, N., 2017. Biocatalysis with Unnatural Amino Acids: Enzymology Meets Xenobiology. Angewandte Chemie International Edition 56, 9680–9703. https://doi.org/10.1002/anie.201610129

Anderson, J.C., Clarke, E.J., Arkin, A.P., Voigt, C.A., 2006. Environmentally Controlled Invasion of Cancer Cells by Engineered Bacteria. Journal of Molecular Biology 355, 619–627. https://doi.org/10.1016/j.jmb.2005.10.076

Anderson, J.C., Dueber, J.E., Leguia, M., Wu, G.C., Goler, J.A., Arkin, A.P., Keasling, J.D., 2010. BglBricks: A flexible standard for biological part assembly. Journal of Biological Engineering 4, 1. https://doi.org/10.1186/1754-1611-4-1

Anderson, R., Bayer, P.E., Edwards, D., 2020. Climate change and the need for agricultural adaptation. Current Opinion in Plant Biology, Biotic interactions 56, 197–202. https://doi.org/10.1016/j.pbi.2019.12.006

Arendt, P., Miettinen, K., Pollier, J., De Rycke, R., Callewaert, N., Goossens, A., 2017. An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metabolic Engineering 40, 165–175. https://doi.org/10.1016/j.ymben.2017.02.007

Arnesen, J.A., Borodina, I., 2022. Engineering of Yarrowia lipolytica for terpenoid production. Metabolic Engineering Communications 15, e00213. https://doi.org/10.1016/j.mec.2022.e00213

Arnesen, J.A., Jacobsen, I.H., Dyekjær, J.D., Rago, D., Kristensen, M., Klitgaard, A.K., Randelovic, M., Martinez, J.L., Borodina, I., 2022. Production of abscisic acid in the oleaginous yeast Yarrowia lipolytica. FEMS Yeast Research 22, foac015. https://doi.org/10.1093/femsyr/foac015

Atkinson, F.S., Villar, A., Mulà, A., Zangara, A., Risco, E., Smidt, C.R., Hontecillas, R., Leber, A., Bassaganya-Riera, J., 2019. Abscisic Acid Standardized Fig (Ficus carica) Extracts Ameliorate Postprandial Glycemic and Insulinemic Responses in Healthy Adults. Nutrients 11, 1757. https://doi.org/10.3390/nu11081757

Audenaert, K., Meyer, G.B.D., Höfte, M.M., 2002. Abscisic Acid Determines Basal Susceptibility of Tomato to Botrytis cinerea and Suppresses Salicylic Acid-Dependent Signaling Mechanisms. Plant Physiology 128, 491–501. https://doi.org/10.1104/pp.010605

Bajpai, P., 2021. Historical Perspectives, in: Bajpai, P. (Ed.), Developments in Bioethanol, Green Energy and Technology. Springer, Singapore, pp. 15–20. https://doi.org/10.1007/978-981-15-8779-5_2

Baker, M., 2016. 1,500 scientists lift the lid on reproducibility. Nature 533, 452–454. https://doi.org/10.1038/533452a

Ban, T., Ishimaru, M., Kobayashi, S., Goto-Yamamoto, N., Horiuchi, S., 2003. Abscisic acid and 2,4-dichlorophenoxyacetic acid affect the expression of anthocyanin biosynthetic pathway genes in 'Kyoho' grape berries. The Journal of Horticultural Science and Biotechnology 78, 586–589. https://doi.org/10.1080/14620316.2003.11511668

Bar-Even, A., Noor, E., Savir, Y., Liebermeister, W., Davidi, D., Tawfik, D.S., Milo, R., 2011. The Moderately Efficient Enzyme: Evolutionary and Physicochemical Trends Shaping Enzyme Parameters. Biochemistry 50, 4402–4410. https://doi.org/10.1021/bi2002289

Barrell, B.G., Bankier, A.T., Drouin, J., 1979. A different genetic code in human mitochondria. Nature 282, 189–194. https://doi.org/10.1038/282189a0

Beal, J., Goñi-Moreno, A., Myers, C., Hecht, A., Vicente, M. del C., Parco, M., Schmidt, M., Timmis, K., Baldwin, G., Friedrichs, S., Freemont, P., Kiga, D., Ordozgoiti, E., Rennig, M., Rios, L., Tanner, K., Lorenzo, V.,

Porcar, M., 2020. The long journey towards standards for engineering biosystems. EMBO reports 21, e50521. https://doi.org/10.15252/embr.202050521

Beaucage, S.L., Caruthers, M.H., 1981. Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Letters 22, 1859–1862. https://doi.org/10.1016/S0040-4039(01)90461-7

Beltrán, J., Steiner, P.J., Bedewitz, M., Wei, S., Peterson, F.C., Li, Z., Hughes, B.E., Hartley, Z., Robertson, N.R., Medina-Cucurella, A.V., Baumer, Z.T., Leonard, A.C., Park, S.-Y., Volkman, B.F., Nusinow, D.A., Zhong, W., Wheeldon, I., Cutler, S.R., Whitehead, T.A., 2022. Rapid biosensor development using plant hormone receptors as reprogrammable scaffolds. Nature Biotechnology 1–7. https://doi.org/10.1038/s41587-022-01364-5

Benner, S.A., Sismour, A.M., 2005. Synthetic biology. Nature Reviews Genetics 6, 533–543. https://doi.org/10.1038/nrg1637

Berche, P., 2012. Louis Pasteur, from crystals of life to vaccination. Clinical Microbiology and Infection 18, 1–6. https://doi.org/10.1111/j.1469-0691.2012.03945.x

Berens, M.L., Berry, H.M., Mine, A., Argueso, C.T., Tsuda, K., 2017. Evolution of Hormone Signaling Networks in Plant Defense. Annual Review of Phytopathology 55, 401–425. https://doi.org/10.1146/annurev-phyto-080516-035544

Björkeroth, J., Campbell, K., Malina, C., Yu, R., Di Bartolomeo, F., Nielsen, J., 2020. Proteome reallocation from amino acid biosynthesis to ribosomes enables yeast to grow faster in rich media. Proceedings of the National Academy of Sciences 117, 21804–21812. https://doi.org/10.1073/pnas.1921890117

Botstein, D., Fink, G.R., 2011. Yeast: An Experimental Organism for 21st Century Biology. Genetics 189, 695–704. https://doi.org/10.1534/genetics.111.130765

Bowman, E.K., Wagner, J.M., Yuan, S.-F., Deaner, M., Palmer, C.M., D'Oelsnitz, S., Cordova, L., Li, X., Craig, F.F., Alper, H.S., 2021. Sorting for secreted molecule production using a biosensor-in-microdroplet approach. Proceedings of the National Academy of Sciences 118, e2106818118. https://doi.org/10.1073/pnas.2106818118

Brower, K.K., Carswell-Crumpton, C., Klemm, S., Cruz, B., Kim, G., Calhoun, S.G.K., Nichols, L., Fordyce, P.M., 2020a. Double emulsion flow cytometry with high-throughput single droplet isolation and nucleic acid recovery. Lab on a Chip. https://doi.org/10.1039/d0lc00261e

Brower, K.K., Khariton, M., Suzuki, P.H., Still, C., Kim, G., Calhoun, S.G.K., Qi, L.S., Wang, B., Fordyce, P.M., 2020b. Double Emulsion Picoreactors for High-Throughput Single-Cell Encapsulation and Phenotyping via FACS. Analytical Chemistry 92, 13262–13270. https://doi.org/10.1021/acs.analchem.0c02499

Bruzzone, S., Ameri, P., Briatore, L., Mannino, E., Basile, G., Andraghetti, G., Grozio, A., Magnone, M., Guida, L., Scarfì, S., Salis, A., Damonte, G., Sturla, L., Nencioni, A., Fenoglio, D., Fiory, F., Miele, C., Beguinot, F., Ruvolo, V., Bormioli, M., Colombo, G., Maggi, D., Murialdo, G., Cordera, R., De Flora, A., Zocchi, E., 2012. The plant hormone abscisic acid increases in human plasma after hyperglycemia and stimulates glucose consumption by adipocytes and myoblasts. FASEB journal 26, 1251–1260. https://doi.org/10.1096/fj.11-190140

Bruzzone, S., Bodrato, N., Usai, C., Guida, L., Moreschi, I., Nano, R., Antonioli, B., Fruscione, F., Magnone, M., Scarfì, S., Flora, A., Zocchi, E., 2008. Abscisic Acid Is an Endogenous Stimulator of Insulin Release from Human Pancreatic Islets with Cyclic ADP Ribose as Second Messenger. Journal of Biological Chemistry 283, 32188–32197. https://doi.org/10.1074/jbc.m802603200

Bruzzone, S., Moreschi, I., Usai, C., Guida, L., Damonte, G., Salis, A., Scarfì, S., Millo, E., Flora, A.D., Zocchi, E., 2007. Abscisic acid is an endogenous cytokine in human granulocytes with cyclic ADP-ribose as second messenger. Proceedings of the National Academy of Sciences 104, 5759–5764. https://doi.org/10.1073/pnas.0609379104

Burdach, K.F., 1800. Propädeutik zum Studium der gesammten Heilkunst, 1. Auflage. ed. Breitkopf & Härtel, Leipzig.

Cao, M., Liu, X., Zhang, Y., Xue, X., Zhou, X.E., Melcher, K., Gao, P., Wang, F., Zeng, L., Zhao, Y., Zhao, Y., Deng, P., Zhong, D., Zhu, J.-K., Xu, H.E., Xu, Y., 2013. An ABA-mimicking ligand that reduces water loss and promotes drought resistance in plants. Cell Research 23, 1043–1054. https://doi.org/10.1038/cr.2013.95

Carman, G.M., Han, G.-S., 2009. Regulation of phospholipid synthesis in yeast. Journal of Lipid Research 50, S69–S73. https://doi.org/10.1194/jlr.R800043-JLR200

Cases, I., de Lorenzo, V., 2005. Genetically modified organisms for the environment: stories of success and failure and what we have learned from them. International Microbiology 8, 213–222.

Chaney, J.L., Steele, A., Carmichael, R., Rodriguez, A., Specht, A.T., Ngo, K., Li, J., Emrich, S., Clark, P.L., 2017. Widespread position-specific conservation of synonymous rare codons within coding sequences. PLoS Computational Biology 13, e1005531. https://doi.org/10.1371/journal.pcbi.1005531

Chang, H.-J., Voyvodic, P.L., Zúñiga, A., Bonnet, J., 2017. Microbially derived biosensors for diagnosis, monitoring and epidemiology. Microbial Biotechnology 10, 1031–1035. https://doi.org/10.1111/1751-7915.12791

Chaqour, J., Lee, S., Ravichandra, A., Chaqour, B., 2018. Abscisic acid – an anti-angiogenic phytohormone that modulates the phenotypical plasticity of endothelial cells and macrophages. Journal of Cell Science 131, jcs210492. https://doi.org/10.1242/jcs.210492

Chen, X., Zhang, C., Too, H.-P., 2017. Multienzyme Biosynthesis of Dihydroartemisinic Acid. Molecules 22, 1422. https://doi.org/10.3390/molecules22091422

Chen, Y., Nielsen, J., 2016. Biobased organic acids production by metabolically engineered microorganisms. Current Opinion in Biotechnology, Food biotechnology / Plant biotechnology 37, 165–172. https://doi.org/10.1016/j.copbio.2015.11.004

Christenhusz, M.J.M., Byng, J.W., 2016. The number of known plants species in the world and its annual increase. Phytotaxa 261, 201. https://doi.org/10.11646/phytotaxa.261.3.1

Coelho, P.S., Brustad, E.M., Kannan, A., Arnold, F.H., 2013. Olefin Cyclopropanation via Carbene Transfer Catalyzed by Engineered Cytochrome P450 Enzymes. Science 339, 307–310. https://doi.org/10.1126/science.1231434

Cohen, A.C., Travaglia, C.N., Bottini, R., Piccoli, P.N., 2009. Participation of abscisic acid and gibberellins produced by endophytic Azospirillum in the alleviation of drought effects in maize. Botany 87, 455–462. https://doi.org/10.1139/B09-023

Collins, D.J., Neild, A., deMello, A., Liu, A.-Q., Ai, Y., 2015. The Poisson distribution and beyond: methods for microfluidic droplet production and single cell encapsulation. Lab on a Chip 15, 3439–3459. https://doi.org/10.1039/c5lc00614g

Convery, N., Gadegaard, N., 2019. 30 years of microfluidics. Micro and Nano Engineering 2, 76–91. https://doi.org/10.1016/j.mne.2019.01.003

Cournoyer, J.E., Altman, S.D., Gao, Y., Wallace, C.L., Zhang, D., Lo, G.-H., Haskin, N.T., Mehta, A.P., 2022. Engineering artificial photosynthetic life-forms through endosymbiosis. Nature Communications 13, 2254. https://doi.org/10.1038/s41467-022-29961-7

Crick, F., 1970. Central Dogma of Molecular Biology. Nature 227, 561–563. https://doi.org/10.1038/227561a0

Crick, F., 1968. The origin of the genetic code. Journal of Molecular Biology 38, 367–379. https://doi.org/10.1016/0022-2836(68)90392-6

Curvers, K., Seifi, H., Mouille, G., Rycke, R. de, Asselbergh, B., Hecke, A.V., Vanderschaeghe, D., Höfte, H., Callewaert, N., Breusegem, F.V., Höfte, M., 2010. Abscisic Acid Deficiency Causes Changes in Cuticle Permeability and Pectin Composition That Influence Tomato Resistance to Botrytis cinerea. Plant Physiology 154, 847–860. https://doi.org/10.1104/pp.110.158972

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., Abrams, S.R., 2010. Abscisic Acid: Emergence of a Core Signaling Network. Annu. Rev. Plant Biol. 61, 651–679. https://doi.org/10.1146/annurev-arplant-042809-112122

Czechowski, T., Weathers, P.J., Brodelius, P.E., Brown, G.D., Graham, I.A., 2020. Editorial: Artemisinin—From Traditional Chinese Medicine to Artemisinin Combination Therapies; Four Decades of Research on the Biochemistry, Physiology, and Breeding of Artemisia annua. Frontiers in Plant Science 11.

Dai, Z., Huang, M., Chen, Y., Siewers, V., Nielsen, J., 2018. Global rewiring of cellular metabolism renders Saccharomyces cerevisiae Crabtree negative. Nature Communications 9, 3059. https://doi.org/10.1038/s41467-018-05409-9

Dar, N.A., Amin, I., Wani, W., Wani, S.A., Shikari, A.B., Wani, S.H., Masoodi, K.Z., 2017. Abscisic acid: A key regulator of abiotic stress tolerance in plants. Plant Gene 11, 106–111. https://doi.org/10.1016/j.plgene.2017.07.003

David, F., Nielsen, J., Siewers, V., 2016. Flux Control at the Malonyl-CoA Node through Hierarchical Dynamic Pathway Regulation in Saccharomyces cerevisiae. ACS Synthetic Biology 5, 224–233. https://doi.org/10.1021/acssynbio.5b00161

de Graeff, N., Jongsma, K.R., Johnston, J., Hartley, S., Bredenoord, A.L., 2019. The ethics of genome editing in non-human animals: a systematic review of reasons reported in the academic literature. Philosophical Transactions of the Royal Society B: Biological Sciences 374, 20180106. https://doi.org/10.1098/rstb.2018.0106

de Lorenzo, V., Schmidt, M., 2018. Biological standards for the Knowledge-Based BioEconomy: What is at stake. New Biotechnology, Bioeconomy 40, 170–180. https://doi.org/10.1016/j.nbt.2017.05.001

Dean, R., Van Kan, J. a. L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J., Foster, G.D., 2012. The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology 13, 414–430. https://doi.org/10.1111/j.1364-3703.2011.00783.x

Dejonghe, W., Cutler, S.R., 2019. Abscisic acid as a gateway for the crops of tomorrow. Advances in Botanical Research. https://doi.org/10.1016/bs.abr.2019.09.015

Devereux, S., Edwards, J., 2004. Climate Change and Food Security. IDS Bulletin 35, 22–30. https://doi.org/10.1111/j.1759-5436.2004.tb00130.x

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., Church, G.M., 2013. Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Research 41, 4336–4343. https://doi.org/10.1093/nar/gkt135

Dumitriu, A., May, A., Ata, Ö., Mattanovich, D., 2021. Fermenting Futures: an artistic view on yeast biotechnology. FEMS Yeast Research 21. https://doi.org/10.1093/femsyr/foab042

Eagles, C.F., Wareing, P.F., 1963. Dormancy Regulators in Woody Plants: Experimental Induction of Dormancy in Betula pubescens. Nature 199, 874–875. https://doi.org/10.1038/199874a0

Edwards, H.H., 1983. Effect of Kinetin, Abscisic Acid, and Cations on Host-Parasite Relations of Barley Inoculated with Erysiphe graminis f. sp. hordei. Journal of Phytopathology 107, 22–30. https://doi.org/10.1111/jph.1983.107.1.22

Elad, Y., Pertot, I., Cotes Prado, A.M., Stewart, A., 2016. Plant Hosts of Botrytis spp., in: Fillinger, S., Elad, Y. (Eds.), Botrytis – the Fungus, the Pathogen and Its Management in Agricultural Systems. Springer International Publishing, pp. 413–486. https://doi.org/10.1007/978-3-319-23371-0_20

Elshout, P.M.F., van Zelm, R., van der Velde, M., Steinmann, Z., Huijbregts, M.A.J., 2019. Global relative species loss due to first-generation biofuel production for the transport sector. GCB Bioenergy 11, 763–772. https://doi.org/10.1111/gcbb.12597

Emenecker, R.J., Strader, L.C., 2020. Auxin-Abscisic Acid Interactions in Plant Growth and Development. Biomolecules 10, 281. https://doi.org/10.3390/biom10020281

Endy, D., 2005. Foundations for engineering biology. Nature 438, 449–453. https://doi.org/10.1038/nature04342

Engler, C., Gruetzner, R., Kandzia, R., Marillonnet, S., 2009. Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes. PLoS ONE 4, e5553. https://doi.org/10.1371/journal.pone.0005553

Engler, C., Kandzia, R., Marillonnet, S., 2008. A One Pot, One Step, Precision Cloning Method with High Throughput Capability. PLOS ONE 3, e3647. https://doi.org/10.1371/journal.pone.0003647

Espinosa-Fernández, V., Mañas-Ojeda, A., Pacheco-Herrero, M., Castro-Salazar, E., Ros-Bernal, F., Sánchez-Pérez, A.M., 2019. Early intervention with ABA prevents neuroinflammation and memory impairment in a triple transgenic mice model of Alzheimer´s disease. Behavioural Brain Research 374, 112106. https://doi.org/10.1016/j.bbr.2019.112106

Fakas, S., 2017. Lipid biosynthesis in yeasts: A comparison of the lipid biosynthetic pathway between the model nonoleaginous yeast Saccharomyces cerevisiae and the model oleaginous yeast Yarrowia lipolytica. Engineering in Life Sciences 17, 292–302. https://doi.org/10.1002/elsc.201600040

Fields, S., Song, O., 1989. A novel genetic system to detect protein–protein interactions. Nature 340, 245–246. https://doi.org/10.1038/340245a0

Finkelstein, R., 2013. Abscisic Acid Synthesis and Response. The Arabidopsis Book 2013. https://doi.org/10.1199/tab.0166

Förster, J., Famili, I., Fu, P., Palsson, B.Ø., Nielsen, J., 2003. Genome-Scale Reconstruction of the Saccharomyces cerevisiae Metabolic Network. Genome Res. 13, 244–253. https://doi.org/10.1101/gr.234503

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.-Y., Cutler, S.R., Sheen, J., Rodriguez, P.L., Zhu, J.-K., 2009. In vitro Reconstitution of an ABA Signaling Pathway. Nature 462, 660–664. https://doi.org/10.1038/nature08599

Galanie, S., Thodey, K., Trenchard, I.J., Interrante, M.F., Smolke, C.D., 2015. Complete biosynthesis of opioids in yeast. Science 349, 1095–1100. https://doi.org/10.1126/science.aac9373

Gambacorta, F.V., Dietrich, J.J., Yan, Q., Pfleger, B.F., 2020. Rewiring yeast metabolism to synthesize products beyond ethanol. Current Opinion in Chemical Biology, Mechanistic Biology / Energy 59, 182–192. https://doi.org/10.1016/j.cbpa.2020.08.005

Gao, F., Hu, T., Tan, W., Yu, C., Li, Z., Zhang, L., Duan, L., 2016. Photoprotectant improves photostability and bioactivity of abscisic acid under UV radiation. Journal of Photochemistry and Photobiology B: Biology 158, 99–104. https://doi.org/10.1016/j.jphotobiol.2016.02.016

Gell-Mann, M., 1995. What is complexity? Remarks on simplicity and complexity by the Nobel Prize-winning author of The Quark and the Jaguar. Complexity 1, 16–19. https://doi.org/10.1002/cplx.6130010105

Gentile, A.C., 1954. Carbohydrate Metabolism and Oxalic Acid Synthesis by Botrytis cinerea. Plant Physiology 29, 257–261.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., André, B., Arkin, A.P., Astromoff, A., El Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K.-D., Flaherty, P., Foury, F., Garfinkel, D.J., Gerstein, M., Gotte, D., Güldener, U., Hegemann, J.H., Hempel, S., Herman, Z., Jaramillo, D.F., Kelly, D.E., Kelly, S.L., Kötter, P., LaBonte, D., Lamb, D.C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Ooi, S.L., Revuelta, J.L., Roberts, C.J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D.D., Sookhai-Mahadeo, S., Storms, R.K., Strathern, J.N., Valle, G., Voet, M., Volckaert, G., Wang, C., Ward, T.R., Wilhelmy, J., Winzeler, E.A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J.D., Snyder, M., Philippsen, P., Davis, R.W., Johnston, M., 2002. Functional profiling of the Saccharomyces cerevisiae genome. Nature 418, 387–391. https://doi.org/10.1038/nature00935

Gibson, D.G., Glass, J.I., Lartigue, C., Noskov, V.N., Chuang, R.-Y., Algire, M.A., Benders, G.A., Montague, M.G., Ma, L., Moodie, M.M., Merryman, C., Vashee, S., Krishnakumar, R., Assad-Garcia, N., Andrews-Pfannkoch, C., Denisova, E.A., Young, L., Qi, Z.-Q., Segall-Shapiro, T.H., Calvey, C.H., Parmar, P.P., Hutchison, C.A., Smith, H.O., Venter, J.C., 2010. Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome. Science 329, 52–56. https://doi.org/10.1126/science.1190719

Gietler, M., Fidler, J., Labudda, M., Nykiel, M., 2020. Abscisic Acid—Enemy or Savior in the Response of Cereals to Abiotic and Biotic Stresses? International Journal of Molecular Sciences 21, 4607. https://doi.org/10.3390/ijms21134607

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., Oliver, S.G., 1996. Life with 6000 Genes. Science 274, 546–567. https://doi.org/10.1126/science.274.5287.546

Gong, T., Shu, D., Zhao, M., Zhong, J., Deng, H.-Y., Tan, H., 2014. Isolation of genes related to abscisic acid production in Botrytis cinerea TB-3-H8 by cDNA-AFLP. Journal of Basic Microbiology 54, 204–214. https://doi.org/10.1002/jobm.201200311

Gong, Z., Nielsen, J., Zhou, Y.J., 2017. Engineering Robustness of Microbial Cell Factories. Biotechnology Journal 12, 1700014. https://doi.org/10.1002/biot.201700014

Gross, M., 2021. Magical mysteries of marine sponges. Current Biology 31, R51–R54. https://doi.org/10.1016/j.cub.2020.12.031

Guo, J., Suástegui, M., Sakimoto, K.K., Moody, V.M., Xiao, G., Nocera, D.G., Joshi, N.S., 2018. Light-driven fine chemical production in yeast biohybrids. Science 362, 813–816. https://doi.org/10.1126/science.aat9777

Guri, A.J., Evans, N.P., Hontecillas, R., Bassaganya-Riera, J., 2011. T cell PPAR γ is required for the anti-inflammatory efficacy of abscisic acid against experimental IBD. The Journal of nutritional biochemistry 22, 812–819. https://doi.org/10.1016/j.jnutbio.2010.06.011

Guri, A.J., Hontecillas, R., Si, H., Liu, D., Bassaganya-Riera, J., 2007. Dietary abscisic acid ameliorates glucose tolerance and obesity-related inflammation in db/db mice fed high-fat diets. Clinical Nutrition 26, 107–116. https://doi.org/10.1016/j.clnu.2006.07.008

Guri, A.J., Misyak, S.A., Hontecillas, R., Hasty, A., Liu, D., Si, H., Bassaganya-Riera, J., 2010. Abscisic acid ameliorates atherosclerosis by suppressing macrophage and CD4+ T cell recruitment into the aortic wall. The Journal of Nutritional Biochemistry 21, 1178–1185. https://doi.org/10.1016/j.jnutbio.2009.10.003

Hadadi, N., Hatzimanikatis, V., 2015. Design of computational retrobiosynthesis tools for the design of de novo synthetic pathways. Current Opinion in Chemical Biology 28, 99–104. https://doi.org/10.1016/j.cbpa.2015.06.025

Hardy, K., Buckley, S., Collins, M.J., Estalrrich, A., Brothwell, D., Copeland, L., García-Tabernero, A., García-Vargas, S., de la Rasilla, M., Lalueza-Fox, C., Huguet, R., Bastir, M., Santamaría, D., Madella, M., Wilson, J., Cortés, Á.F., Rosas, A., 2012. Neanderthal medics? Evidence for food, cooking, and medicinal plants entrapped in dental calculus. Naturwissenschaften 99, 617–626. https://doi.org/10.1007/s00114-012-0942-0

Hartung, W., 2010. The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. Functional Plant Biology 37, 806. https://doi.org/10.1071/FP10058

Hauser, F., Waadt, R., Schroeder, J.I., 2011. Evolution of Abscisic Acid Synthesis and Signaling Mechanisms. Current Biology 21, R346–R355. https://doi.org/10.1016/j.cub.2011.03.015

Heel, T., McIntosh, J.A., Dodani, S.C., Meyerowitz, J.T., Arnold, F.H., 2014. Non-natural Olefin Cyclopropanation Catalyzed by Diverse Cytochrome P450s and Other Hemoproteins. ChemBioChem 15, 2556–2562. https://doi.org/10.1002/cbic.201402286

Heim, R., Prasher, D.C., Tsien, R.Y., 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proceedings of the National Academy of Sciences of the United States of America 91, 12501–12504. https://doi.org/10.1073/pnas.91.26.12501

Henry, S.A., Kohlwein, S.D., Carman, G.M., 2012. Metabolism and Regulation of Glycerolipids in the Yeast Saccharomyces cerevisiae. Genetics 190, 317–349. https://doi.org/10.1534/genetics.111.130286

Hershey, A.D., Chase, M., 1952. Independent Functions of Viral Protein and Nucleic Acid in Growth of Bacteriaophage. Journal of General Physiology 36, 39–56. https://doi.org/10.1085/jgp.36.1.39

Hicks, D.R., Luckhart, S., Adams, L.G., Dehesh, K., Glennon, E.K.K., 2016. Supplementation with Abscisic Acid Reduces Malaria Disease Severity and Parasite Transmission. The American Journal of Tropical Medicine and Hygiene 94, 1266–1275. https://doi.org/10.4269/ajtmh.15-0904

Hicks, M., Bachmann, T.T., Wang, B., 2020. Synthetic Biology Enables Programmable Cell-Based Biosensors. ChemPhysChem 21, 132–144. https://doi.org/10.1002/cphc.201900739

Hofer, M., Lutolf, M.P., 2021. Engineering organoids. Nature Reviews Materials 6, 402–420. https://doi.org/10.1038/s41578-021-00279-y

Hontecillas, R., Roberts, P.C., Carbo, A., Vives, C., Horne, W.T., Genis, S., Velayudhan, B., Bassaganya-Riera, J., 2013. Dietary abscisic acid ameliorates influenza-virus-associated disease and pulmonary immunopathology through a PPARγ-dependent mechanism. The Journal of Nutritional Biochemistry 24, 1019–1027. https://doi.org/10.1016/j.jnutbio.2012.07.010

Houseknecht, K.L., Cole, B.M., Steele, P.J., 2002. Peroxisome proliferator-activated receptor gamma (PPARγ) and its ligands: A review. Domestic Animal Endocrinology 22, 1–23. https://doi.org/10.1016/S0739-7240(01)00117-5

Hu, B., Deng, F., Chen, G., Chen, X., Gao, W., Long, L., Xia, J., Chen, Z.-H., 2020. Evolution of Abscisic Acid Signaling for Stress Responses to Toxic Metals and Metalloids. Frontiers in Plant Science 11, 909. https://doi.org/10.3389/fpls.2020.00909

Hu, B., Zhao, X., Wang, E., Zhou, J., Li, J., Chen, J., Du, G., 2022. Efficient heterologous expression of cytochrome P450 enzymes in microorganisms for the biosynthesis of natural products. Critical Reviews in Biotechnology 0, 1–15. https://doi.org/10.1080/07388551.2022.2029344

Hughes, T.R., 2002. Yeast and drug discovery. Functional & Integrative Genomics 2, 199–211. https://doi.org/10.1007/s10142-002-0059-1

Inomata, M., Hirai, N., Yoshida, R., Ohigashi, H., 2004. The biosynthetic pathway to abscisic acid via ionylideneethane in the fungus Botrytis cinerea. Phytochemistry 65, 2667–2678. https://doi.org/10.1016/j.phytochem.2004.08.025

Ishchuk, O.P., Domenzain, I., Sánchez, B.J., Muñiz-Paredes, F., Martínez, J.L., Nielsen, J., Petranovic, D., 2022. Genome-scale modeling drives 70-fold improvement of intracellular heme production in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences 119, e2108245119. https://doi.org/10.1073/pnas.2108245119

Izquierdo-Bueno, I., González-Rodríguez, V.E., Simon, A., Dalmais, B., Pradier, J.-M., Le Pêcheur, P., Mercier, A., Walker, A.-S., Garrido, C., Collado, I.G., Viaud, M., 2018. Biosynthesis of abscisic acid in fungi: identification of a sesquiterpene cyclase as the key enzyme in Botrytis cinerea. Environmental Microbiology 20, 2469–2482. https://doi.org/10.1111/1462-2920.14258

Jackson, D.A., Symons, R.H., Berg, P., 1972. Biochemical Method for Inserting New Genetic Information into DNA of Simian Virus 40: Circular SV40 DNA Molecules Containing Lambda Phage Genes and the Galactose Operon of Escherichia coli. Proceedings of the National Academy of Sciences 69, 2904–2909. https://doi.org/10.1073/pnas.69.10.2904

Jacob, F., 1977. Evolution and Tinkering. Science 196, 1161–1166. https://doi.org/10.1126/science.860134

Jiang, K., Koob, J., Chen, X.D., Krajeski, R.N., Zhang, Y., Villiger, L., Zhou, W., Abudayyeh, O.O., Chen, F., Gootenberg, J.S., 2022. Programmable eukaryotic protein expression with RNA sensors. bioRxiv 2022.01.26.477951. https://doi.org/10.1101/2022.01.26.477951

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816–821. https://doi.org/10.1126/science.1225829

Jones, A.M., Danielson, J.A., Manojkumar, S.N., Lanquar, V., Grossmann, G., Frommer, W.B., 2014. Abscisic acid dynamics in roots detected with genetically encoded FRET sensors. eLife 3. http://dx.doi.org/10.7554/eLife.01741.001.

Jones, G.M., Stalker, J., Humphray, S., West, A., Cox, T., Rogers, J., Dunham, I., Prelich, G., 2008. A systematic library for comprehensive overexpression screens in Saccharomyces cerevisiae. Nature Methods 5, 239–241. https://doi.org/10.1038/nmeth.1181

Jung, Y.K., Kim, T.Y., Park, S.J., Lee, S.Y., 2010. Metabolic engineering of Escherichia coli for the production of polylactic acid and its copolymers. Biotechnology and Bioengineering 105, 161–171. https://doi.org/10.1002/bit.22548

Jurgensen, C.W., Madsen, A., 2009. Exposure to the airborne mould Botrytis and its health effects. Annals of agricultural and environmental medicine 16, 183–196.

Kachroo, A.H., Laurent, J.M., Yellman, C.M., Meyer, A.G., Wilke, C.O., Marcotte, E.M., 2015. Evolution. Systematic humanization of yeast genes reveals conserved functions and genetic modularity. Science 348, 921–925. https://doi.org/10.1126/science.aaa0769

Kan, S.B.J., Lewis, R.D., Chen, K., Arnold, F.H., 2016. Directed Evolution of Cytochrome c for Carbon–Silicon Bond Formation: Bringing Silicon to Life. Science 354, 1048–1051. https://doi.org/10.1126/science.aah6219

Karppinen, K., Tegelberg, P., Häggman, H., Jaakola, L., 2018. Abscisic Acid Regulates Anthocyanin Biosynthesis and Gene Expression Associated With Cell Wall Modification in Ripening Bilberry (Vaccinium myrtillus L.) Fruits. Frontiers in Plant Science 9.

Keasling, J.D., 2008. Synthetic Biology for Synthetic Chemistry. ACS Chemical Biology 3, 64–76. https://doi.org/10.1021/cb7002434

Kettner, J., Dörffling, K., 1995. Biosynthesis and metabolism of abscisic acid in tomato leaves infected with Botrytis cinerea. Planta 196. https://doi.org/10.1007/bf00197324

Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial Cell Factories 17, 36. https://doi.org/10.1186/s12934-018-0879-x

Kilby, J.S., 1964. Miniaturized electronic circuits. US3138743A.

Kim, J.-E., Jang, I.-S., Son, S.-H., Ko, Y.-J., Cho, B.-K., Kim, S.C., Lee, J.Y., 2019. Tailoring the Saccharomyces cerevisiae endoplasmic reticulum for functional assembly of terpene synthesis pathway. Metabolic Engineering 56, 50–59. https://doi.org/10.1016/j.ymben.2019.08.013

Kim, S.W., Goossens, A., Libert, C., Van Immerseel, F., Staal, J., Beyaert, R., 2020. Phytohormones: Multifunctional nutraceuticals against metabolic syndrome and comorbid diseases. Biochemical Pharmacology 175, 113866. https://doi.org/10.1016/j.bcp.2020.113866

Kimura, T., 木村拓平, Shirai, M., 白井真, Tsutsui, H., 筒井浩己, 1994. Production of natural abscisic acid. JPH06197775A.

Knight, T., 2003. Idempotent Vector Design for Standard Assembly of Biobricks. MIT Artificial Intelligence Laboratory; MIT Synthetic Biology Working Group.

Korolev, N., Rav David, D., Elad, Y., 2008. The role of phytohormones in basal resistance and Trichoderma-induced systemic resistance to Botrytis cinerea in Arabidopsis thaliana. BioControl 53, 667–683. https://doi.org/10.1007/s10526-007-9103-3

Kung, S.H., Lund, S., Murarka, A., McPhee, D., Paddon, C.J., 2018. Approaches and Recent Developments for the Commercial Production of Semi-synthetic Artemisinin. Frontiers in Plant Science 9.

Kützing, F., 1837. Microscopische Untersuchungen über die Hefe und Essigmutter, nebst mehreren andern dazu gehörigen vegetabilischen Gebilden. Journal für Praktische Chemie 11, 385–409. https://doi.org/10.1002/prac.18370110181

Kwak, S., Jin, Y.-S., 2017. Production of fuels and chemicals from xylose by engineered Saccharomyces cerevisiae: a review and perspective. Microbial Cell Factories 16, 82. https://doi.org/10.1186/s12934-017-0694-9

Le Page-Degivry, M.T., Bidard, J.N., Rouvier, E., Bulard, C., Lazdunski, M., 1986. Presence of abscisic acid, a phytohormone, in the mammalian brain. Proceedings of the National Academy of Sciences 83, 1155–1158. https://doi.org/10.1073/pnas.83.4.1155

Lee, J.W., Yook, S., Koh, H., Rao, C.V., Jin, Y.-S., 2021. Engineering xylose metabolism in yeasts to produce biofuels and chemicals. Current Opinion in Biotechnology 67, 15–25. https://doi.org/10.1016/j.copbio.2020.10.012

Lee, M.E., DeLoache, W.C., Cervantes, B., Dueber, J.E., 2015. A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. ACS Synthetic Biology 4, 975–986. https://doi.org/10.1021/sb500366v

Lee, S.C., Luan, S., 2012. ABA signal transduction at the crossroad of biotic and abiotic stress responses. Plant, Cell & Environment 35, 53–60. https://doi.org/10.1111/j.1365-3040.2011.02426.x

Leisen, T., Bietz, F., Werner, J., Wegner, A., Schaffrath, U., Scheuring, D., Willmund, F., Mosbach, A., Scalliet, G., Hahn, M., 2020. CRISPR/Cas with ribonucleoprotein complexes and transiently selected telomere vectors allows highly efficient marker-free and multiple genome editing in Botrytis cinerea. PLOS Pathogens 16, e1008326. https://doi.org/10.1371/journal.ppat.1008326

Leng, P., Yuan, B., Guo, Y., 2014. The role of abscisic acid in fruit ripening and responses to abiotic stress. Journal of Experimental Botany 65, 4577–4588. https://doi.org/10.1093/jxb/eru204

Lennox, J., 2021. Aristotle's Biology, in: Zalta, E.N. (Ed.), The Stanford Encyclopedia of Philosophy. Metaphysics Research Lab, Stanford University.

Li, A., Heath, M.C., 1990. Effect of plant growth regulators on the interactions between bean plants and rust fungi non-pathogenic on beans. Physiological and Molecular Plant Pathology 37, 245–254. https://doi.org/10.1016/0885-5765(90)90074-8

Li, X., Chen, Y., Nielsen, J., 2019. Harnessing xylose pathways for biofuels production. Current Opinion in Biotechnology 57, 56–65. https://doi.org/10.1016/j.copbio.2019.01.006

Liang, J.C., Chang, A.L., Kennedy, A.B., Smolke, C.D., 2012. A high-throughput, quantitative cell-based screen for efficient tailoring of RNA device activity. Nucleic Acids Research 40, e154–e154. https://doi.org/10.1093/nar/gks636

Lievens, L., Pollier, J., Goossens, A., Beyaert, R., Staal, J., 2017. Abscisic Acid as Pathogen Effector and Immune Regulator. Frontiers in Plant Science 8. https://doi.org/10.3389/fpls.2017.00587

Linshiz, G., Jensen, E., Stawski, N., Bi, C., Elsbree, N., Jiao, H., Kim, J., Mathies, R., Keasling, J.D., Hillson, N.J., 2016. End-to-end automated microfluidic platform for synthetic biology: from design to functional analysis. Journal of Biological Engineering 10, 3. https://doi.org/10.1186/s13036-016-0024-5

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews 23, 3–25. https://doi.org/10.1016/S0169-409X(96)00423-1

Liu, Q., Liu, Y., Li, G., Savolainen, O., Chen, Y., Nielsen, J., 2021. De novo biosynthesis of bioactive isoflavonoids by engineered yeast cell factories. Nature Communications 12, 6085. https://doi.org/10.1038/s41467-021-26361-1

Lu, H., Kerkhoven, E.J., Nielsen, J., 2022. Multiscale models quantifying yeast physiology: towards a whole-cell model. Trends in Biotechnology 40, 291–305. https://doi.org/10.1016/j.tibtech.2021.06.010

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., Grill, E., 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science 324, 1064–8. https://doi.org/10.1126/science.1172408

Maddox, B., 2003. The double helix and the "wronged heroine." Nature 421, 407–408. https://doi.org/10.1038/nature01399

Magner, L.N., 2002. A History of the Life Sciences, Revised and Expanded. CRC Press.

Magnone, M., Ameri, P., Salis, A., Andraghetti, G., Emionite, L., Murialdo, G., Flora, A.D., Zocchi, E., 2015. Microgram amounts of abscisic acid in fruit extracts improve glucose tolerance and reduce insulinemia in rats and in humans. The FASEB Journal 29, 4783–4793. https://doi.org/10.1096/fj.15-277731

Magnone, M., Sturla, L., Jacchetti, E., Scarfì, S., Bruzzone, S., Usai, C., Guida, L., Salis, A., Damonte, G., De Flora, A., Zocchi, E., 2012. Autocrine abscisic acid plays a key role in quartz-induced macrophage activation. The FASEB Journal 26, 1261–1271. https://doi.org/10.1096/fj.11-187351

Magyar, I., 2011. Chapter 6 - Botrytized Wines, in: Jackson, R.S. (Ed.), Advances in Food and Nutrition Research, Speciality Wines. Academic Press, pp. 147–206. https://doi.org/10.1016/B978-0-12-384927-4.00006-3

Mahler, L., Tovar, M., Weber, T., Brandes, S., Rudolph, M.M., Ehgartner, J., Mayr, T., Figge, M.T., Roth, M., Zang, E., 2015. Enhanced and homogeneous oxygen availability during incubation of microfluidic droplets. RSC Advances 101871–101878. https://doi.org/10.1039/c5ra20118g

Mahr, R., Frunzke, J., 2016. Transcription factor-based biosensors in biotechnology: current state and future prospects. Applied Microbiology and Biotechnology 100, 79–90. https://doi.org/10.1007/s00253-015-7090-3

Malara, A., Fresia, C., Buduo, C., Soprano, P., Moccia, F., Balduini, C., Zocchi, E., Flora, A., Balduini, A., 2017. The Plant Hormone Abscisic Acid Is a Prosurvival Factor in Human and Murine Megakaryocytes. Journal of Biological Chemistry 292, 3239–3251. https://doi.org/10.1074/jbc.m116.751693

Malyshev, D.A., Dhami, K., Lavergne, T., Chen, T., Dai, N., Foster, J.M., Corrêa, I.R., Romesberg, F.E., 2014. A Semi-Synthetic Organism with an Expanded Genetic Alphabet. Nature 509, 385–388. https://doi.org/10.1038/nature13314

Manz, A., Graber, N., Widmer, H.M., 1990. Miniaturized total chemical analysis systems: A novel concept for chemical sensing. Sensors and Actuators B: Chemical 1, 244–248. https://doi.org/10.1016/0925-4005(90)80209-I

Mark, D., Haeberle, S., Roth, G., von Stetten, F., Zengerle, R., 2010. Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications. Chemical Society Reviews 39, 1153. https://doi.org/10.1039/b820557b

Martignago, D., Siemiatkowska, B., Lombardi, A., Conti, L., 2020. Abscisic Acid and Flowering Regulation: Many Targets, Different Places. International Journal of Molecular Sciences 21, 9700. https://doi.org/10.3390/ijms21249700

Maxam, A.M., Gilbert, W., 1977. A new method for sequencing DNA. Proceedings of the National Academy of Sciences 74, 560–564.

McBride, L.J., Caruthers, M.H., 1983. An investigation of several deoxynucleoside phosphoramidites useful for synthesizing deoxyoligonucleotides. Tetrahedron Letters 24, 245–248. https://doi.org/10.1016/S0040-4039(00)81376-3

McGovern, P.E., Zhang, J., Tang, J., Zhang, Z., Hall, G.R., Moreau, R.A., Nuñez, A., Butrym, E.D., Richards, M.P., Wang, Chen-shan, Cheng, G., Zhao, Z., Wang, Changsui, 2004. Fermented beverages of pre- and proto-historic China. Proceedings of the National Academy of Sciences 101, 17593–17598. https://doi.org/10.1073/pnas.0407921102

Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R.H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., Zhao, L., Dasika, M.S., Murarka, A., Lenihan, J., Eng, D., Leng, J.S., Liu, C.-L., Wenger, J.W., Jiang, H., Chao, L., Westfall, P., Lai, J., Ganesan, S., Jackson, P., Mans, R., Platt, D., Reeves, C.D., Saija, P.R., Wichmann, G., Holmes, V.F., Benjamin, K., Hill, P.W., Gardner, T.S., Tsong, A.E., 2016. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 537, 694. https://doi.org/10.1038/nature19769

Meng, F., Ellis, T., 2020. The second decade of synthetic biology: 2010–2020. Nature Communications 11, 5174. https://doi.org/10.1038/s41467-020-19092-2

Michener, J.K., Nielsen, J., Smolke, C.D., 2012. Identification and treatment of heme depletion attributed to overexpression of a lineage of evolved P450 monooxygenases. Proceedings of the National Academy of Sciences 109, 19504–19509. https://doi.org/10.1073/pnas.1212287109

Mutschler, H., Robinson, T., Tang, T.-Y.D., Wegner, S., 2019. Special Issue on Bottom-Up Synthetic Biology. ChemBioChem 20, 2533–2534. https://doi.org/10.1002/cbic.201900507

Nagamune, K., Hicks, L.M., Fux, B., Brossier, F., Chini, E.N., Sibley, L.D., 2008. Abscisic acid controls calcium-dependent egress and development in Toxoplasma gondii. Nature 451, 207–210. https://doi.org/10.1038/nature06478

Nambara, E., Marion-Poll, A., 2005. Abscisic acid biosynthesis and catabolism. Annual review of plant biology 56, 165–85. https://doi.org/10.1146/annurev.arplant.56.032604.144046

Negri, P., Maggi, M.D., Ramirez, L., De Feudis, L., Szwarski, N., Quintana, S., Eguaras, M.J., Lamattina, L., 2015. Abscisic acid enhances the immune response in Apis mellifera and contributes to the colony fitness. Apidologie 46, 542–557. https://doi.org/10.1007/s13592-014-0345-7

Nielsen, J., 2019. Yeast Systems Biology: Model Organism and Cell Factory. Biotechnology Journal 14, 1800421. https://doi.org/10.1002/biot.201800421

Nielsen, J., 2013. Production of biopharmaceutical proteins by yeast. Bioengineered 4, 207–211. https://doi.org/10.4161/bioe.22856

Nielsen, J., 2003. It Is All about Metabolic Fluxes. Journal of Bacteriology 185, 7031–7035. https://doi.org/10.1128/JB.185.24.7031-7035.2003

Nielsen, J., Keasling, J.D., 2016. Engineering Cellular Metabolism. Cell 164, 1185–1197. https://doi.org/10.1016/j.cell.2016.02.004

Nielsen, J., Keasling, J.D., 2011. Synergies between synthetic biology and metabolic engineering. Nature Biotechnology 29, 693–695. https://doi.org/10.1038/nbt.1937

Nielsen, J., Tillegreen, C.B., Petranovic, D., 2022. Innovation trends in industrial biotechnology. Trends in biotechnology. https://doi.org/10.1016/j.tibtech.2022.03.007

Niemiec, E., Howard, H.C., 2020. Ethical issues related to research on genome editing in human embryos. Computational and Structural Biotechnology Journal 18, 887–896. https://doi.org/10.1016/j.csbj.2020.03.014

Normile, D., 2018. Shock greets claim of CRISPR-edited babies. Science 362, 978–979. https://doi.org/10.1126/science.362.6418.978

Oh, H.D., Yu, D.J., Chung, S.W., Chea, S., Lee, H.J., 2018. Abscisic acid stimulates anthocyanin accumulation in 'Jersey' highbush blueberry fruits during ripening. Food Chemistry 244, 403–407. https://doi.org/10.1016/j.foodchem.2017.10.051

Ohkuma, K., Lyon, J.L., Addicott, F.T., Smith, O.E., 1963. Abscisin II, an Abscission-Accelerating Substance from Young Cotton Fruit. Science 142, 1592–1593. https://doi.org/10.1126/science.142.3599.1592

Olds, C.L., Glennon, E., Luckhart, S., 2018. Abscisic acid: new perspectives on an ancient universal stress signaling molecule. Microbes and Infection. https://doi.org/10.1016/j.micinf.2018.01.009

Olivares-Yañez, C., Sánchez, E., Pérez-Lara, G., Seguel, A., Camejo, P.Y., Larrondo, L.F., Vidal, E.A., Canessa, P., 2021. A comprehensive transcription factor and DNA-binding motif resource for the construction of gene regulatory networks in Botrytis cinerea and Trichoderma atroviride. Computational and Structural Biotechnology Journal 19, 6212–6228. https://doi.org/10.1016/j.csbj.2021.11.012

Osawa, S., Jukes, T.H., Watanabe, K., Muto, A., 1992. Recent evidence for evolution of the genetic code. Microbiological Reviews 56, 229–264. https://doi.org/10.1128/mr.56.1.229-264.1992

Paddon, C.J., Keasling, J.D., 2014. Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nature Reviews Microbiology 12, 355–367. https://doi.org/10.1038/nrmicro3240

Paddon, C.J., Westfall, P.J., Pitera, D.J., Benjamin, K., Fisher, K., McPhee, D., Leavell, M.D., Tai, A., Main, A., Eng, D., Polichuk, D.R., Teoh, K.H., Reed, D.W., Treynor, T., Lenihan, J., Fleck, M., Bajad, S., Dang, G., Dengrove, D., Diola, D., Dorin, G., Ellens, K.W., Fickes, S., Galazzo, J., Gaucher, S.P., Geistlinger, T., Henry, R., Hepp, M., Horning, T., Iqbal, T., Jiang, H., Kizer, L., Lieu, B., Melis, D., Moss, N., Regentin, R., Secrest, S., Tsuruta, H., Vazquez, R., Westblade, L.F., Xu, L., Yu, M., Zhang, Y., Zhao, L., Lievense, J., Covello, P.S., Keasling, J.D., Reiling, K.K., Renninger, N.S., Newman, J.D., 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496, 528–32. https://doi.org/10.1038/nature12051

Pandi, A., Koch, M., Voyvodic, P.L., Soudier, P., Bonnet, J., Kushwaha, M., Faulon, J.-L., 2019. Metabolic perceptrons for neural computing in biological systems. Nature Communications 10, 3880. https://doi.org/10.1038/s41467-019-11889-0

Papagiannidis, D., Bircham, P.W., Lüchtenborg, C., Pajonk, O., Ruffini, G., Brügger, B., Schuck, S., 2021. Ice2 promotes ER membrane biogenesis in yeast by inhibiting the conserved lipin phosphatase complex. The EMBO Journal 40, e107958. https://doi.org/10.15252/embj.2021107958

Parapouli, M., Vasileiadis, A., Afendra, A.-S., Hatziloukas, E., 2020. Saccharomyces cerevisiae and its industrial applications. AIMS Microbiology 6, 1–31. https://doi.org/10.3934/microbiol.2020001

Park, S.-Y.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.-F.F., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Desveaux, D., Rodriguez, P.L., McCourt, P., Zhu, J.-K.K., Schroeder, J.I., Volkman, B.F., Cutler, S.R., 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science 324, 1068–71. https://doi.org/10.1126/science.1173041

Parry, I.W.H., Black, S., Vernon, N., 2021. Still Not Getting Energy Prices Right: A Global and Country Update of Fossil Fuel Subsidies. IMF Working Papers.

Pausch, M.H., 1997. G-protein-coupled receptors in Saccharomyces cerevisiae: high-throughput screening assays for drug discovery. Trends in Biotechnology 15, 487–494. https://doi.org/10.1016/S0167-7799(97)01119-0

Peplow, M., 2016. Synthetic biology's first malaria drug meets market resistance. Nature 530, 389–390. https://doi.org/10.1038/530390a

Pereira, R., Ishchuk, O.P., Li, X., Liu, Q., Liu, Y., Otto, M., Chen, Y., Siewers, V., Nielsen, J., 2021. Metabolic Engineering of Yeast, in: Metabolic Engineering. pp. 689–733. https://doi.org/10.1002/9783527823468.ch18

Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergström, A., Sigwalt, A., Barre, B., Freel, K., Llored, A., Cruaud, C., Labadie, K., Aury, J.-M., Istace, B., Lebrigand, K., Barbry, P., Engelen, S., Lemainque, A., Wincker, P., Liti, G., Schacherer, J., 2018. Genome evolution across 1,011 Saccharomyces cerevisiae isolates. Nature 556, 339–344. https://doi.org/10.1038/s41586-018-0030-5

Presidential Commission for the Study of Bioethical Issues, 2010. New Directions: The Ethics of Synthetic Biology and Emerging Technologies, https://bioethicsarchive.georgetown.edu/pcsbi/sites/default/files/PCSBI-Synthetic-Biology-Report-12.16.10_0.pdf.

PubChem, 2022. Abscisic acid [WWW Document]. URL https://pubchem.ncbi.nlm.nih.gov/compound/5280896 (accessed 1.21.23).

Purnick, P.E.M., Weiss, R., 2009. The second wave of synthetic biology: from modules to systems. Nature Reviews Molecular Cell Biology 10, 410–422. https://doi.org/10.1038/nrm2698

Pydi, S.P., Jaggupilli, A., Nelson, K.M., Abrams, S.R., Bhullar, R.P., Loewen, M.C., Chelikani, P., 2015. Abscisic Acid Acts as a Blocker of the Bitter Taste G Protein-Coupled Receptor T2R4. Biochemistry 54, 2622–31. https://doi.org/10.1021/acs.biochem.5b00265

Qi, C.-C., Shu, Y.-M., Chen, F.-H., Ding, Y.-Q., Zhou, J.-N., 2016. Sensitivity during the forced swim test is a key factor in evaluating the antidepressant effects of abscisic acid in mice. Behavioural Brain Research 300, 106–113. https://doi.org/10.1016/j.bbr.2015.12.009

Qi, C.-C., Zhang, Z., Fang, H., Liu, J., Zhou, N., Ge, J.-F., Chen, F.-H., Xiang, C.-B., Zhou, J.-N., 2015. Antidepressant Effects of Abscisic Acid Mediated by the Downregulation of Corticotrophin-Releasing Hormone Gene Expression in Rats. International Journal of Neuropsychopharmacology 18, pyu006. https://doi.org/10.1093/ijnp/pyu006

Quinn, R., 2013. Rethinking Antibiotic Research and Development: World War II and the Penicillin Collaborative. American Journal of Public Health 103, 426–434. https://doi.org/10.2105/AJPH.2012.300693

Ramirez, L., Negri, P., Sturla, L., Guida, L., Vigliarolo, T., Maggi, M., Eguaras, M., Zocchi, E., Lamattina, L., 2017. Abscisic acid enhances cold tolerance in honeybee larvae. Proceedings. Biological Sciences 284, 20162140. https://doi.org/10.1098/rspb.2016.2140

Rantasalo, A., Kuivanen, J., Penttilä, M., Jäntti, J., Mojzita, D., 2018. Synthetic Toolkit for Complex Genetic Circuit Engineering in Saccharomyces cerevisiae. ACS Synthetic Biology 7, 1573–1587. https://doi.org/10.1021/acssynbio.8b00076

Raser, J.M., O'Shea, E.K., 2004. Control of Stochasticity in Eukaryotic Gene Expression. Science 304, 1811–1814. https://doi.org/10.1126/science.1098641

Rebatchouk, D., Daraselia, N., Narita, J.O., 1996. NOMAD: a versatile strategy for in vitro DNA manipulation applied to promoter analysis and vector design. Proceedings of the National Academy of Sciences 93, 10891–10896. https://doi.org/10.1073/pnas.93.20.10891

Ribes-Navarro, A., Atef, M., Sánchez-Sarasúa, S., Beltrán-Bretones, M.T., Olucha-Bordonau, F., Sánchez-Pérez, A.M., 2019. Abscisic Acid Supplementation Rescues High Fat Diet-Induced Alterations in Hippocampal Inflammation and IRSs Expression. Molecular Neurobiology 56, 454–464. https://doi.org/10.1007/s12035-018-1091-z

Richardson, S.M., Mitchell, L.A., Stracquadanio, G., Yang, K., Dymond, J.S., DiCarlo, J.E., Lee, D., Huang, C.L.V., Chandrasegaran, S., Cai, Y., Boeke, J.D., Bader, J.S., 2017. Design of a synthetic yeast genome. Science 355, 1040–1044. https://doi.org/10.1126/science.aaf4557

Ro, D.-K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J.D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940. https://doi.org/10.1038/nature04640

Rondon, R.E., Groseclose, T.M., Short, A.E., Wilson, C.J., 2019. Transcriptional programming using engineered systems of transcription factors and genetic architectures. Nature Communications 10, 4784. https://doi.org/10.1038/s41467-019-12706-4

Ruggiero, M.A., Gordon, D.P., Orrell, T.M., Bailly, N., Bourgoin, T., Brusca, R.C., Cavalier-Smith, T., Guiry, M.D., Kirk, P.M., 2015. A Higher Level Classification of All Living Organisms. PLOS ONE 10, e0119248. https://doi.org/10.1371/journal.pone.0119248

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N., 1985. Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia. Science 230, 1350–1354. https://doi.org/10.1126/science.2999980

Sakthivel, P., Sharma, N., Klahn, P., Gereke, M., Bruder, D., 2016. Abscisic Acid: A Phytohormone and Mammalian Cytokine as Novel Pharmacon with Potential for Future Development into Clinical Applications. Current Medicinal Chemistry 23, 1549–1570. https://doi.org/10.2174/0929867323666160405113129

Saltepe, B., Wang, L., Wang, B., 2022. Synthetic biology enables field-deployable biosensors for water contaminants. Trends in Analytical Chemistry 146, 116507. https://doi.org/10.1016/j.trac.2021.116507

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences 74, 5463–5467.

Sarrion-Perdigones, A., Falconi, E.E., Zandalinas, S.I., Juárez, P., Fernández-del-Carmen, A., Granell, A., Orzaez, D., 2011. GoldenBraid: An Iterative Cloning System for Standardized Assembly of Reusable Genetic Modules. PLOS ONE 6, e21622. https://doi.org/10.1371/journal.pone.0021622

Sauer, M., 2016. Industrial production of acetone and butanol by fermentation—100 years later. FEMS Microbiology Letters 363, fnw134. https://doi.org/10.1093/femsle/fnw134

Saxena, S., Tripathi, J., Chatterjee, S., Gautam, S., 2017. Natural Predominance of Abscisic Acid in Pongammia pinnata ("Karanj") Honey Contributed to its Strong Antimutagenicity. Journal of Agricultural and Food Chemistry 65, 4624–4633. https://doi.org/10.1021/acs.jafc.7b01529

Scarfì, S., Ferraris, C., Fruscione, F., Fresia, C., Guida, L., Bruzzone, S., Usai, C., Parodi, A., Millo, E., Salis, A., Burastero, G., De Flora, A., Zocchi, E., 2008. Cyclic ADP-Ribose-Mediated Expansion and Stimulation of Human Mesenchymal Stem Cells by the Plant Hormone Abscisic Acid. Stem Cells 26, 2855–2864. https://doi.org/10.1634/stemcells.2008-0488

Scarfì, S., Fresia, C., Ferraris, C., Bruzzone, S., Fruscione, F., Usai, C., Benvenuto, F., Magnone, M., Podestà, M., Sturla, L., Guida, L., Albanesi, E., Damonte, G., Salis, A., De Flora, A., Zocchi, E., 2009. The Plant Hormone Abscisic Acid Stimulates the Proliferation of Human Hemopoietic Progenitors through the Second Messenger Cyclic ADP-Ribose. Stem Cells 27, 2469–2477. https://doi.org/10.1002/stem.173

Schäfer, M., Fischer, C., Baldwin, I.T., Meldau, S., 2011. Grasshopper oral secretions increase salicylic acid and abscic acid levels in wounded leaves of Arabidopsis thaliana. Plant Signaling & Behavior 6, 1256–1258. https://doi.org/10.4161/psb.6.9.16552

Schendzielorz, G., Dippong, M., Grünberger, A., Kohlheyer, D., Yoshida, A., Binder, S., Nishiyama, C., Nishiyama, M., Bott, M., Eggeling, L., 2014. Taking Control over Control: Use of Product Sensing in Single Cells to Remove Flux Control at Key Enzymes in Biosynthesis Pathways. ACS Synthetic Biology 3, 21–29. https://doi.org/10.1021/sb400059y

Schlenk, F., 1985. Early research on fermentation — a story of missed opportunities. Trends in Biochemical Sciences 10, 252–254. https://doi.org/10.1016/0968-0004(85)90145-8

Schoch, G.A., Attias, R., Belghazi, M., Dansette, P.M., Werck-Reichhart, D., 2003. Engineering of a Water-Soluble Plant Cytochrome P450, CYP73A1, and NMR-Based Orientation of Natural and Alternate Substrates in the Active Site. Plant Physiology 133, 1198–1208. https://doi.org/10.1104/pp.103.020305

Schwann, Th., 1837. Vorläufige Mittheilung, betreffend Versuche über die Weingährung und Fäulniss. Annalen der Physik 117, 184–193. https://doi.org/10.1002/andp.18371170517

Schwille, P., 2011. Bottom-Up Synthetic Biology: Engineering in a Tinkerer's World. Science 333, 1252–1254. https://doi.org/10.1126/science.1211701

Scott, L.H., Wigglesworth, M.J., Siewers, V., Davis, A.M., David, F., 2022. Genetically Encoded Whole Cell Biosensor for Drug Discovery of HIF-1 Interaction Inhibitors. ACS Synthetic Biology 11, 3182–3189. https://doi.org/10.1021/acssynbio.2c00274

Semkiv, M.V., Ruchala, J., Dmytruk, K.V., Sibirny, A.A., 2020. 100 Years Later, What Is New in Glycerol Bioproduction? Trends in Biotechnology 38, 907–916. https://doi.org/10.1016/j.tibtech.2020.02.001

Shao, Y., Lu, N., Wu, Z., Cai, C., Wang, S., Zhang, L.-L., Zhou, F., Xiao, S., Liu, L., Zeng, X., Zheng, H., Yang, C., Zhao, Z., Zhao, G., Zhou, J.-Q., Xue, X., Qin, Z., 2018. Creating a functional single-chromosome yeast. Nature 560, 331–335. https://doi.org/10.1038/s41586-018-0382-x

Sharma, B., Shukla, P., 2022. Futuristic avenues of metabolic engineering techniques in bioremediation. Biotechnology and Applied Biochemistry 69, 51–60. https://doi.org/10.1002/bab.2080

Shemer, B., Palevsky, N., Yagur-Kroll, S., Belkin, S., 2015. Genetically engineered microorganisms for the detection of explosives' residues. Frontiers in Microbiology 6, 1175. https://doi.org/10.3389/fmicb.2015.01175

Shen, X., Song, S., Li, C., Zhang, J., 2022. Synonymous mutations in representative yeast genes are mostly strongly non-neutral. Nature 606, 725–731. https://doi.org/10.1038/s41586-022-04823-w

Siedler, S., Khatri, N.K., Zsohár, A., Kjærbølling, I., Vogt, M., Hammar, P., Nielsen, C.F., Marienhagen, J., Sommer, M.O.A., Joensson, H.N., 2017. Development of a Bacterial Biosensor for Rapid Screening of Yeast p -Coumaric Acid Production. ACS Synthetic Biology 6, 1860–1869. https://doi.org/10.1021/acssynbio.7b00009

Siewers, V., Kokkelink, L., Smedsgaard, J., Tudzynski, P., 2006. Identification of an Abscisic Acid Gene Cluster in the Grey Mold Botrytis cinerea. Applied and Environmental Microbiology 72, 4619–4626. https://doi.org/10.1128/AEM.02919-05

Siewers, V., Smedsgaard, J., Tudzynski, P., 2004. The P450 Monooxygenase BcABA1 Is Essential for Abscisic Acid Biosynthesis in Botrytis cinerea. Applied and Environmental Microbiology 70. https://doi.org/10.1128/AEM.70.7.3868-3876.2004

Singh, A.H., Kaufmann-Malaga, B.B., Lerman, J.A., Dougherty, D.P., Zhang, Y., Kilbo, A.L., Wilson, E.H., Ng, C.Y., Erbilgin, O., Curran, K.A., Reeves, C.D., Hung, J.E., Mantovani, S., King, Z.A., Ayson, M.J., Denery, J.R., Lu, C.-W., Norton, P., Tran, C., Platt, D.M., Cherry, J.R., Chandran, S.S., Meadows, A.L., 2023. An Automated Scientist to Design and Optimize Microbial Strains for the Industrial Production of Small Molecules. bioRxiv 2023.01.03.521657. https://doi.org/10.1101/2023.01.03.521657

Singh, S., Yap, W.S., Ge, X.Y., Min, V.L.X., Choudhury, D., 2022. Cultured meat production fuelled by fermentation. Trends in Food Science & Technology 120, 48–58. https://doi.org/10.1016/j.tifs.2021.12.028

Siu, K.-H., Chen, R.P., Sun, Q., Chen, L., Tsai, S.-L., Chen, W., 2015. Synthetic scaffolds for pathway enhancement. Current Opinion in Biotechnology, Pathway engineering 36, 98–106. https://doi.org/10.1016/j.copbio.2015.08.009

Slovik, S., Baier, M., Hartung, W., 1992. Compartmental distribution and redistribution of abscisic acid in intact leaves : I. Mathematical formulation. Planta 187, 14–25. https://doi.org/10.1007/BF00201619

Smith, H.O., Welcox, K.W., 1970. A Restriction enzyme from Hemophilus influenzae: I. Purification and general properties. Journal of Molecular Biology 51, 379–391. https://doi.org/10.1016/0022-2836(70)90149-X

Smith, M.G., Snyder, M., 2006. Yeast as a Model for Human Disease. Current Protocols in Human Genetics 48, 15.6.1-15.6.8. https://doi.org/10.1002/0471142905.hg1506s48

Spence, C.A., Lakshmanan, V., Donofrio, N., Bais, H.P., 2015. Crucial Roles of Abscisic Acid Biogenesis in Virulence of Rice Blast Fungus Magnaporthe oryzae. Frontiers in plant science 6, 1082. https://doi.org/10.3389/fpls.2015.01082

Stableford, B.M., 2004. Historical Dictionary of Science Fiction Literature. Scarecrow Press.

Starr, B., 2016. A Nobel Prize for Work in Yeast. Again! [WWW Document]. URL https://www.yeastgenome.org/blog/a-nobel-prize-for-work-in-yeast-again (accessed 12.6.22).

Stephanopoulos, G., 2012. Synthetic Biology and Metabolic Engineering. ACS Synthetic Biology 1, 514–525. https://doi.org/10.1021/sb300094q

Šuchová, K., Fehér, C., Ravn, J.L., Bedő, S., Biely, P., Geijer, C., 2022. Cellulose- and xylan-degrading yeasts: Enzymes, applications and biotechnological potential. Biotechnology Advances 59, 107981. https://doi.org/10.1016/j.biotechadv.2022.107981

Sweet, R.G., 1965. High Frequency Recording with Electrostatically Deflected Ink Jets. Review of Scientific Instruments 36, 131–136. https://doi.org/10.1063/1.1719502

Szita, N., Polizzi, K., Jaccard, N., Baganz, F., 2010. Microfluidic approaches for systems and synthetic biology. Current Opinion in Biotechnology, Nanobiotechnology – Systems biology 21, 517–523. https://doi.org/10.1016/j.copbio.2010.08.002

Takino, J., Kozaki, T., Ozaki, T., Liu, C., Minami, A., Oikawa, H., 2019. Elucidation of biosynthetic pathway of a plant hormone abscisic acid in phytopathogenic fungi. Bioscience, Biotechnology, and Biochemistry 83, 1642–1649. https://doi.org/10.1080/09168451.2019.1618700

Takino, J., Kozaki, T., Sato, Y., Liu, C., Ozaki, T., Minami, A., Oikawa, H., 2018. Unveiling Biosynthesis of the Phytohormone Abscisic Acid in Fungi: Unprecedented Mechanism of Core Scaffold Formation Catalyzed by an Unusual Sesquiterpene Synthase. Journal of the American Chemical Society. https://doi.org/10.1021/jacs.8b08925

Tan, S.Z., Prather, K.L., 2017. Dynamic pathway regulation: recent advances and methods of construction. Current Opinion in Chemical Biology 41, 28–35. https://doi.org/10.1016/j.cbpa.2017.10.004

Terry, S.C., Jerman, J.H., Angell, J.B., 1979. A gas chromatographic air analyzer fabricated on a silicon wafer. IEEE Transactions on Electron Devices 26, 1880–1886. https://doi.org/10.1109/T-ED.1979.19791

Thorp, H.H., Yaffe, M.B., 2023. Seeing is great, understanding is better. Science Signaling 16, eadg2868. https://doi.org/10.1126/scisignal.adg2868

Tossi, V., Cassia, R., Bruzzone, S., Zocchi, E., Lamattina, L., 2012. ABA says NO to UV-B: a universal response? Trends in Plant Science 17, 510–517. https://doi.org/10.1016/j.tplants.2012.05.007

Tudzynski, B., Sharon, A., 2002. Biosynthesis, Biological Role and Application of Fungal Phytohormones, in: Osiewacz, H.D. (Ed.), Industrial Applications. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 183–211. https://doi.org/10.1007/978-3-662-10378-4_9

Veber, D.F., Johnson, S.R., Cheng, H.-Y., Smith, B.R., Ward, K.W., Kopple, K.D., 2002. Molecular Properties That Influence the Oral Bioavailability of Drug Candidates. Journal of Medicinal Chemistry 45, 2615–2623. https://doi.org/10.1021/jm020017n

Venter, C., Cohen, D., 2004. The Century of Biology. New Perspectives Quarterly 21, 73–77. https://doi.org/10.1111/j.1540-5842.2004.00701.x

Verma, V., Ravindran, P., Kumar, P.P., 2016. Plant hormone-mediated regulation of stress responses. BMC Plant Biology 16, 86. https://doi.org/10.1186/s12870-016-0771-y

Visscher, S.N., 1980. Regulation of grasshopper fecundity, longevity and egg viability by plant growth hormones. Experientia 36, 130–131. https://doi.org/10.1007/BF02004017

Waadt, R., Hitomi, K., Nishimura, N., Hitomi, C., Adams, S.R., Getzoff, E.D., Schroeder, J.I., 2014. FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. eLife 3, e01739. https://doi.org/10.7554/elife.01739

Wang, C., Liwei, M., Park, J.-B., Jeong, S.-H., Wei, G., Wang, Y., Kim, S.-W., 2018. Microbial Platform for Terpenoid Production: Escherichia coli and Yeast. Frontiers in Microbiology 9. https://doi.org/10.3389/fmicb.2018.02460

Wang, L., Brock, A., Herberich, B., Schultz, P.G., 2001. Expanding the Genetic Code of Escherichia coli. Science 292, 498–500. https://doi.org/10.1126/science.1060077

Watson, J.D., Crick, F.H.C., 1953. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Nature 171, 737–738. https://doi.org/10.1038/171737a0

Weber, E., Engler, C., Gruetzner, R., Werner, S., Marillonnet, S., 2011. A Modular Cloning System for Standardized Assembly of Multigene Constructs. PLoS ONE 6, e16765. https://doi.org/10.1371/journal.pone.0016765

Wenjian, L., Xiaoqiang, H., Yumei, X., Jinlong, F., Yuanzhi, Z., Huizhe, L., Mingan, W., Zhaohai, Q., 2013. Synthesis, photostability and bioactivity of 2,3-cyclopropanated abscisic acid. Phytochemistry 96, 72–80. https://doi.org/10.1016/j.phytochem.2013.09.029

Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. Genome Biology 1, reviews3003.1. https://doi.org/10.1186/gb-2000-1-6-reviews3003

Weyrich, L.S., Duchene, S., Soubrier, J., Arriola, L., Llamas, B., Breen, J., Morris, A.G., Alt, K.W., Caramelli, D., Dresely, V., Farrell, M., Farrer, A.G., Francken, M., Gully, N., Haak, W., Hardy, K., Harvati, K., Held, P., Holmes, E.C., Kaidonis, J., Lalueza-Fox, C., de la Rasilla, M., Rosas, A., Semal, P., Soltysiak, A., Townsend, G., Usai, D., Wahl, J., Huson, D.H., Dobney, K., Cooper, A., 2017. Neanderthal behaviour, diet, and disease inferred from ancient DNA in dental calculus. Nature 544, 357–361. https://doi.org/10.1038/nature21674

Wirth, T., Parker, N., Ylä-Herttuala, S., 2013. History of gene therapy. Gene 525, 162–169. https://doi.org/10.1016/j.gene.2013.03.137

Woolley, A.T., Mathies, R.A., 1994. Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. Proceedings of the National Academy of Sciences 91, 11348–11352. https://doi.org/10.1073/pnas.91.24.11348

Xiong, D., Lu, S., Wu, J., Liang, C., Wang, Wei, Wang, Wenzhao, Jin, J.-M., Tang, S.-Y., 2017. Improving key enzyme activity in phenylpropanoid pathway with a designed biosensor. Metabolic Engineering 40, 115–123. https://doi.org/10.1016/j.ymben.2017.01.006

Xu, X., Liu, Y., Du, G., Ledesma-Amaro, R., Liu, L., 2020. Microbial Chassis Development for Natural Product Biosynthesis. Trends in Biotechnology 38, 779–796. https://doi.org/10.1016/j.tibtech.2020.01.002

Yabusaki, Y., 1995. Artificial P450/reductase fusion enzymes: What can we learn from their structures? Biochimie 77, 594–603. https://doi.org/10.1016/0300-9084(96)88175-2

Yadav, V.G., De Mey, M., Giaw Lim, C., Kumaran Ajikumar, P., Stephanopoulos, G., 2012. The future of metabolic engineering and synthetic biology: Towards a systematic practice. Metabolic Engineering 14, 233–241. https://doi.org/10.1016/j.ymben.2012.02.001

Yang, Y., Arnold, F.H., 2021. Navigating the Unnatural Reaction Space: Directed Evolution of Heme Proteins for Selective Carbene and Nitrene Transfer. Accounts of Chemical Research 54, 1209–1225. https://doi.org/10.1021/acs.accounts.0c00591

Zaidi, S.S.-A., Mahas, A., Vanderschuren, H., Mahfouz, M.M., 2020. Engineering crops of the future: CRISPR approaches to develop climate-resilient and disease-resistant plants. Genome Biology 21, 289. https://doi.org/10.1186/s13059-020-02204-y

Zalucki, Y.M., Beacham, I.R., Jennings, M.P., 2009. Biased codon usage in signal peptides: a role in protein export. Trends in Microbiology 17, 146–150. https://doi.org/10.1016/j.tim.2009.01.005

Zangar, R., 2004. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. Toxicology and Applied Pharmacology 199, 316–331. https://doi.org/10.1016/j.taap.2004.01.018

Zhang, C., Yan, K., Fu, C., Peng, H., Hawker, C.J., Whittaker, A.K., 2022. Biological Utility of Fluorinated Compounds: from Materials Design to Molecular Imaging, Therapeutics and Environmental Remediation. Chemical Reviews 122, 167–208. https://doi.org/10.1021/acs.chemrev.1c00632

Zhang, G., Wang, H., Zhang, Z., Verstrepen, K.J., Wang, Q., Dai, Z., 2022. Metabolic engineering of Yarrowia lipolytica for terpenoids production: advances and perspectives. Critical Reviews in Biotechnology 42, 618–633. https://doi.org/10.1080/07388551.2021.1947183

Zhang, J., Hansen, L.G., Gudich, O., Viehrig, K., Lassen, L.M.M., Schrübbers, L., Adhikari, K.B., Rubaszka, P., Carrasquer-Alvarez, E., Chen, L., D'Ambrosio, V., Lehka, B., Haidar, A.K., Nallapareddy, S., Giannakou, K., Laloux, M., Arsovska, D., Jørgensen, M.A.K., Chan, L.J.G., Kristensen, M., Christensen, H.B., Sudarsan, S., Stander, E.A., Baidoo, E., Petzold, C.J., Wulff, T., O'Connor, S.E., Courdavault, V., Jensen, M.K., Keasling, J.D., 2022. A microbial supply chain for production of the anti-cancer drug vinblastine. Nature 609, 341–347. https://doi.org/10.1038/s41586-022-05157-3

Zhang, K., Li, H., Cho, K.M., Liao, J.C., 2010. Expanding metabolism for total biosynthesis of the nonnatural amino acid L-homoalanine. Proceedings of the National Academy of Sciences 107, 6234–6239. https://doi.org/10.1073/pnas.0912903107

Zhao, F., Bai, P., Liu, T., Li, D., Zhang, X., Lu, W., Yuan, Y., 2016. Optimization of a cytochrome P450 oxidation system for enhancing protopanaxadiol production in Saccharomyces cerevisiae. Biotechnology and Bioengineering 113, 1787–1795. https://doi.org/10.1002/bit.25934

Zhou, M., Wang, T., Fu, J., Xiao, G., Liu, Y., 2015. Nonoptimal codon usage influences protein structure in intrinsically disordered regions. Molecular Microbiology 97, 974–987. https://doi.org/10.1111/mmi.13079

Zhou, N., Yao, Y., Ye, H., Zhu, W., Chen, L., Mao, Y., 2016. Abscisic-acid-induced cellular apoptosis and differentiation in glioma via the retinoid acid signaling pathway. International Journal of Cancer 138, 1947–1958. https://doi.org/10.1002/ijc.29935

Zimmerman, S.B., Little, J.W., Oshinsky, C.K., Gellert, M., 1967. Enzymatic joining of DNA strands: a novel reaction of diphosphopyridine nucleotide. Proceedings of the National Academy of Sciences 57, 1841–1848. https://doi.org/10.1073/pnas.57.6.1841

Zimran, G., Feuer, E., Pri-Tal, O., Shpilman, M., Mosquna, A., 2022. Directed Evolution of Herbicide Biosensors in a Fluorescence-Activated Cell-Sorting-Compatible Yeast Two-Hybrid Platform. ACS Synthetic Biology. https://doi.org/10.1021/acssynbio.2c00297

Zocchi, E., Carpaneto, A., Cerrano, C., Bavestrello, G., Giovine, M., Bruzzone, S., Guida, L., Franco, L., Usai, C., 2001. The temperature-signaling cascade in sponges involves a heat-gated cation channel, abscisic acid, and cyclic ADP-ribose. Proceedings of the National Academy of Sciences 98, 14859–14864. https://doi.org/10.1073/pnas.261448698