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Biosynthetic production of retinoic acid from β-carotene

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AFFIDAVIT

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Abstract

9-cis retinoic acid is a derivative of vitamin A which plays an important role in physiological processes. In medicine, there is a need for this compound for the treatment of chronic hand eczema and as anti-cancer agent. Currently retinoic acids are obtained chemically. As an alternative a biosynthetic route for *9-cis* retinoic acid production was sought for.

Based on information from literature, a possible pathway extension from β-carotene to *9-cis* retinoic acid was proposed. For this purpose, the activity of three enzymes needed to be combined: β-carotene isomerase, β-carotene 15'-15' monooxygenase and retinal dehydrogenase. Based on an already existing β-carotene producing *Pichia pastoris* strain such enzymes from various organisms were evaluated to establish and implement a retinoic acid biosynthetic pathway in *P.pastoris*.

In a first step, the pathway enzymes were separately produced in *P. pastoris* and tested for their activity *in vivo*. Then enzyme combinations were tested with either by gene linkage by bidirectional promoters or T2A peptide linkers between the genes.

At the end a pathway to produce *all-trans* retinoic acid in *P. pastoris* with β-carotene 15'-15' monooxygenase from chicken and retinal dehydrogenase from rat was generated. A quantitative analysis showed that 0.10 mg/g (cell dry weight) *all-trans* retinoic acid were successful produced. In addition, the presence of an unknown by-product was observed.

The isomerases did not show any activity *in vivo*. Chemically catalysed isomerization of the biologically produced *all-trans* retinoic acid might be an alternative approach to obtain the API in the correct configuration.

Kurzfassung

9-cis Retinsäure ist ein Derivat von Vitamin A und hat eine wichtige Rolle in physiologischen Prozessen. Im medizinischen Bereich gibt es Bedarf für diese Verbindung zur Behandlung von chronischen Handekzemen und auch als Antikrebsmittel. Derzeit wird Retinsäure chemisch hergestellt. Als Alternative wurde eine biosynthetische Route zur 9-*cis* Retinsäureproduktion angestrebt.

Auf Basis von Informationen aus der Literatur, wurde eine mögliche des Biosynthesewegs für die Produktion von *9-cis* Retinsäure aus β-Carotin vorgeschlagen. Dafür mussten die Aktivitäten von drei Enzymen kombiniert werden: β-Carotin Isomerase, 15'-15' Monooxygenase und Retinal Dehydrogenase. Aufbauend auf einem bereits existierenden β-Carotin produzierenden *Pichia pastoris* Stamm, wurden solche Enzyme aus verschiedenen Organismen evaluiert, um einen Retinsäure Syntheseweg in *P. pastoris* zu implementierten.

Im ersten Schritt wurden die Enzyme des Stoffwechselwegs getrennt exprimiert und auf deren Aktivität *in vivo* getestet. Nach den ersten Analysen wurden Enzymkombinationen sowohl mit bidirektionalen Promotern als auch koexprimiert durch T2A Peptidlinker getestet.

Am Ende der Arbeit konnte ein Syntheseweg zur Herstellung von *all-trans* Retinsäure mit Hilfe von 15'-15' Monooxygenase aus dem Huhn und Retinal Dehydrogenase aus der Ratte etabliert werden. Eine quantitative Analyse zeigte eine spezifische Produktivität von 0,10 mg/g (Zelltrockengewicht) *all-trans* Retinsäure. Zusätzlich wurde ein unbekanntes Nebenprodukt festgestellt.

Die Isomerasen zeigten keine Aktivität *in vivo* für *all-trans* β-Carotin. Eine chemisch katalysierte Isomerisierung von der biologisch produzierten *all-trans* Retinsäure könnte eine Alternative sein, um das API in der korrekten Konfiguration zu produzieren.

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1. Introduction

1.1. Synthetic biology

Synthetic biology defines the engineering of complex biologically systems which have functions that do not exist in nature [1].

Synthetic biology is often based on systems biology, that deals with the understanding of existing biological systems, starting from interactions of genes and proteins, cell networks to circuits at cellular, tissues and whole-organism level. However, the most important difference between these systems is, that synthetic biology is focused on engineering and synthesizing novel functions for these systems [1] [2].

Such engineering can be applied at genetic level, for example the design and synthesis of genes and proteins (expand and modify the genetic code) or the interaction between these proteins (regulation mechanisms, signal sensing or enzymatic reactions). Furthermore, multi-component modules can be created or even engineered cells. In general, it is a new way to produce molecules and the possibilities of this designed way will increase enormously by adaption of natural biological mechanisms to engineering approaches [2].

The field of synthetic biology is interdisciplinary and involves computer modelling, information technology, control theory, chemistry and nanotechnology. It's a full complementation of each other and can be seen as a new discipline which is a systematic application-driven engineering [2].

Synthetic biology can be applied in biomedicine, for example for the production of smart drugs or vectors for gene therapy or personalized medicine. Other application fields involve the environment and energy sector e.g. bioremediation or energy production. By design a set of chemical pathways which are able to convert available solar energy and natural or waste materials to e.g. biofuels, energy can be generated. The production of smart materials and biomaterials, where the engineered proteins or organisms are used to develop new materials such as silk-like or bioadhesive domains as well as the synthesis of biopharmaceuticals such as artemisinin can be realized with synthetic biology [2].

1.2. Carotenoid derivatives

Carotenoids are tetra terpenoids consisting of 40 carbons. Carotenoids are also precursors of vitamin A and have many benefits for human health due to their activity as antioxidants. Furthermore, they are important for the food and cosmetic industry for example as nutritional supplements [3].

Some carotenoids can be derived in sufficient amount and quality from natural sources, but there are also carotenoid compounds which have to be mainly synthesized from petroleum by organic synthesis such as canthaxanthin or astaxanthin [3]. If the carotenoids are produced via chemical synthesis, a lot of hazardous waste is generally generated [4]. Furthermore, complex purification steps and formation of by-products are involved. Therefore, the biotechnological production of carotenoids by engineered microbial hosts attracts increasing interest, since it is safer and might allow production at lower costs due to the use of low-cost substrates.

Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the precursors in the biosynthesis of carotenoids. They can be produced by two different pathways, either the mevalonate pathway (MVA) or the non-mevalonate pathway (MEP) [3]. The first one is used by yeasts while the MEP is employed by plant plastids or most bacteria. These precursors form then farnesyl pyrophosphate (FFP), which is the starting point of the carotenoid pathway. Next the geranylgeranyl pyrophosphate (GGPP) is formed by c*rt*E, the GGPP synthase. The formation of phytoene is catalysed from two GGPP molecules and by *crt*B, the phytoene synthase. The carotene desaturase (*crt*I) forms the lycopene via desaturation and isomerization. The last step is catalysed by lycopene β-cyclase (*crt*Y) and is the circularisation of the terminal isoprene groups of lycopene to form β-carotene [3].

The genes required for this pathway, *crt*E, *crt*B, *crt*I and *crt*Y, were isolated from *Erwinia* and *Agrobacterium* [4] [5]. In Figure 1 the biosynthesis of β-carotene by *Erwinia* species is shown.

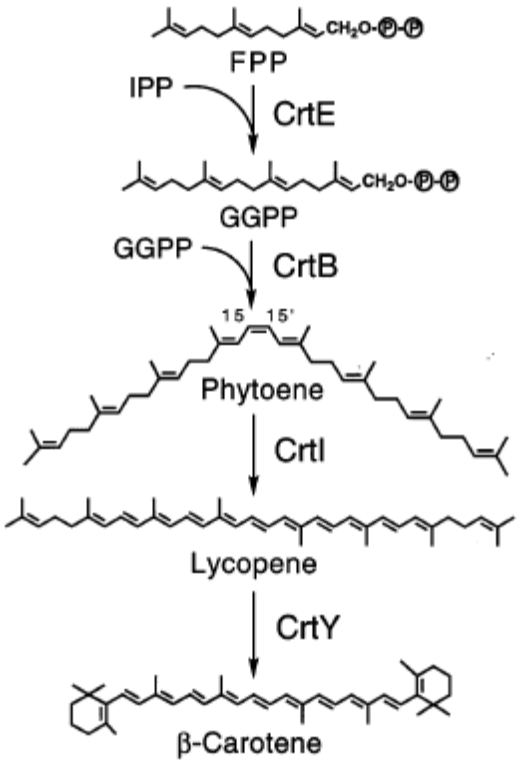

Figure 1: Carotenoid biosynthesis with the gene clusters from *Erwinia* species. FPP farnesyl pyrophosphate, isopentenyl diphosphate (IPP), geranylgeranyl diphosphate (GGPP), *CrtE* GGPP synthase, *CrtB* phytoene synthase, *CrtI* carotene desaturase, *CrtY* lycopene cyclase [3] [4].

In this study a ß-carotene pathway extension was tested to obtain *9-cis* retinoic acid from β-carotene via retinal.

Retinal is the aldehyde of vitamin A and currently produced chemically. The focus for retinal production are the carotene monooxygenases because the synthesis with chemicals has disadvantages as described above [6].

All-trans retinoic acid and *9-cis* retinoic acid (alitretinoin) are small lipid molecules and are derivatives of vitamin A. They play an important role in cell development, nervous system function, cell proliferation and differentiation, reproduction and immune response including effects on T-cells, monocytes and dendritic cells. By binding to the nuclear retinoic acid receptors (RAR) and retinoic X receptors (RXR), they activate their expression for their target genes. Whereas *all-trans* retinoic acid binds only to RAR, *9-cis* retinoic acid can bind to both receptors with high affinity [7] [8].

9-cis retinoic acid is needed for oral treatment of severe chronic hand eczema. Approximately 0.5 % to 0.7 % of the population suffer from this health problem. Due to 10-30 mg of alitretinoin are recommended daily for the patients, high amounts are needed [9]. Therefore, the focus is on synthetic production. In addition, *9-cis* retinoic acid is indicated to act as cancer agent.

Currently *9-cis* retinoic acid is produced chemically by a Wittig reaction. This chemical way has many disadvantages such as the use of solvents (e.g. methylene chloride), atom inefficiency or the generation of unwanted waste [10].

9-cis retinoic acid exists in nature and the mechanism of its bio-synthesis is so far known and described subsequently [11].

1.3. Biosynthetic route to retinoic acid

The starting point for the *9-cis* retinoic acid pathway was the *P. pastoris* producing β-carotene strain (pPp_T4_Kan_S_crtEBIY_T2A, clone 4A). This strain was chosen because high levels of carotenoids were reached, e.g. i.e. more than 5 mg/g cell dry weight of β-carotene were produced, previously [12].

One potential biosynthetic route is known from literature [11] and the second has been proposed by GSK to produce *9-cis* retinoic acid from β-carotene. Each of the routes involves one unproven bio catalytic transformation.

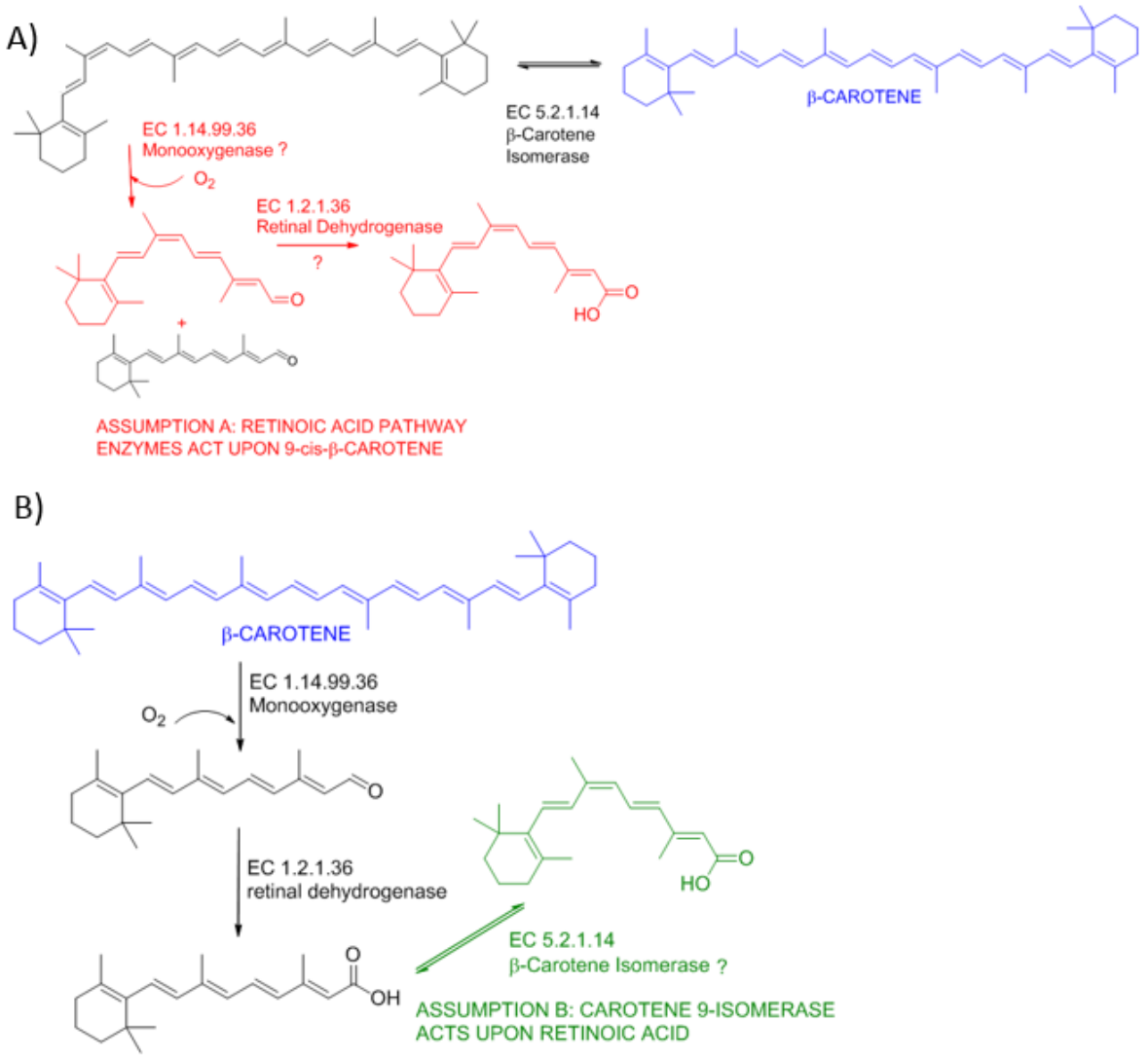

Figure 2: Proposed biosynthetic routes for the synthesis of *9-cis* retinoic acid. A) First step is the isomerization to *9-cis* β-carotene; cleavage to one molecule *all-trans* retinal and one molecule *9-cis* retinal. Finally the retinal dehydrogenase converts the retinal into *9-cis* retinoic acid. B) The monooxygenase cleaves the *all-trans* β-carotene into two molecules *all-trans* retinal; dehydrogenase catalyses the reaction to *all-trans* retinoic acid and here is the last step the isomerization. [10].

Route A starts with the isomerisation of *all-trans* β-carotene to its *9-cis* isomer which is catalysed by a β-carotene isomerase. In the BRENDA database there are only two entries for such β-carotene isomerases with little knowledge about these enzymes.

The next step is the central cleavage of *9-cis* β-carotene into one molecule of *all-trans* retinal and one molecule of *9-cis* retinal. In the end the retinal dehydrogenase catalyses the oxidation of *9-cis* retinal to *9-cis* retinoic acid. As the *all-trans* retinal is a side product in the second reaction step, the product yield is only 50 % compared to route B [10].

The second route starts with the cleavage of *all-trans* β-carotene into two molecules of *all-trans* retinal by a β-carotene monooxygenase. The retinal dehydrogenase converts the retinal

molecules to *all-trans* retinoic acid. Finally the isomerization step into *9-cis* retinoic acid is catalysed by β-carotene isomerase. In comparison to route A, this way converts all molecules of retinal to *all-trans* retinoic acid [10]. However, the question is if there is an isomerase which accepts retinoic acid as a substrate.

1.3.1. β-carotene isomerase

β-carotene isomerases (E.C 5.2.1.14) catalyse the reversible isomerization of the C9-C10 double bound in *all-trans* β-carotene to *9-cis* β-carotene. In BRENDA database isomerases from only two organisms are described, i.e. *Arabidopsis thaliana* and *Oryza sativa*. These enzymes are ferrous binding proteins and have signal sequences for targeting to chloroplasts.

1.3.2. β-carotene 15, 15´- monooxygenases

The cleavage of β-carotene into retinal is an important step in vitamin A synthesis and also for the production of retinoic acid. There are two cleavage strategies possible as β-carotene is a symmetrical molecule. The first one is the central cleavage, which occurs at the 15, 15' double bound and results in two molecules of retinal whereas the eccentric cleavage yields to β-apo-carotenals with different chain lengths [13] [14]. In this study β-carotene 15, 15´-monooxygenases were used.

Activity of β-carotene 15, 15´- monooxygenases was previously found in the intestinal mucosa and jejunum enterocytes and in the liver, lung, kidney and brain of different animal species such as rats, chickens, mice, hogs or humans [15] [16].

Both, *all-trans* β-carotene and *9-cis* β-carotene are accepted by the murine BCMO1 protein, which is the monooxygenase from mouse. In comparison to *9-cis* β-carotene which yields *all-trans* retinal and *9-cis* retinal in a molar ratio of 3:1, the *all-trans* β-carotene is mainly converted to *all-trans* retinal and 5 time faster [17].

The monooxygenase from chicken showed the highest efficiency for β-carotene (yielding the highest reported conversion of 60 % (w/w) of ß-carotene to retinal so far) in comparison to other carotenoid substrates, such as α-carotene or β-cryptoxanthin [13][18].

The only bacterial β-carotene 15, 15´- monooxygenase known to convert β-carotene into retinal was made from a gene of an uncultured marine bacterium 66A03. It is a bacterial

bacteriorhodopsin-related protein like homolog (Blh) protein shown to act as β-carotene 15, 15´-dioxygenases. The highest activity was found for β-carotene, but it also accepted other substrates such as β-cryptoxanthin, β-apo-4' –carotenal, α-carotene or γ-carotene. Enzyme activity is only shown if the substrate has one unsubstituted β-ionone ring and a molecular mass greater than C35. [13] [14].

While β-carotene exists predominantly in the *all-trans* form, the reaction processes in vitamin A synthesis increase the amount of *cis* isomers such as *9-cis* and *13-cis*. The monooxygenase from human was described not to accept *9-cis* β-carotene and therefore this enzyme was not relevant for the establishment of our proposed pathway [19].

1.3.3. Retinal dehydrogenase

Retinal dehydrogenases belong to the class of aldehyde dehydrogenases. Aldehyde dehydrogenases are NAD(P)+ dependent enzymes which oxidize aldehydes to their corresponding acids. They play an important role for the detoxification of aldehydes that are formed endogenously or introduced from outside [20]. Based on sequence similarity there are three classes of aldehyde dehydrogenases. Class I and III contain constitutive and inducible expressed cytosolic enzymes, whereas class II enzymes are expressed constitutively and in mitochondria. [21] [22] [23].

High efficiency and activity towards the *9-cis* retinal was observed for human and rat aldehyde dehydrogenase 1 [24] [25]. Also the retinal dehydrogenase 4 from mouse showed two-orders of magnitude more activity for *9-cis* retinal than for *all-trans* retinal [26].

1.4. *Pichia pastoris* as host system for the expression of heterologous pathways

Pichia pastoris (Komagataella phaffi) is a eukaryote and thus has some advantages of higher eukaryotic expression systems such as the ability to perform typical eukaryotic protein processing and posttranslational modifications, such as disulphide bond formation, or glycosylation. Furthermore, it is able to grow to high cell densities, able to produce high

amounts of proteins and there are strong and tightly regulated promoters available. *P. pastoris* is a methylotrophic yeast, which is able to metabolize methanol as a sole carbon source.

The *AOX1* and *AOX2* genes encode the 2 alcohol oxidases (AOX). The oxidase AOX1 accounts for the majority of the alcohol oxidase activity, typically >35% of total soluble protein in cells grown on methanol [28]. The poor affinity of the enzyme for O_2 is compensated by high protein expression in case of need. Although *AOX2* is very similar to *AOX1*, growth on methanol is much slower when just *AOX2* is expressed (Mut_S phenotype). This is the case when the *AOX1* gene is replaced by the coding sequence of heterologous proteins. Their gene expression is controlled at transcriptional level [27] [28] [29].

1.4.1. Expression of multi-enzyme pathways in yeasts

Many secondary metabolites with value for the pharmaceutical and food industry are produced by plants, but very often only in small amounts and at specific time points during development. To avoid these limitations nowadays it is possible to implement the corresponding pathways into microorganisms. Some examples are the production of artemisinic acid, the antioxidant resveratrol or the flavour component vanillin in yeasts [30].

For efficient bioconversions, it is Important to express pathway genes in sufficient amounts and to balance the activity of each enzyme to avoid the accumulation of intermediates. However, a highly expressed foreign pathway could cause stress to the cell. Thus, decreasing the expression of some enzymes might lead to a higher product yield, which underlines again the importance of a balanced pathway expression [31].

One example of a multigene expression in *P. pastoris* is the five gene (*vioABDEC*) violacein pathway. Violacein is a violet pigment and isolated from the gram-negative betaproteobacterium, *Chromobacterium violaceum*. One reason for implementing this pathway into microorganisms is the capability of violacein to kill bacteria and to induce apoptosis in various cancer cells [32]. However, due to the different intense colours of violacein and some precursor molecules it developed to be a preferred model pathway in synthetic biology, too.

The β-carotene pathway described in chapter 1.2. is another example for multigene expression in microorganisms.

Multigene expression is often based on co-expression constructs harbouring the pathways genes under the separate control of the same promoter and terminator. This strategy increases the size of the expression construct and might result in decreased transformation rates. In addition, the costs of labour and materials are increasing. Furthermore, recombination events can occur if repetitive homologous sequences are used leading to strain instability [33].

To overcome the homologous recombination different promoters and terminators for each gene can be used. To avoid bottlenecks in the pathway, balancing the catalysts at transcriptional level is possible [33]. *Vogl et al* described various promoters that differ in their DNA sequence to increase the genetic stability while show strong coexpression [12].

A further strategy to decrease the size of the expression cassette and to increase transformation efficiency is the use of bidirectional promoters which are short regulatory DNA sequences and capable of initiating transcription in both directions. The promoter is in between two genes which are expressed [34] [35].

In addition, multigene pathways can be expressed from one polycistronic transcript. In contrast to prokaryotes, eukaryotes generally do not express polycistronic operons. However, there are different ways to achieve polycistronic expression. Internal ribosome binding sites (IRES) can be used which are capable to initiate translation at internal sites. However, these sequences are large and result in lower expression of the downstream encoded protein. Hence, this strategy is not suitable for multigene pathways [33] [36] [37] [38].

Another way is to use 2A sequences which are short peptides (up to 20 amino acids) and have their origin from viral polyproteins. The advantages are the size of the expression construct due to the reduction of regulatory elements as well as the prevention of homologous sequences resulting in improved strain stability. Furthermore, the establishment of multi-enzyme pathways based on 2A sequences is simple and quick. *Geier et al* showed the successful expression of nine genes in *P. pastoris* from a single transcript [33].

The combination of bidirectional promoters and T2A sequences is also possible.

2. Aim of this thesis

The objective of this master thesis was to produce *9-cis* retinoic acid in a biological way in *Pichia pastoris* or *E. coli*. The main focus for the production was *P. pastoris* as there are different strategies for pathway design in yeast, i.e. strong promoters, bidirectional promoters or 2A sequences. In addition, eukaryotic expression hosts are generally more successful for the production of proteins originating from higher eukaryotes.

Based on the β-carotene producing yeast strain, three more enzymes were needed to obtain the desired product. Therefore, each enzyme was expressed and their activity was analysed. The experiments were started with the β-carotene isomerases and β-carotene 15'-15' monooxygenases. At last the retinal dehydrogenases was tested in combination with the different monooxygenases.

Additionally, the isomerases were also expressed in *E. coli* BL21 pC21e1 crtEBIY for SDS-Page. Due to the activity of the monooxygenases and retinal dehydrogenases in *P. pastoris*, these enzymes were not analyzed in E. coli.

For first investigations, the single genes were assembled with the pPpT4_S vector and used to transform the ß-carotene producing *P. pastoris* crtEBIY strain. To assess the *in vivo* activity of the additional enzymes, HPLC methods for the analysis of the expected products were developed.

After first activity determinations of the single enzymes, enzyme combinations were tested to obtain retinoic acid by using T2A sequences and bidirectional promoters. Retinoic acid formation was quantified by cultivating the resulting strains in shake flasks.

3. Materials and Methods

3.1. Enzymes

Table 1: Enzymes used during this thesis

Enzyme	Company
FastDigest *Bgl*II	Thermo Scientific – USA
FastDigest *Eco*RI	Thermo Scientific - USA
FastDigest *Not*I	Thermo Scientific – USA
FastDigest *Pst*I	Thermo Scientific – USA
FastDigest *Sal*I	Thermo Scientific – USA
FastDigest *Smi*I	Thermo Scientific – USA
FastDigest *Spe*I	Thermo Scientific – USA
DNA blunting enzyme	Thermo Scientific – USA
FastAP Thermosensitive Alkaline Phosphatase	Thermo Scientific - USA
PhusionTM high fidelity DNA polymerase	Thermo Scientific – USA
T4 DNA ligase	Thermo Scientific – USA
Zymolyase	Gerbu Biotechnik GmbH

3.2. PCR amplification of genes

The genes were codon optimized for *P. pastoris* and *E. coli* and were provided from GSK in pUC57. The sequences are shown in the appendix.

Due to the presence of signal peptides for chloroplasts, the truncated versions were tested too because *P. pastoris and E. coli* do not possess chloroplasts.

The codon optimized genes of the isomerases, monooxygenases and retinal dehydrogenases for *P. pastoris* were amplified via PCR. The primers for the amplification of the genes included the *EcoRI* and *NotI* restriction sites for cloning into the pPp T4_S vector and are listed in S 1 (supplementary data). The amplification of these genes was performed using the PhusionTM high fidelity DNA polymerase. The mixtures for the PCR reactions included 2 µL of the corresponding forward and reverse primer (5 µM), 5 µL dNTP's (2 mM), 10 µL 5x HF buffer, 10 ng template DNA and 1 U PhusionTM high fidelity DNA polymerase. The total volume was 50 µL. In Table 2 the PCR temperature profile is shown. The elongation time depended on the

length of the fragments, 15 sec were used for 1 kb. The used device was a GeneAmp® PCR System 2700 from Applied Biosystems.

Table 2: General PCR temperature program.

Time	Temperature	
30 sec	98 °C	
10 sec	98 °C	30 x
20 sec	58 °C	
15 sec / 1 kb	72 °C	
7 min	72 °C	
∞	4 °C	

3.3. Restriction enzyme based cloning

For cloning the amplified genes into the T4_S vector, 100 ng digested vector were ligated with the insert in a ratio of 1:3. The amount of insert was calculated with the ligation calculator of the University of Düsseldorf (http://www.insilico.uni-duesseldorf.de/Lig_Input.html). The mixture for ligation included 0.2 µL T4 DNA ligase (5 U), 2 µL 10x T4 DNA ligase buffer, 100 ng vector, calculated amount of insert and ddH_2O in a total volume of 20 µL. The reaction mixture was either incubated for two hours at room temperature or overnight at 16 °C and was inactivated at 65 °C for 10 minutes.

3.4. Overlap extension PCR

The overlap extension PCR is a two-step reaction. First the desired genes were linked together and afterwards the full-length products were amplified.

For easy handling the genes were diluted to 1 ng/µL. The reaction mixture included the DNA fragments to be assembled, with a concentration of 10 ng/µL for the biggest fragment. Furthermore 5 µL dNTPs (2mM), 10 µL 5x HF buffer and 1 U Phusion TM high fidelity DNA polymerase were added in a total volume of 50 µL. The elongation time depended on the length of the fragments, 15 sec were used for 1 kb. The PCR temperature profile for the first 15 cycles is shown in Table 2.

After 15 cycles, 4 µL of forward and reverse primers (5 µM), 1.5 µL dNTPs (2mM), 4 µL 5x HF buffer and 0.5 U PhusionTM high fidelity DNA polymerase were added in a total volume of 20 µL. The temperature profile was the same as before, except that the PCR reaction was performed for 20 cycles.

3.5. Gibson Assembly and *E. coli* transformation

For Gibson Assembly [39] the entry vector and inserts were mixed in an equimolar ratio and added to the Gibson Assembly master mix to obtain a total volume of 20 µL. The mixture was incubated at 50 °C for one hour. The content of the Gibson Assembly mix is listed in Table 3.

Table 3: Components of Gibson Assembly master mix [39]

Components for Gibson Assembly master mix	Volume
5x isothermal (ISO) reaction buffer [39]	4 µL
T5 exonuclease (10 U/µL)	0.008 µL
PhusionTM high fidelity DNA polymerase (2 U/µL)	0.25 µL
Taq DNA ligase (40 U/µL)	2 µL
Sterile ddH_2O	8.742 µL

Prior to the *E. coli* transformation, 3 µL of the assembled DNA were desalted for 20 minutes.

3.6. *E. coli* BL 21 *and* crtEBIY transformation

Preparation of electrocompetent *E. coli* cells:

Two precultures were inoculated with a single colony of the *E. coli* wildtype strain BL21 and the carotenoid strain *E.* coli BL21 pC21e1 crtEBIY in 30 ml sterile LB medium and grown over night at 37 °C and 120 rpm. The main cultures were inoculated with the precultures to a starting OD_{600} of 0.05 – 0.1. The final OD_{600} should be between 0.6-0.8 and was reached after approximately 2-3 hours. The main cultures were centrifuged for 10 minutes at 4 °C and 4000 rpm. The supernatant was discarded and the pellet was resuspended in 50 ml ice cold 10% glycerol. The centrifugation step was repeated. The supernatant was discarded again and the pellet was resuspended in 1 mL ice cold 10% glycercol. 80 µl of the *E. coli* electrocompetent

cells were mixed with about 2 µg of DNA or 3 µL desalted DNA in electroporation cuvettes and were incubated on ice for 5 minutes. The transformation was performed at 2.5 kV. For regeneration 900 µL SOC were added and the cells were incubated for 1 hours at 37 °C and 700 rpm. Aliquots of the regeneration mixture were plated on LB agar plates containing 25 µg/ml Zeocin for *E. coli* Top 10F cells or 100 µg/mL Ampicillin for *E. coli* BL21 strain or 100 µg/mL Ampicillin and 100 µg/mL Kanamycin for *E. coli* crtEBIY strain, respectively. The plates were incubated at 37 °C for one day.

Single colonies were streaked out on LB plates for subsequent plasmid preparation.

3.7. *P. pastoris* transformation

Preparation of DNA:

Each expression construct was linearized with the restriction enzyme *SwaI*. The fast digest included the plasmid DNA obtained by one plasmid preparation, 2.5 µL *SwaI* and 5.5 µL FastDigest White buffer in a total volume of about 53 µL. This mixture was incubated for one to two hours at 37 °C and was heat inactivated at 65 °C for 15 minutes. The linearized constructs were purified and concentrated with the Wizard® SV Gel and PCR Clean-Up System.

Preparation of electrocompetent *P. pastoris* cells [40]:

Two precultures were inoculated with a single colony of the *P. pastoris* wildtype strain CBS 7435 and the carotenoid strain crtEBIY in 50 ml sterile YPD medium and grown over night at 28 °C and 90 rpm. The main cultures were inoculated with the precultures to a starting OD_{600} of 0.2. The final OD_{600} should be between 0.8-1.0 and was reached after approximately four hours. The main cultures were centrifuged at 500xg, RT for 5 minutes. The supernatant was discarded and the pellet was resuspended in 9 mL ice cold BEDS (see appendix) and 1mL 1 M DTT. The suspension was shaken for about 5 minutes until it was lukewarm. Then the centrifugation step was repeated. The supernatant was discarded again and the pellet was resuspended in 1 mL ice cold BEDS.

P. pastoris transformation:

80 µL of the *P. pastoris* electrocompetent cells were mixed with about 2 µg of linearized DNA in electroporation cuvettes and were incubated on ice for 5 minutes. The transformation was performed at 2 kV. For regeneration 500 µL sterile YPD medium and 500 µL 1M sorbitol were added and the cells were incubated for 2 hours at 28 °C and 120 rpm. The regenerated cells were plated on YPD agar plates containing 100 µg/mL Zeocin for the *P. pastoris* CBS7435 strain and containing 100 µg/mL Zeocin and 300 µg/mL Geneticin for the *P. pastoris* crtEBIY strain, respectively. The plates were incubated at 28 °C for two days.

3.8. Small scale cultivation of recombinant *P. pastoris* strains

For screening recombinant *P. pastoris* strain in small scale format, each well of a 96- well deep well plate was filled with 250 µL BMD1% (see supplementary data S3) and inoculated with a single transformant. Colonies of the *P. pastoris* wildtype strain, the crtEBIY strain and different controls were also included on each plate. The residual wells of the plates were left empty and served as sterile controls. The cells were grown for two days at 28 °C and 320 rpm before induction with methanol.

After 60 hours the deep well plate cultures were induced with 250 µL BMM2 (see supplementary data S3) and were incubated again at 28 °C and 320 rpm. About eight hours later, 50 µL of BMM10 (see supplementary data S3) were added per well. The cultures in the deep well plates were induced on two more days in the morning and in the afternoon with BMM10. The incubation was always performed at 28 °C and 320 rpm [41].

3.9. *P. pastoris* cultivation in shake flasks

For cultivation in baffled shake flasks (250 mL) each was filled with 50 mL buffered minimal dextrose media (BMD1%) and inoculated with recombinant strains. The *P. pastoris* wildtype strain and the carotene producing *P. pastoris* strain were also included to serve as negative controls. The cells were grown for two days at 28 °C and 120 rpm before induction with methanol. After 60 hours the flask cultures were induced with 25 mL buffered minimal methanol medium (1% methanol, BMM2) and were incubated again at 28 °C and 120 rpm.

About eight hours later, 2 mL of methanol were added to the flasks. The cultures were induced two more times with 2 mL methanol. The incubation was always at 28 °C and 120 rpm.

3.10. *E. coli* cultivation in shake flasks

Two precultures were inoculated with a recombinant strain in 4 mL sterile 2x YT medium and grown at 37 °C and 160 rpm. The *E. coli* BL21 strain and the *E. coli* BL21 pC21e1 crtEBIY strain were also included to serve as negative controls. The main cultures were inoculated with the precultures to a starting OD_{600} of 0.1. After approximately 2 hours, an OD_{600} of 0.6-0.8 was reached and the cells based on the recombinant *E. coli* BL21 strain were induced with 0.2 % arabinose and the cells based on the recombinant *E. coli* BL21 pC21e1 crtEBIY strain with 0.2 % arabinose and 1 mM IPTG. The induced cultures were incubated for 24 hours at 30 °C and 160 rpm.

3.11. Cell lysis and Bradford protein assay

From each culture, cells corresponding to an OD_{600} of 250 were harvested and were lysed. Therefore, 500 µL glass beads with a diameter of about 300 µm and 750 µL buffer A (appendix) supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride) were mixed with the cell pellets and disrupted in the TissueLyser II from Qiagen three times for 2 minutes. Between the steps the samples were cooled down on ice. By centrifugation at 4° C and full speed for 10 minutes the disrupted cells were pelleted. The supernatant of each sample was used in the Bio-Rad protein assay to determine the protein concentrations. This assay is based on the Bradford test.

First the samples and the dye reagents were prepared. Therefore two dilutions of each sample were made, 1:2 and 1:10. The Bio-Rad reagent was diluted 1:5. The diluted samples and protein standards were measured in duplicates. The protein standards were different concentrations of bovine serum albumin (BSA): 0 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.5 mg/mL, 1 mg/mL. 10 µL of each sample were pipetted into a separate well from a microtiter plate. By adding 200 µl of the diluted Bio-Rad reagent in each well the reaction was started and incubated for 5 minutes. The absorbance was measured at 595 nm.

Employing standard curve the protein concentrations were determined.

3.12. SDS-Page

15 µg total protein of each *P. pastoris* expression construct were mixed with NuPAGE® LDS Sample Buffer (4X) and NuPAGE® Sample Reducing Agent (10X) and incubated for 10 minutes at 70°C. After short centrifugation, the samples and the PageRuler Prestained Protein Ladder were loaded onto a Bis-Tris Gel from NuPAGE®.

For the SDS-Page for proteins of *E. coli* strains, the cells corresponding to an OD_{600} of 0.5 were harvested before induction, after 4.5 hours of induction and after 24 hours of. Cells were pelleted and resuspended in 37.5 µL H_2O. 12.5 µL 4x Loading Dye were added and the mix was cooked at 95 °C for 10 minutes. After spinning down, 10 µL of each sample and the PageRuler Prestained Protein Ladder were loaded onto a Bis-Tris Gel from NuPAGE®.

The gel was run in an XCell SureLock® Mini-Cell gel running tank with NuPAGE® MOPS SDS Running Buffer (20X) from Invitrogen life technologies for about one hour and 250 V. The content of the MOPS buffer is listed in S2.

To detect the proteins the gel was stained with Coomassie Brilliant Blue and was decolorized overnight.

3.13. eGFP measurements

For the measurement of eGFP (enhanced green fluorescence protein) fluorescence, microtiter plates were filled with 190 µL dH_2O and 10 µL of the recombinant *P. pastoris* cultures from deep well plates. The screening was performed with the synergyMx plate reader from Biotek at 488/507 nm.

3.14. Carotenoid extraction and HPLC analysis

For product analysis, carotenoids were extracted from recombinant *P. pastoris* strains. Therefore, the respective strains were cultivated (3.8. and 3.9.) and cells corresponding to an OD_{600} of 100 were harvested. The cells were centrifuged at full speed for 5 minutes. The supernatant were discarded and afterwards the pellets were resuspended in 1 mL yeast lysis buffer (1 M sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol) supplemented with 100 µL of

a zymolyase stock solution (1000 U/mL). This mixture was incubated at 30°C for 30 minutes. For carotenoid extraction, the spheroplasts were centrifuged at full speed for 5 minutes and resuspended in 100 µL ddH_2O and 500 µL methanol for the first time and in 250 µL methanol and 250 µL chloroform for second and third time. The incubation was always done at 60°C for 15 minutes. The supernatant of every extraction step was collected and in the end they were combined and dried with a steam of dry nitrogen gas. For HPLC analysis the samples were dissolved in 300 µL methanol.

For the *E. coli* samples the cultivation is described in 3.10. For carotenoid extraction, the methanol and chloroform was directly pipetted to the cells.

HPLC analysis was done with a HPLC device from Agilent Technologies 1100 series and a reversed phase column Carotenoid S-5 µm of YMC. Mobile phase A was a 4:96 mixture of ddH_2O and methanol and the second mobile phase was methanol and Methyl-*tert*-butylether (MTBE) in a ratio of 5:95. The method is shown in Table 4. The injection was 5 µL for each analysis and the flow rate 0.75 mL/min. After 30 minutes the analysis of one sample was stopped. The maximum pressure of the column is 200 bar. The detection wavelengths are following: 450 nm for β-carotene, 380 nm for retinal and 345 nm for retinoic acid.

Table 4: HPLC analysis method for beta-carotenoid derivatives

Time [min]	Solvent A	Solvent B
0	95%	5%
20	35%	65%
25	35%	65%
25.01	95%	5%

Calibration curves of *all-trans* β-carotene and *all-trans* retinoic acid were determined by using following concentrations of the corresponding reference material: 0.007813 mg/mL, 0.015625 mg/mL, 0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL for *all-trans* β-carotene and 0.001 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL and for *all-trans* retinoic acid.

The references of *all-trans* β-carotene, *9-cis* β-carotene, *all-trans* retinal, *all-trans* retinoic acid and 9-cis retinoic acid were from Sigma-Aldrich.

The calibration curves are shown in S4.

3.15. Design of carotenoid constructs

In the beginning, expression constructs for each β-carotene isomerase, 15'-15' monooxygenase and combinations with aldehyde dehydrogenase for the retinoic acid pathway were designed *in silico* using CLC workbench.

After cloning the constructs were sent for sequencing to Microsynth AG or LGC Genomics GmbH.

3.15.1. Construction of plasmids expressing the β-carotene isomerase, 15'-15' monooxygenase and retinal dehydrogenase

For the first attempt, each gene optimized for *P. pastoris* encoded for the truncated (signal sequence for chloroplast was removed) and full-length β-carotene isomerase, 15'-15' monooxygenase and retinal dehydrogenase was assembled with the T4_S vector under the control of the *AOX1* promoter by restriction enzyme based cloning. Therefore, each gene was amplified by PCR with primers including the *EcoR*I and *Not*I restriction sites and additionally the optimized Kozak sequence (CGAAACG). The primers are listed in Table 5. For assembly, the T4_S vector was digested with the restriction enzymes *Eco*RI and *Not*I. Subsequently, the prepared vector and the insert were ligated as described previously. The isomerase of *A. thaliana* was assembled with the T4_S vector by Gibson Assembly as described before.

The *E. coli* optimized genes for the truncated and full-length isomerases were also amplified by using PCR. Each gene was ligated with the T4_S vector and with the BAD vector by Gibson Assembly.

Table 5: Primers used for assembling the T4_S vector with isomerases, monooxygenases and dehydrogenases

Primer #	Primer name	Primer sequence (5'-3')
C587	Iso_Rice_Pp_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGG AAA CTA CAA CCT TGG TCT TGC TTT TGC
C588	Iso_Rice_Pp_NotI_rev	ATA TGC GGC CGC TTA GAT AGA ACA ATT AAC ACC GTG GTT CTG TTT AGC G
C589	Iso_Arabid_Pp_EcoRI_fw	ATA TGA ATT CCG AAA CGA TGA ACA CTA AGT TGT CAC TTA GTC AAA CC
C590	Iso_Arabid_Pp_NotI_rev	TATTGCGGCCGCTTAGTGCTTAACACCGTAGGACTTGTTAGAC

C591	Monoox_marbac_PP_EcoRI_fw	AAT AGA ATT CCG AAA CGA TGG GAC TTA TGT TGA TTG ATT GGT GTG C
C592	Monoox_marbac_PP_Not_rev	TAT AGC GGC CGC TTA ATT TTT GAT CTT AAT TCT GGA AGA GTG AGG TCT G
C593	Monoox_mouse_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGG AAA TCA TCT TCG GTC AAA ACA AGA AAG AAC
C594	Monoox_mouse_PP_NotI_rev	AAT TGC GGC CGC TTA CAA GGA AGA TCC ACC ATG ACC TGC
C595	Monoox_chicken_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGG AAA CAA TTT TTA ACA GAA ACA AGG AAG AGC AC
C596	Monoox_chicken_PP_NotI_fw	TAT TGC GGC CGC TTA CTC AGT TTC TGC TCC CAA GTC ATT TTG AG
C597	Monoox_rat_PP_EcoRI_fw	ATA TGA ATT CCG AAA CGA TGG AAA TCA TCT TCG GTA GAA ACA AGA AAG AAC
C598	Monoox_rat_PP_NotI_fw	TAT TGC GGC CGC TTA CAA AGA CTT TCC ACC ATG ACC AGC AG
C599	retDH_mouse_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGG CTG GTA AAA GAG AAT TGC TTA TGT TGG
C600	retDH_mouse_PP_NotI_rev	TAT TGC GGC CGC TTA ATA TTT GAT AGT AAT GGT CTT AAT CTC AGT GAA AAA GTC G
C601	retDH_human_PP_EcoRI_fw	ATT AGA ATT CCG AAA CGA TGT CTT CCT CAG GTA CAC CAG ACC
C602	retDH_human_PP_NotI_rev	TAT TGC GGC CGC TTA AGA GTT CTT TTG GGA AAT CTT AAC AGT GAC GG
C603	retDH_chicken_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGA AGA AAC AAG GTT CTC CAT CCA ACC C
C604	retDH_chicken_PP_NotI_rev	TAT TGC GGC CGC TTA AGA GTT TTT CTG AGG GAT CTT AAT GGT AAC TG
C605	retDH_rat_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGT CTT CCC CAG CAC AAC CTG CTG
C606	retDH_rat_PP_NotI_rev	TAT TGC GGC CGC TTA AGA GTT CTT TTG AGA AAT CTT CAT AGC AAC AG
C609	Iso_Arabid_Trunc_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGG CTG CCA AAG AAA CAG CTA GAA TTG AGA C
C610	Iso_Rice_Trunc_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGG CAG TCA TGG CTA GAC CAC AAG AGG CC
C614	GSK12_Gibson fw	AACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCcgaaacgATGAACACTAAGTTGTCACTTAGTCAAACCAAAATTTTTAC
C615	GSK12_Gibson rev	CTC AGG CAA ATG GCA TTC TGA CAT CCT CTT GAG CGG CCG CTT AGT GCT TAA CAC CGT AGG ACT TGT TAG AC
C628	GSK1_EcoRI_fwd	ataaGAATTCcgaaacg ATG GAA ACC ACC ACC CTG GTG C
C629	GSK1_NotI_rev	taac GCG GCC GCT TAG ATG CTA CAA TTG ACG CCG TGG TTT TGT TTT GC
C630	GSK2_EcoRI_fwd	tattGAATTCcgaaacg ATGAACACCAAACTGTCACTGTCGC
C631	GSK2_NotI_rev	aataGCG GCC GCT TAG TGT TTC ACG CCG TAC GAT TTG TTT GAT TTG

C653	GSK1_gibsT4_fwd	ACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCCG AAACG ATG GAA ACC ACC ACC CTG GTG C
C654	GSK1_gibsT4_rev	TCT CTC AGG CAA ATG GCA TTC TGA CAT CCT CTT GAG CGG CCG CTT AGA TGC TAC AAT TGA CGC CGT GGT TTT GTT TTG C
C655	GSK1_trunc_gibsT4 fwd	ACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCcg aaacgATG GCG GTT ATG GCA CGC CC
C656	GSK2_gibsT4_fwd	ACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCcg aaacg ATGAACACCAAACTGTCACTGTCGC
C657	GSK2_gibsT4_rev	TCT CTC AGG CAA ATG GCA TTC TGA CAT CCT CTT GAG CGG CCG CTT AGT GTT TCA CGC CGT ACG ATT TGT TTG ATT TGC
C658	GSK2_trunc_gibsT4 fwd	ACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCcg aaacgATG GCG GCC AAA GAA ACC GCA C
C661	GSK1_ara_gib_fwd	AGCCTTTCGTTTTATTTGATGCCTGATGGTCGACTTTTAGATGC TACAATTGACGCCGTGGTTTTGTTTTGC
C662	GSK1_ara_gib_rev	GCT TTT TAT CGC AAC TCT CTA CTG TTG GAG CTC GAG GCC TCG TCG ACT TAA AGA GGA GAA ATT AAC TAT GGA AAC CAC
C663	GSK1t_ara_gib_fwd	TCGTTTTATTTGATGCCTGATGGTCGACTTATTAGATGCTACAA TTGACGCCGTGGTTTTGTTTTGC
C664	GSK1t_ara_gib_rev	TTA TCG CAA CTC TCT ACT GTT GGA GCT CGA GGC CTC GTC GAC TTA AAG AGG AGA AAT TAA CTA TGG CGG TTA TGG CAC
C665	GSK2_ara_gib_fwd	TCGTTTTATTTGATGCCTGATGGTCGACTTATTAGTGTTTCACG CCGTACGATTTGTTTGATTTGC
C666	GSK2_ara_gib_rev	TAT CGC AAC TCT CTA CTG TTG GAG CTC GAG GCC TCG TCG ACT TAA AGA GGA GAA ATT AAC TAT GAA CAC CAA ACT GTC
C667	GSK2t_ara_gib_fwd	CGTTTTATTTGATGCCTGATGGTCGACTTATTAGTGTTTCACGC CGTACGATTTGTTTGATTTGC
C668	GSK2t_ara_gib_rev	TAT CGC AAC TCT CTA CTG TTG GAG CTC GAG GCC TCG TCG ACT TAA AGA GGA GAA ATT AAC TAT GGC GGC CAA AGA AAC
C713	Iso_Brassrap_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGA TGA ATA CAA AAT TGT CCC TTT CAC AAA CC
C714	Iso_Brassrap_PP_NotI_rev	ATT TGC GGC CGC TTA GTG CTT AAC ACC GTA GGA TCT GTT GG
C715	Iso_Camsat_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGA TGA ATA CCA AGT TGT CAC TTA GTC ATA C
C716	Iso_Camsat_PP_NotI_rev	ATA TGC GGC CGC TTA GTG TTT AAC ACC ATA GGA TTT GTT AGA CTT AC
C717	Iso_Micpuss_PP_EcoRI_fw	TAA TGA ATT CCG AAA CGA TGT CTT ACT CCT TTT TGG GTC AAT CTA C
C718	Iso_Micpuss_PP_NotI_rev	TAT TGC GGC CGC TTA TCC TCT AAG CAA ATC ACA TGT CTC ATT AGC

3.15.2. Construction of plasmids expressing β-carotene isomerase eGFP fusions with different N-terminal modifications under the control of different promoters

Different strategies were applied to improve the expression of the isomerases. First, the N-terminal signal peptide of the isomerases were replaced by either the maltose binding protein (MBP) or the signal sequence of alternative oxidase (AOD). Furthermore, four expression constructs with different promoters were assembled, two constitutive versions and two inducible versions. For the constitutive plasmids the *TPI* and *ADH2* promoter were chosen and for the inducible plasmids the *CAT* and *AOX1* promoter were used. In addition, the eGFP was fused C-terminal to each promoter construct and to each N-terminal modification.

The eGFP (sequence shown in appendix; provided from Pia Fauland), the full-length and truncated isomerases were amplified by PCR using primers with a linker (see appendix) to eGFP and with *Spe*I and *Not*I restriction sites. Primers are listed in Table 6. Via overlap extension PCR the desired isomerases and the eGFP were linked together. For assembly, the T4_S vector was digested with *Spe*I and *Not*I restriction enzymes. The ligation of the vector and the isomerase-eGFP fusions were performed as described before.

Table 6: Primers used for assembling the T4_S vector with isomerases fused to eGFP

Primer #	Primer name	Primer sequence (5'-3')
C16	NotI_eGFP_rev	TATAGCGGCCGCTTACTTGTACAATTCATCCATGCCATGTG
C673	Linker_eGFP_fw	GCTGCTGTTGATCCAAAGTTGTTGGGTGCTATGGCTAGCAAAGG AGAAGAACTTTTCAC
C674	Iso_Arab_Pp_SpeI_fw	ATA TAC TAG TCG AAA CGA TGA ACA CTA AGT TGT CAC TTA GTC AAA CC
C675	Iso_Arab_Pp_Linker_rev	CAC CCA ACA ACT TTG GAT CAA CAG CAG CAG CGT GCT TAA CAC CGT AGG ACT TGT TAG AC
C676	Iso_tr_Arab_Pp_SpeI_fw	TAT AAC TAG TCG AAA CGA TGG CTG CCA AAG AAA CAG CTA GAA TTG AGA C
C684	Iso_Rice_Pp_SpeI_fw	ATA TAC TAG TCG AAA CGA TGG AAA CTA CAA CCT TGG TCT TGC TTT TGC
C685	Iso_Rice_Pp_Linker_rev	ACA ACT TTG GAT CAA CAG CAG CAG CGA TAG AAC AAT TAA CAC CGT GGT TCT GTT TAG CG
C686	Iso_tr_Rice_Pp_SpeI_fw	ATA TAC TAG TCG AAA CGA TGG CAG TCA TGG CTA GAC CAC AAG AG
C713	Iso_Brassrap_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGA TGA ATA CAA AAT TGT CCC TTT CAC AAA CC
C715	Iso_Camsat_PP_EcoRI_fw	TAT AGA ATT CCG AAA CGA TGA TGA ATA CCA AGT TGT CAC TTA GTC ATA C

C717	Iso_Micpuss_PP_EcoRI_fw	TAA TGA ATT CCG AAA CGA TGT CTT ACT CCT TTT TGG GTC AAT CTA C
C749	Iso_CamSat_Linker_rev	ACT TTG GAT CAA CAG CAG CAG CGT GTT TAA CAC CAT AGG ATT TGT TAG ACT TAC AGA AC
C750	Iso_MicoPus_Linker_rev	CAA CAA CTT TGG ATC AAC AGC AGC AGC TCC TCT AAG CAA ATC ACA TGT CTC ATT AGC TC
C751	Iso_Brassica_Linker_rev	AGC ACC CAA CAA CTT TGG ATC AAC AGC AGC AGC GTG CTT AAC ACC GTA GGA TCT GTT GG

The AOD signal sequence (see appendix) linked to the *AOX1* promoter was provided from Peter Kusstatscher. This template and the truncated isomerase-eGFP constructs were amplified by PCR using primers to create overhangs between them and with *the Smi*I restriction site. Primers are listed in Table 7. By overlap extension PCR the AOD-AOX sequence and the truncated isomerase-eGFP constructs were linked together. The T4_S vector was prepared by the restriction enzymes *Smi*I and *Not*I. For assembly a restriction enzyme cloning was performed as described before.

Table 7: Primers used for assembling the T4_S vector with isomerases fused to AOD

Primer #	Primer name	Primer sequence (5'-3')
C16	NotI_eGFP_rev	TATAGCGGCCGCTTACTTGTACAATTCATCCATGCCATGTG
C678	SmiI_pAOX1_fw	ATA TAT TTA AAT AAC ATC CAA AGA CGA AAG GTT GAA TGA AAC C
C679	mit_tr_IsoArab_fw	GTG GCT TCC ACT TTC AGG CAC TAT GCT GCC AAA GAA ACA GCT AGA ATT GAG AC
C680	mit_AOD_IsoArab_rev	GTC TCA ATT CTA GCT GTT TCT TTG GCA GCA TAG TGC CTG AAA GTG GAA GCC AC
C687	mit_tr_IsoRice_fw	GTG GCT TCC ACT TTC AGG CAC TAT GCA GTC ATG GCT AGA CCA CAA GAG G
C688	mit_AOD_IsoRice_rev	CCT CTT GTG GTC TAG CCA TGA CTG CAT AGT GCC TGA AAG TGG AAG CCA C

The template for the MBP included a linker (sequence see appendix) and a protease site and was provided by Christian Schmid. For amplification of this template a PCR was performed with primers including the *Spe*I restriction site. The existing isomerase-eGFP construct was amplified with primers including the *Not*I restriction site. Primers are listed in Table 8. By overlap extension PCR the prepared DNA fragments were linked together first and then amplified. The T4_S vector was digested with the restriction enzymes *Spe*I and *Not*I. For assembly, a ligation was performed as described previously.

The *E. coli* optimized genes coding for the truncated isomerases were also fused to MBP. The MBP template for *E. coli* was provided from Corinna Odar. The truncated isomerases were amplified by PCR using primers to create overhangs to MBP. For fusion an overlap extension PCR was performed. The truncated isomerases-MBP fusions were linked with the BAD vector by Gibson Assembly.

Table 8: Primers used for assembling the T4_S vector with isomerases fused to MBP

Primer #	Primer name	Primer sequence (5'-3')
C16	NotI_eGFP_rev	TATAGCGGCCGCTTACTTGTACAATTCATCCATGCCATGTG
C661	GSK1_ara_gib_fwd	AGCCTTTCGTTTTATTTGATGCCTGATGGTCGACTTTTAGATGCTACAAT TGACGCCGTGGTTTTGTTTTGC
C665	GSK2_ara_gib_fwd	TCGTTTTATTTGATGCCTGATGGTCGACTTATTAGTGTTTCACGCCGTAC GATTTGTTTGATTTGC
C681	MBP_IsoArab_fw	GGT CCT GAG AAC CTT TAC TTT CAA GCT ATG GCT GCC AAA GAA ACA GCT AGA ATT GAG AC
C682	MBP_IsoArab_rev	GTC TCA ATT CTA GCT GTT TCT TTG GCA GCC ATA GCT TGA AAG TAA AGG TTC TCA GGA CC
C683	MBP_SpeI_fw	ATA TAC TAG TCG AAA CGA TGA AGA TCG AGG AAG GTA AGT TAG TGA TTT GG
C689	MBP_IsoRice_fw	GGT CCT GAG AAC CTT TAC TTT CAA GCT ATG GCA GTC ATG GCT AGA CCA CAA GAG
C690	MBP_IsoRice_rev	CTC TTG TGG TCT AGC CAT GAC TGC CAT AGC TTG AAA GTA AAG GTT CTC AGG ACC
C703	GSK1_linker_rev	CAA TAA CAA CAA CCT CGG GAT CGA GGG AAG GAT TTC AAT GGA AAC CAC CAC CCT GGT GC
C704	Linker_MBP_fw	TTCCCTCGATCCCGAGGTTGTTGTTATTGTTATTGTTGTTGTTGTTCGAG CTCGAATTAGTCTGCGCGTCTTTCAGGGC
C705	MBP_ara_rev	ACT CTC TAC TGT TGG AGC TCG AGG CCT CGT CGA CTT AAA GAG GAG AAA TTA ACT ATG AAA ATC GAA GAA GGT AAA CTG G
C706	GSK2_linker_rev	TAA CAA CAA CCT CGG GAT CGA GGG AAG GAT TTC AAT GAA CAC CAA ACT GTC ACT GTC GC

The *TPI* and *ADH2* promoters [12] (sequences see appendix) were amplified by PCR using primers with *Spe*I and *Smi*I restriction sites. Primers are listed in Table 9. The *CAT* promoter was prepared from T4_S_CAT. For replacing the *AOX1* promoter, the already generated expression constructs were digested with the restriction enzymes *Spe*I and *Smi*I. The assembly was performed by ligation.

Table 9: Primers used for assembling the T4_S vector different promoters

Primer #	Primer name	Primer sequence (5'-3')
C669	pTPI_SmiI_fw	CAC TAT TTA AAT TCA ACG AGA CAC TCT TCC GTC AGT TCC
C670	pTP1_SpeI_rev	CTC TAC TAG TTG TGT TTG TGA TAG ATC TTG TAT ATC AAT GAA AAA AAC TG

C671	pADH2_SmiI_fw	CTA CAT TTA AAT CGC AGC GTT TTC TGA CGG TAC TAG AG
C672	pADH2_SpeI_rev	TAT CAC TAG TTT TCG TAA AGT AAA TAA GAT AAA AGC TAG TAG CTG ATG GAA G

3.15.3. Construction of plasmids combining the 15'-15' monooxygenase and the retinal dehydrogenase with 2A sequences

For the production of *9-cis* retinoic acid, the 15'-15' monooxygenase and the retinal dehydrogenase were combined. One strategy was to link these enzymes via a 2A peptide sequence. Therefore, the 15'-15' monooxygenase and the retinal dehydrogenase were amplified by PCR using primers that included the DNA coding for the T2A sequence. Primers are listed in Table 10. A restriction digest was performed with the enzymes *EcoR*I and *Not*I for preparing the T4_S vector. The T4_S vector and the combinations were ligated by Gibson Assembly. Figure 3 shows the chicken/rat construct.

Table 10: Primers used for combining the 15'-15' monooxygenase and retinal dehydrogenase with 2A sequences.

Primer #	Primer name	Primer sequence (5'-3')
C616	MonoChick_comb1_fw	AACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCcgaaacgATGGAAACAATTTTTAACAGAAACAAGGAAGAGCAC
C617	MonoChick_comb1_rev	GGG TCC TGG ATT TTC TTC CAC ATC ACC GCA GGT AAG TAA GGA ACC ACG GCC CTC TGC ACG CTC AGT TTC TGC TCC CAA GTC ATT TTG AGG
C618	retDH_rat_comb1_fw	CGTGCAGAGGGCCGTGGTTCCTTACTTACCTGCGGTGATGTGGAAGAAAATCCAGGACCCTCTTCCCCAGCACAACCTGCTGTTC
C619	retDH_rat_comb1_rev	CAT CTC TCA GGC AAA TGG CAT TCT GAC ATC CTC TTG AGC GGC CGC TTA AGA GTT CTT TTG AGA AAT CTT CAT AGC AAC AG
C620	monox_mouse_comb2_fw	CTT GAG AAG ATC AAA AAA CAA CTA ATT ATT GAA AGA ATT CCG AAA CGA TGG AAA TCA TCT TCG GTC AAA AC
C621	monox_mouse_comb2_rev	CTGGATTTTCTTCCACATCACCGCAGGTAAGTAAGGAACCACGGCCCTCTGCACGCAAGGAAGATCCACCATGACCTGC
C622	retDH_mouse_comb2_fw	CCGTGGTTCCTTACTTACCTGCGGTGATGTGGAAGAAAATCCAGGACCCGCTGGTAAAAGAGAATTGCTTATGTTGGAG
C623	retDH_mouse_comb2_rev	GCCTGCATCTCTCAGGCAAATGGCATTCTGACATCCTCTTGAGCGGCCGCTTAATATTTGATAGTAATGGTCTTAATC

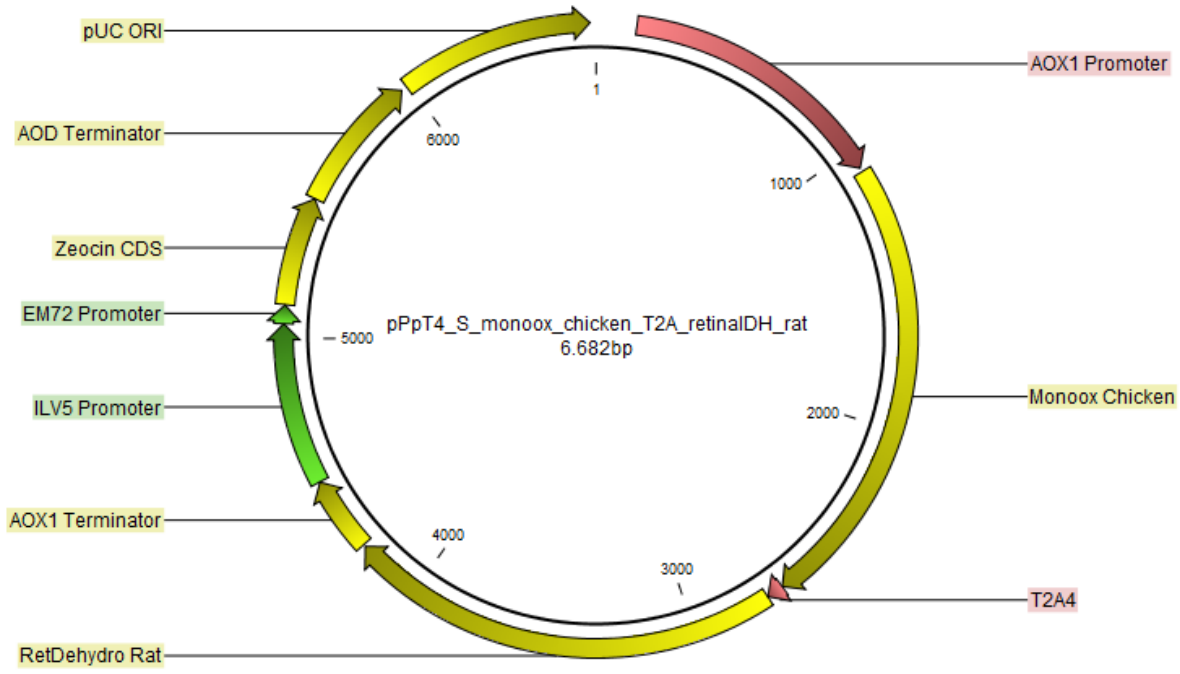

Figure 3: Construct of combined monooxygenase from chicken and retinal dehydrogenase from rat with the 2A sequence.

3.15.4. Construction of plasmids combining the 15'-15' monooxygenase and the retinal dehydrogenase with bidirectional promoters

The second strategy for a combination of the 15'-15' monooxygenase and the retinal dehydrogenase was to use the bidirectional promoter BZ6. The template was #TV0935 of the Thomas Vogl strain collection. For creating overhangs between the enzymes and the promoter, a PCR was performed. Primers are listed in Table 11. The vector #TV0975 was digested with the restriction enzyme *Not*I. The vector and the combined enzymes were assembled by Gibson Assembly. In Figure 4 the construct of the combined monooxygenase from chicken and retinal dehydrogenase from is shown.

Table 11: Primers used for combining the 15'-15' monooxygenase and retinal dehydrogenase with the bidirectional promoter BZ6.

Primer #	Primer name	Primer sequence (5'-3')
C764	RalDH_mouse_BZ6_fw	GTT CTC CAA CAT AAG CAA TTC TCT TTT ACC AGC CAT TGT TGT AGT TTT AAT ATA GTT TGA GTA TGA GAT GGA ACT CAG
C765	RalDH_mouse_BZ6_rev	CTG AGT TCC ATC TCA TAC TCA AAC TAT ATT AAA ACT ACA ACA ATG GCT GGT AAA AGA GAA TTG CTT ATG TTG GAG AAC

C766	DAS1_TT_RalDH_mouse_fw	CCT AAC TAA AAC TGT AAA GAC TTC CCG TGC GGC CGC TTA ATA TTT GAT AGT AAT GGT CTT AAT CTC AGT GAA AAA GTC G
C767	BZ6_monoox_mouse_fw	CTA AAC ACT AAA GTT CAC TCT TAT CAA ACT ATC AAA CAT CAA AAA TGG AAA TCA TCT TCG GTC AAA ACA AGA AAG AAC
C768	BZ6_monoox_mouse_rev	GTT CTT TCT TGT TTT GAC CGA AGA TGA TTT CCA TTT TTG ATG TTT GAT AGT TTG ATA AGA GTG AAC TTT AGT GTT TAG
C769	AOX1_TT_monox_mouse_rev	CAG GCA AAT GGC ATT CTG ACA TCC TCT TGA GCG GCC GCT TAC AAG GAA GAT CCA CCA TGA CCT GC
C770	RalDH_rat_BZ6_fw	GAG GGG CTG GAA CAG CAG GTT GTG CTG GGG AAG ACA TTG TTG TAG TTT TAA TAT AGT TTG AGT ATG AGA TGG AAC TCA G
C771	RalDH_rat_BZ6_rev	CTG AGT TCC ATC TCA TAC TCA AAC TAT ATT AAA ACT ACA ACA ATG TCT TCC CCA GCA CAA CCT GCT GTT CCA GCC CCT C
C772	DAS1_TT_RalDH_rat_fw	GGG CTC CTA ACT AAA ACT GTA AAG ACT TCC CGT GCG GCC GCT TAA GAG TTC TTT TGA GAA ATC TTC ATA GCA ACA G
C773	BZ6_monoox_chicken_fw	TAAACACTAAAGTTCACTCTTATCAAACTATCAAACATCAAAA ATGGAAACAATTTTTAACAGAAACAAGGAAGAGCAC
C774	BZ6_monoox_chicken_rev	TG CTC TTC CTT GTT TCT GTT AAA AAT TGT TTC CAT TTT TGA TGT TTG ATA GTT TGA TAA GAG TGA ACT TTA GTG TTT AG
C775	AOX1_TT_monox_chicken_rev	GCA TCT CTC AGG CAA ATG GCA TTC TGA CAT CCT CTT GAG CGG CCG CTT ACT CAG TTT CTG CTC CCA AGT CAT TTT GAG

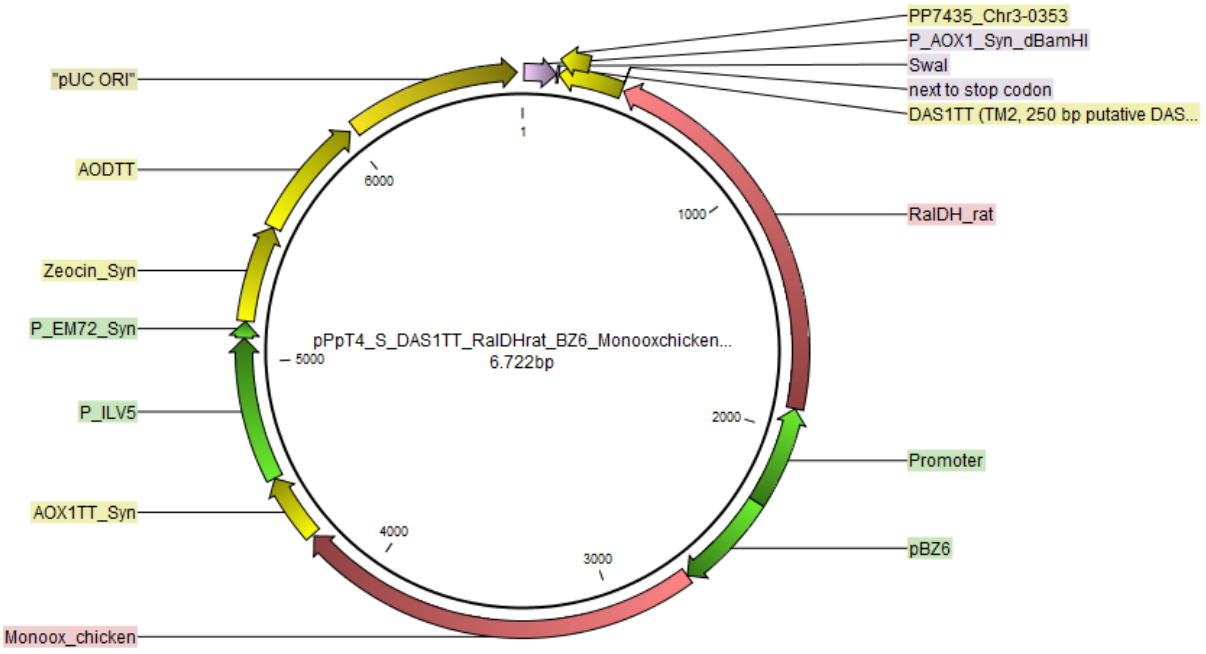

Figure 4: Construct of combined monooxygenase from chicken with retinal dehydrogenase from rat under the control of the bidirectional promoter BZ6

3.15.5. Construction of plasmids combining the β-carotene isomerase, 15'-15' monooxygenase and retinal dehydrogenase

For production of *9-cis* retinoic acid, the β-carotene isomerase was combined with the bidirectional construct of the chicken/rat construct. Therefore, the truncated isomerase from *A. thaliana* under the control of the P_{CAT} was amplified by PCR using primers to create overhangs to the DAS1 terminator and the promoter sequence P_AOX1_Syn, which is a residual promoter and inactive. A Gibson assembly was performed to assemble the isomerase with the bidirectional construct. In Figure 5 this construct is shown.

Table 12: Primers used for combining the β-carotene isomerase, 15'-15' monooxygenase and retinal dehydrogenase

Primer #	Primer name	Primer sequence (5'-3')
C785	3Kombi_pCAT_fw	GAT CGG GAA CAC TGA AAA ATA CAC AGT TAT TAT TCA TTT AAA TTA ATC GAA CTC CGA ATG CGG TTC TCC TGT AAC
C786	3Kombi_AOXTT_rev	AGA ATA GGG ACT CCC AAA GTG TCA GTC ACA AGG GTC GCA CAA ACG AAG GTC TCA CTT AAT CTT CTG TAC TC

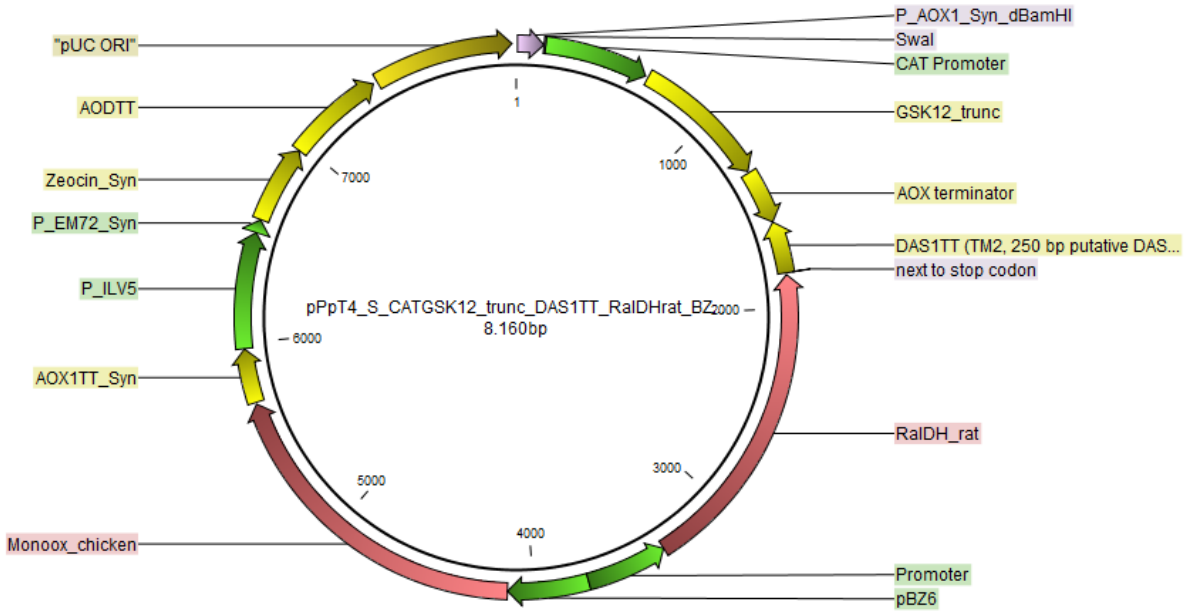

Figure 5: Construct of combined truncated β-carotene isomerase from *A. thaliana* under the control of P_{CAT}, 15'-15' monooxygenase from chicken and retinal dehydrogenase from rat linked with the bidirectional promoter BZ6

The expression constructs created during this master thesis are listed in the appendix.

All constructs were sent for sequencing after transformation into *E. coli* Top 10F'.

3.16. *E. coli* pBAD expression plasmid preparation

Based on the inactive isomerases in *P. pastoris*, the codon optimized genes encoded for isomerases were analysed in *E. coli*. Therefore, a suitable *E. coli* vector had to be prepared. For this purpose, the pREP_pBAD plasmid from Patrik Fladischer was modified, i.e. the eGFP was removed (see appendix). The restriction digest included 47 µL plasmid DNA, 5.8 µL FastDigest White buffer, 2.5 µL *PstI* and 2.5 µL *BglII* and was incubated for 2 hours at 37°C. The DNA was purified via preparative gel electrophoresis and extracted from the agarose with the Wizard® SV Gel and PCR Clean-Up System. To create blunt ends for re-ligation of the plasmid, the reaction mixture was set up on ice and included 10 µL 2x reaction buffer, 2 µL DNA Blunting enzyme and 7 µL DNA sample. After the mix was vortexed and centrifuged shortly, an incubation step followed at 70 °C for 5 minutes. Then 1 µL T4 DNA Ligase was added for circularisation. The ligation mixture was incubated at room temperature for 5 minutes and was used directly to transform *E. coli* Top 10F' cells for sequencing.

For cloning the *E. coli* optimized isomerases Gibson Assembly was used. Therefore the adapted vector pBAD was digested with 2.5 µL *SalI* and 5 µL FastDigest Green buffer. After 1.5 hours 1.5 µL FastAP was added for dephosphorylation. The fragments were electrophoretically separated with 1 % agarose gel and purified and concentrated with the Wizard® SV Gel and PCR Clean-Up System. Gibson Assembly was done as described in chapter 2.3. The ligated constructs were used to transform *E. coli* Top 10F' cells.

The *E. coli* codon optimized genes for monooxygenase and dehydrogenase were not analysed, since these enzymes were active and showed activity in *P. pastoris*.

3.17. Staining with Mitotracker® Red CMXRos

The staining of the mitochondria of the AOD-isomerases-eGFP constructs were performed according the protocol of Invitrogen/LifeTechnologies [42].

In this study the cells were stained with the lowest concentration (50 nM) of the Mitotracker® Red CMXRos and for 15 minutes. This dye is visible at 579/599 nm. The used microscope was a Leica TCS SPE. Armin Erlacher performed the microscopy analysis.

4. Results and Discussion

4.1. Identification of pathway enzymes

The enzymes required for the production of *9-cis* retinoic acid were chosen in cooperation with GSK. Based on a first literature and database search (e.g. BRENDA database) by Martina Geier, eligible enzymes were suggested to GSK. After a joint decision, genes coding for the selected enzymes were ordered by GSK with a codon usage optimized for expression in *P. pastoris* and *E. coli*, respectively. The accession numbers of the β-carotene isomerases, the β-carotene 15,15´-monooxygenases and the retinal dehydrogenases are listed in Table 13.

Table 13: Accession numbers of the β-carotene isomerases, the β-carotene 15,15´-monooxygenases and the retinal dehydrogenases

Enzyme	Accession Number
β-carotene isomerase *A. thaliana*	Q7XA78
β-carotene isomerase *O. sativa*	C7AU21
β-carotene isomerase *C. sativa*	XP_010474908
β-carotene isomerase *B. rapa*	XP_009118720
β-carotene isomerase *M. pusilla*	XP_003064988
β-carotene 15,15´-monooxygenase murine bacterium	Q4PNI0
β-carotene 15,15´-monooxygenase mouse	Q9JJS6
β-carotene 15,15´-monooxygenase chicken	NP_989966 XP_414163
β-carotene 15,15´-monooxygenase rat	Q91XT5
retinal dehydrogenase mouse	NP_848828 XP_994422
retinal dehydrogenase chicken	NP_989908
retinal dehydrogenase rat	NP_071852
retinal dehydrogenase human	AAP36480

A BLAST search was done with the isomerase from *A. thaliana* and *O. sativa* to select the alternative isomerases. For prediction of signal sequences, different tools were used e.g. SignalP, TargetP, PSort and ChloroP.

4.2. Investigating the functional expression of β-carotene isomerase in *P. pastoris* and *E. coli*

4.2.1. Functional expression of *P. pastoris* optimized genes for β-carotene isomerases in *P. pastoris* crtEBIY strain and CBS7435 strain

The β-carotene isomerases from *Arabidopsis thaliana* and *Oryza sativa* are described to convert the *all-trans* β-carotene into *9-cis* β-carotene [BRENDA database]. For a first functional analysis, the enzymes and also the truncated versions of them were recombinantly produced in the *P. pastoris* CBS7435 and in the β-carotene producing *P. pastoris* strain. The latter strain harbours all four genes to produce β-carotene and is thus producing the substrate for the β-carotene isomerases *in vivo*. Due to the presence of signal peptides for chloroplasts, the truncated versions were tested too because *P. pastoris* does not possess chloroplasts. To investigate if the isomerases show activity *in vivo*, a HPLC analysis was performed after cultivation and extraction.

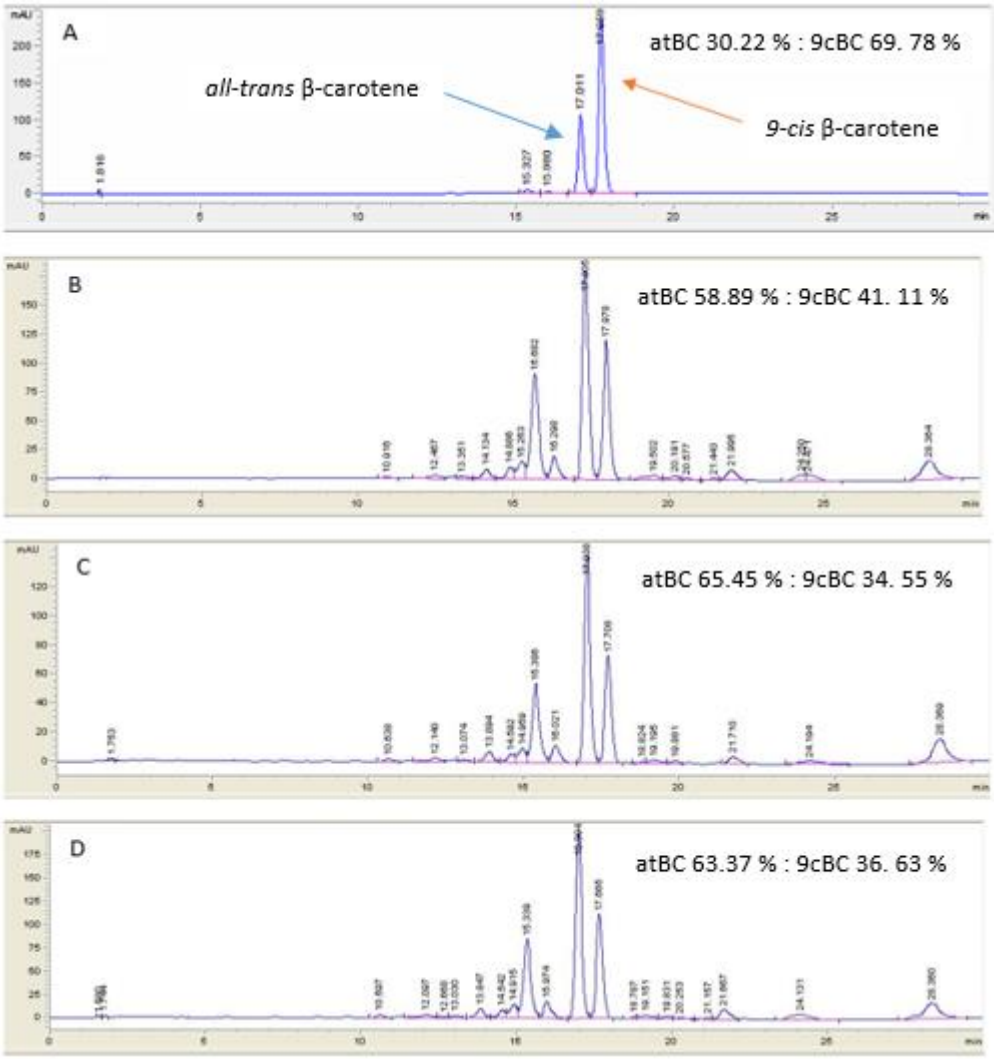

Figure 6: HPLC analysis to assess the *in vivo* activity of the *P. pastoris* optimized isomerases in the *P. pastoris* crtEBIY strain. UV-chromatogram at 450 nm. The β-carotene peaks are detected between minute 17 and 18. A) *9-cis* β-carotene reference B) *P. pastoris* crtEBIY strain [empty control] C) *Oryza sativa* isomerase D) *Arabidopsis thaliana* truncated isomerase. The ratios of *all-trans* and *9-cis* β-carotene detected in the analysed samples and of the crtEBIY strain are indicated. atBC: *all-trans* β-carotene, 9cBC: *9-cis* β-carotene

Figure 6 shows the obtained chromatograms of *9-cis* β-carotene reference material (panel A) and of the analysed recombinant *P. pastoris* strains with the β-carotene isomerase (panel C and D). The analysed isomerases samples did not produce the *9-cis* β-carotene, indicated by the ratios of *all-trans* and *9-cis* β-carotene formed in the analysed samples and of the crtEBIY strain. For calculating the ratios, the peak areas were summed up and were spread in percentage to obviate fluctuations during the extraction steps.

The *9-cis* β-carotene reference material showed a peak for *all-trans* β-carotene too. A reason for the unexpected reaction might be that the isomerization can occur spontaneously.

Beside the *all-trans* and *9-cis* β-carotene, there are other peaks visible that might be other carotenoids such as lycopene or α-carotene or artefacts of the extraction steps. To investigate artefacts derived from sample preparation (3.14.), the *9-cis* β-carotene reference and the *all-trans* β-carotene reference (Figure 7) were also extracted.

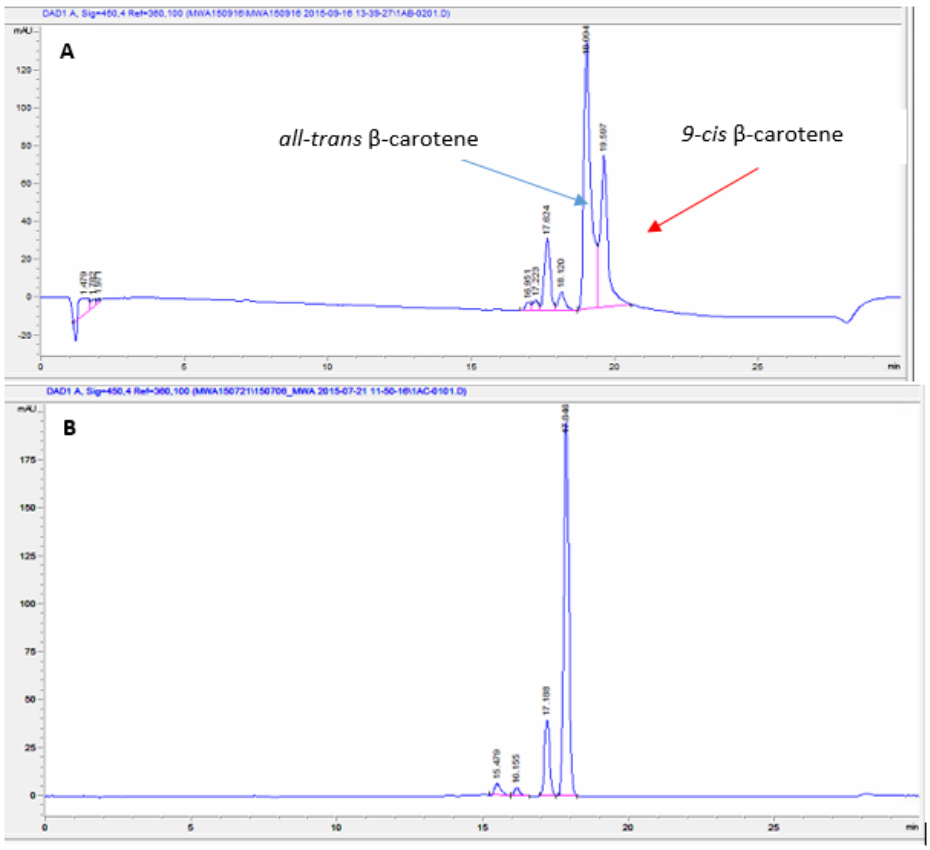

Figure 7: HPLC analysis of extracted *all-trans* reference (A) and *9-cis* β-carotene reference (B). UV-chromatogram at 450 nm. The β-carotene peaks were detected between minute 17 and 20.

The absence of enzyme activity can either be explained by a lack of functional expression or no production of the target enzyme at all.

Due to these facts, a SDS-Page was performed to assess if the enzyme was produced at all. The first gel in Figure 8 shows the enzymes optimized for *P. pastoris.*

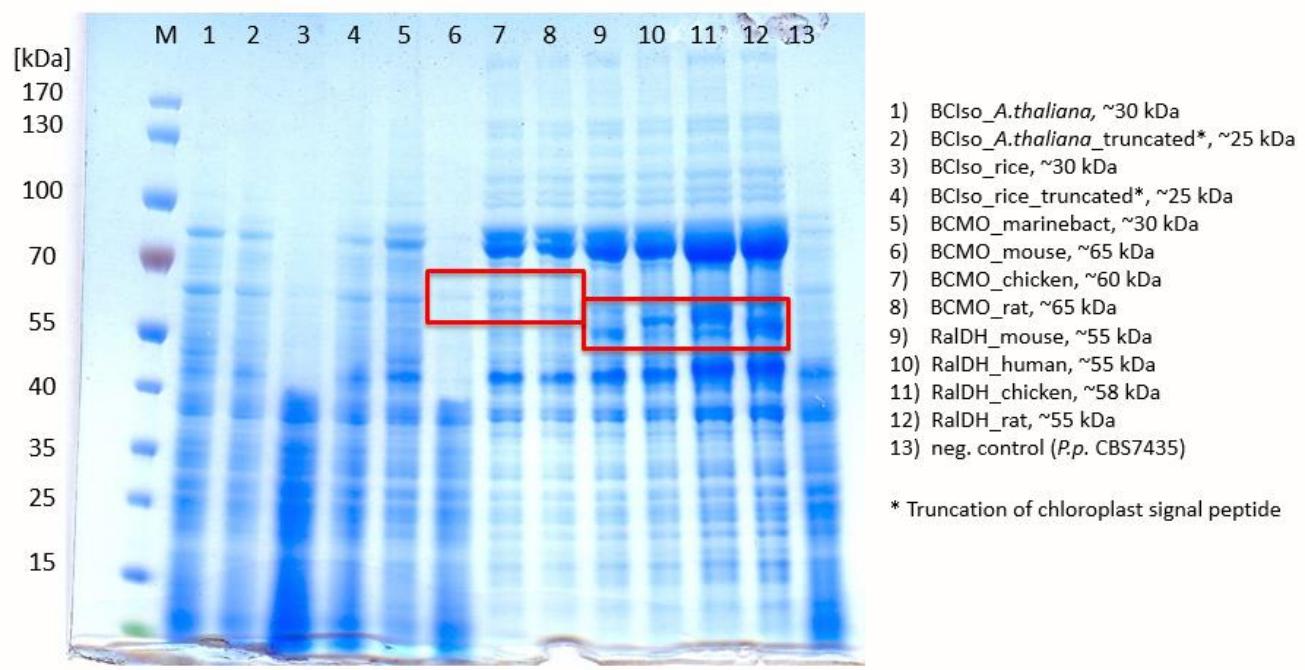

Figure 8: SDS-Page for analysis of the *P. pastoris* optimized genes for isomerases, monooxygenases and retinal dehydrogenases.

The red boxes indicate the bands for the enzymes with the right size. In contrast to the monooxygenases and retinal dehydrogenases, no bands corresponding to the isomerases were observed. This is hard to say because the bands are in the low-molecular area. The smear in lane 3 and 6 below 40 kDa could be a reason for degradation of proteins.

Because the truncated versions weren't active and expressed too, alternative isomerases were expressed and tested for their *in vivo* activity. To identify potential candidates, a BLAST search was done first with the isomerases from *O. sativa* and *A. thaliana* as templates. Isomerases from *Camelina sativa* and *Brassica rapa* showed high sequence identity to *A. thaliana* and *O. sativa* and have a predicted chloroplast signal peptide too. The third isomerase from *Micromonas pusilla* has low sequence identity and has no signal peptide. This enzyme was chosen because maybe the signal sequences influences the activity.

Also the alternative isomerases weren't active *in vivo* too as displayed in Figure 9.

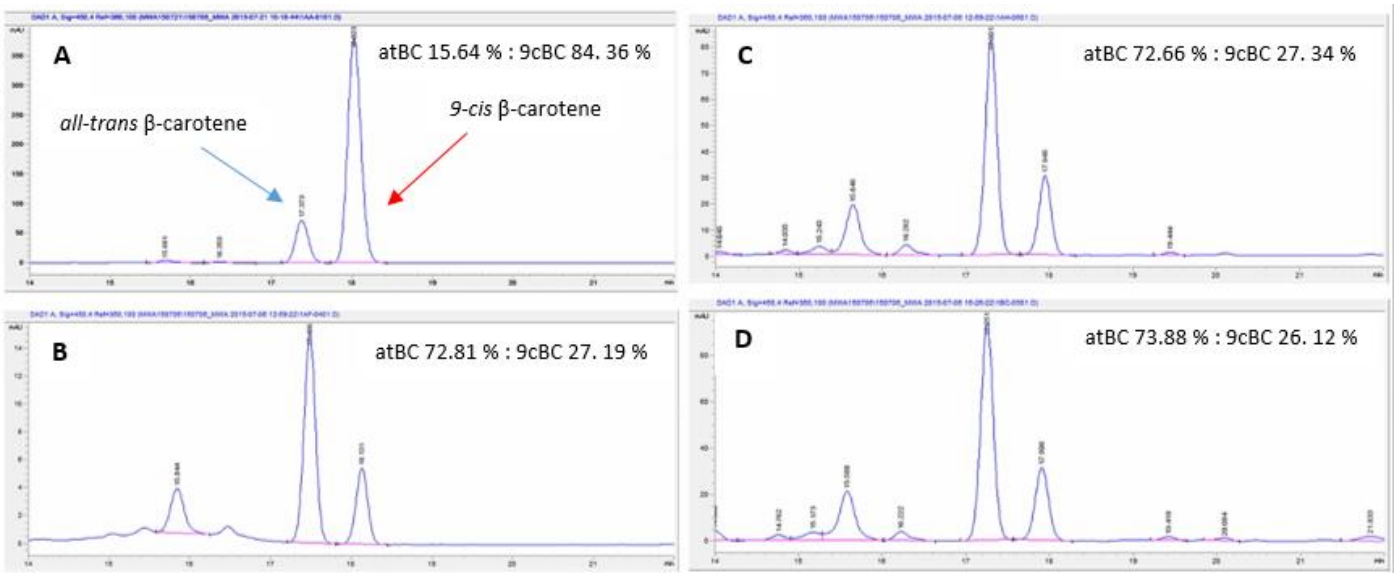

Figure 9: HPLC analysis to assess the *in vivo* activity of the alternative isomerases in the carotene producing *P. pastoris* strain. UV-chromatogram at 450 nm. Peaks were detected between the 17th and 18th minute. A) *9-cis* β-carotene reference B) *Camelina sativa* isomerase C) *Brassica rapa* isomerase D) *Micromonas pusilla* isomerase. The ratios of *all-trans* and *9-cis* β-carotene detected in the analysed samples and of the crtEBIY strain are indicated. atBC: *all-trans* β-carotene, 9cBC: *9-cis* β-carotene

In addition, it seems that the *9-cis* retinoic acid isn't that stable as chromatogram A shows *all-trans* retinoic acid too. A possible explanation for this result might be the occurrence of spontaneous isomerization.

In a next step, the *E. coli* optimized isomerases genes were expressed in the *P. pastoris* producing β-carotene strain. The expression of them might be better because of another codon usage.

As the *P. pastoris* optimized genes coding for the isomerases in Figure 6, the isomerases encoded from *E. coli* optimized genes showed the same results for producing the *9-cis* β-carotene, shown in Figure 10. This analysis was done with a HPLC device from Shimadzu Nexera equipped with an SPD-M20A photodiode array detector. The method is described in 3.14.

There is no reason known why the measurements of the samples were crooked.

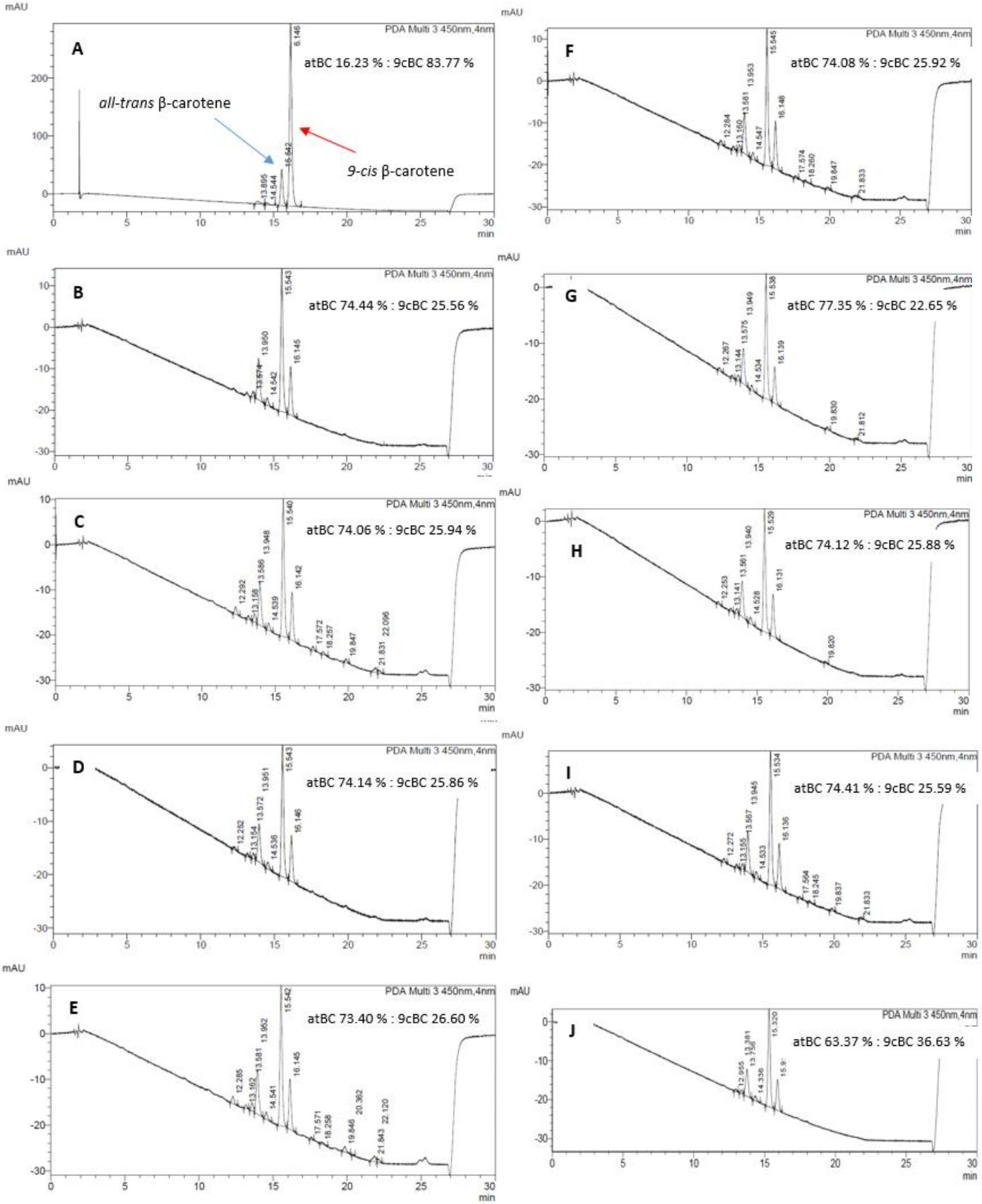

Figure 10: HPLC analysis of *in vivo* activity of the *E. coli* optimized isomerases in the carotene producing *P. pastoris* strain. UV-chromatogram at 450 nm A) 9-cis β-carotene reference B) *O. sativa* isomerase C1 C) *O. sativa* isomerase E6 D) *Osativa* truncated isomerase D12 E) *Osativa* truncated isomerase G10 F) *A. thaliana* isomerase C6 G) *A. thaliana* isomerase F3 H) *A. thaliana* truncated isomerase C9 I) *A. thaliana* truncated isomerase F11 J) *P. pastoris* crtEBIY strain [empty control]. The ratios of *all-trans* and *9-cis* β-carotene detected in the analysed samples and of the crtEBIY strain are indicated. atBC: *all-trans* β-carotene, 9cBC: *9-cis* β-carotene

4.2.2. *In vivo* activity of *E. coli* optimized isomerases in *E. coli* crtEBIY strain

Figure 11 displays the results of the analysed isomerases in the carotene producing *E. coli* strain (*E. coli* BL21 pC21e1 crtEBIY) with an arabinose inducible promoter. As observed for the isomerases produced in *P. pastoris*, also the ones produced in *E. coli* did not show the desired activity. So the production of the enzymes in *E. coli* is problematic too.

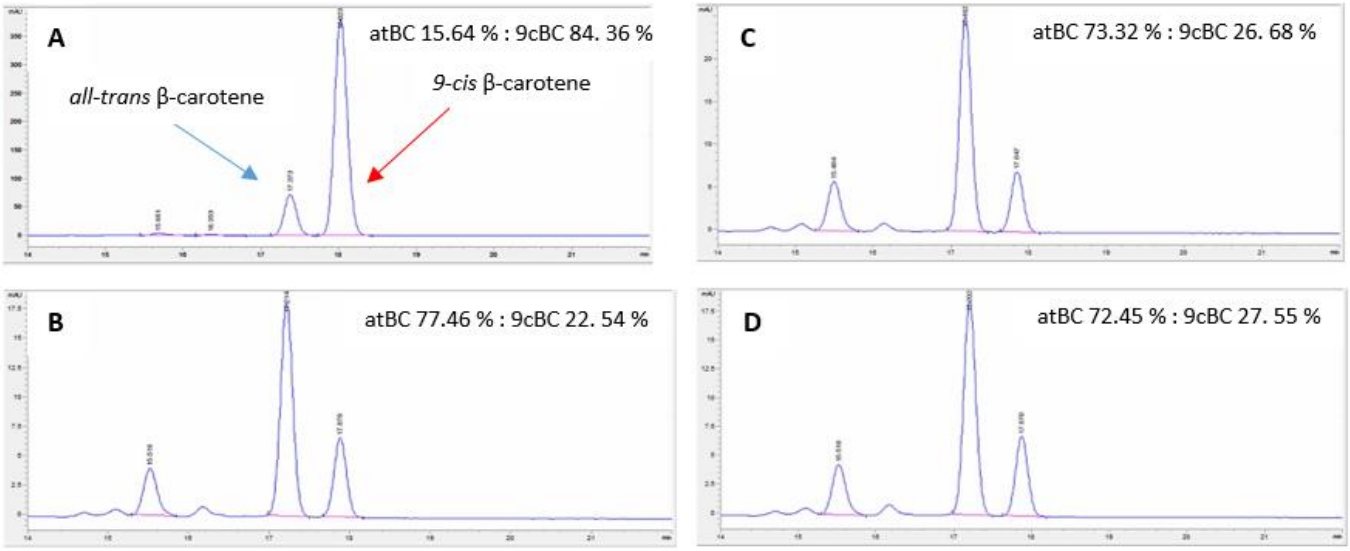

Figure 11: HPLC analysis to assess the *in vivo* activity of the *E. coli* optimized isomerases in the carotene producing *E. coli* strain. UV-chromatogram at 450 nm. Peaks were detected at minute 17 and 18. A) *9-cis* β-carotene reference B) *O. sativa* truncated isomerase C) *A. thaliana* isomerase D) *O. sativa* truncated isomerase fused C-terminally with MBP. The ratios of the *all-trans* and *9-cis* β-carotene of the detected samples are indicated and nearly the same. atBC: *all-trans* β-carotene, 9cBC: *9-cis* β-carotene

For better expression and increasing solubility the maltose binding protein (MBP) was fused N-terminally to the isomerases and replaced the chloroplast signal sequence. The ratios were nearly the same as the results described before and also this strategy wasn't working in our case. The ratios of β-carotene from analysed MBP-isomerase fusions are listed in Table 14.

Table 14: Ratios of β-carotene from analysed MBP-isomerase fusions in *E. coli*

Sample	Ratio [*all-trans* β-carotene : *9-cis* β-carotene]
crtEBIY strain	63.36 % : 36. 63 %
MBP truncated isomerase *O. sativa*	72.45 % : 27.55 %
MBP truncated isomerase *A. thaliana*	73.70 % : 26.30 %

Pryor et al reported that the expression rate of the tested protein-MBP fusions were between 5 and 40 % of the total cell protein. 75 % of these fusions were expressed at high levels (>200 mg/L). But the analysed fusion proteins were no isomerases [43].

Due to the fact that the *E. coli* optimized genes coding for isomerases were not expressed, a SDS-Page was performed with the isomerases and also their MBP fusions.

The cultivation was done as described in 3.10. The result is shown in Figure 12.

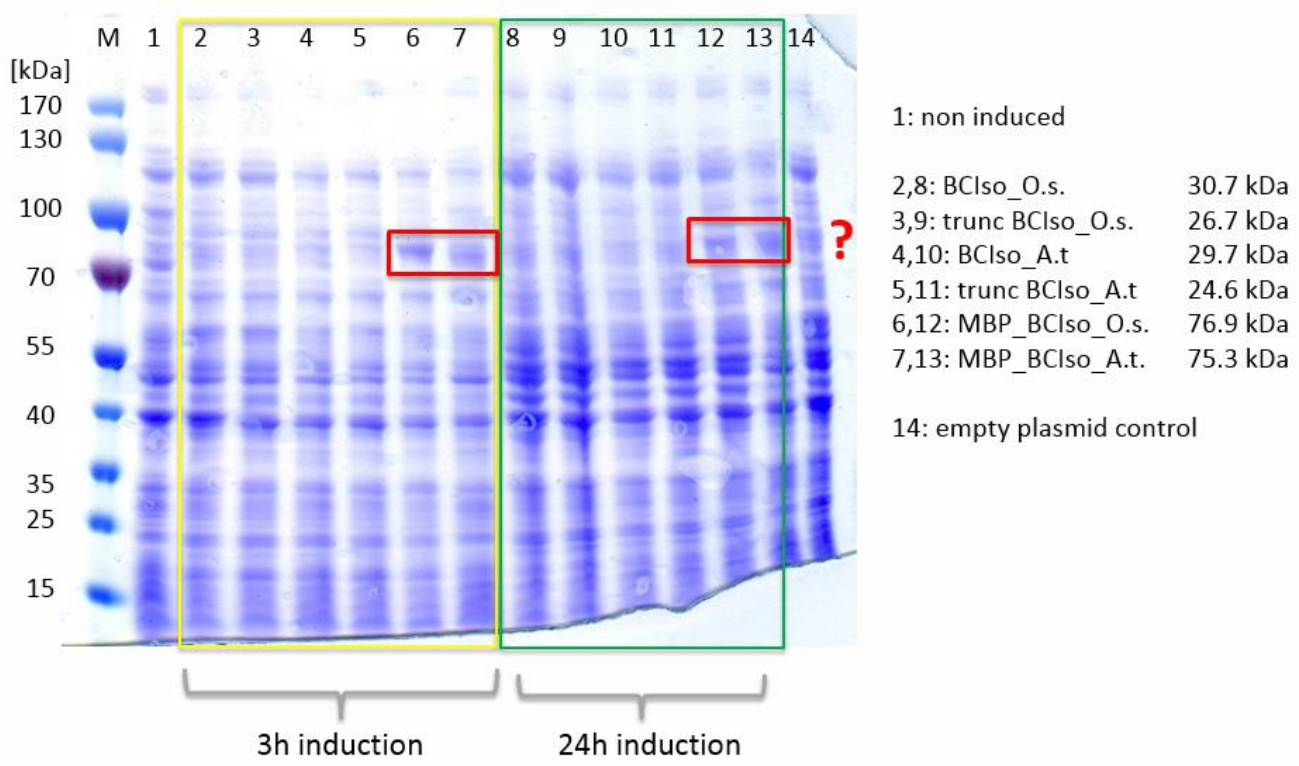

Figure 12: SDS-Page for analysis of *E. coli* optimized genes encoding for isomerases and isomerase-MBP fusions.

The red boxes are showing the isomerases of *O. sativa* and *A. thaliana* fused with maltose binding protein. We aren't sure if these bands are indicating the right proteins because the negative control also shows bands in this region, so this would need to be investigated in more detail e.g. by MS-analysis. A reason for the detection of these fusion proteins could be that the maltose binding protein increases the solubility of proteins and therefore the expression optimized. For future work the activity of the isomerases with MBP fusion has to be tested *in vivo*.

4.3. Optimizing functional isomerase production in *P. pastoris*

4.3.1. Investigating the effect of Fe^{2+} supplementation

β-carotene isomerases are Fe^{2+} dependent enzymes and the incorporation of iron might be important for the correct folding of the proteins. Thus, a possible reason for no observed expression is that the iron available in the cell was to less for sufficient activity. To see if the Fe^{2+} concentration was indeed limiting, Fe_2SO_4 was added during cultivation. Therefore, the cultivations were supplemented once with 10 mM and once with 100 mM of Fe_2SO_4. The induction and carotenoid analysis were the same as described before. The results of the HPLC analysis are shown in Figure 13.

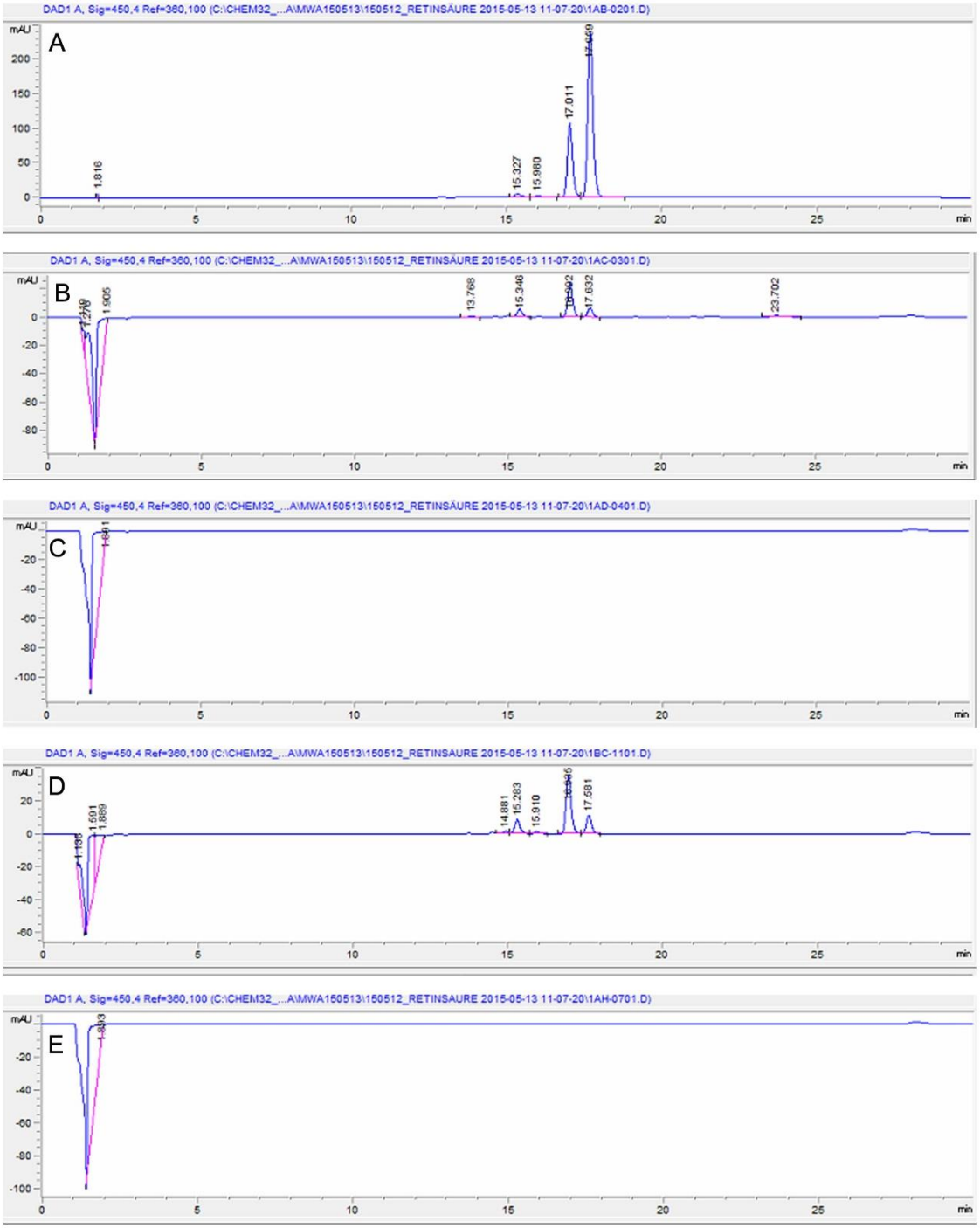

Figure 13: HPLC analysis to assess the *in vivo* activity of the *P. pastoris* optimized isomerases with Fe^{2+} supplementation. UV-chromatogram at 450 nm. Peaks were detected at minute 17 and 18. A) *9-cis* β-carotene reference B) *Oryza sativa* isomerase with 10 mM Fe^{2+} C) *Oryza sativa* isomerase with 100 mM Fe^{2+} D) *Arabidopsis thaliana* truncated isomerase with 10 mM Fe^{2+} E) *Arabidopsis thaliana* truncated isomerase with 100 mM Fe^{2+}. β-carotene is produced at low concentration of Fe^{2+} but not at the high concentration. The peak at minute 1.5 was not identified.

It can be seen that the isomerases produced by *P. pastoris* strains which were cultivated with 10 mM Fe_2SO_4 showed no activity for β-carotene. More importantly, there was less β-carotene produced in comparison to cultivations without iron supplementation. Cells that were grown

in the presence of 100 mM did not even produce the β-carotene. This result might indicate that the high Fe_2SO_4 concentration is problematic for the enzymes of the β-carotene pathway. Supplementation of Fe^{2+} was not successful in the case of β-carotene isomerases. For ongoing studies the β-carotene isomerases could be cultivated with low concentrations of ferric ions.

Iron addition was shown to be important before. *Krainer et al*. tested different medium supplementations to increase the amount of active heme proteins in *P. pastoris*. 5-aminolevulinic acid (ALA) is the heme precursor and traditionally used but did not enhance the yield of active product. In contrast to ALA, medium supplementation with $FeSO_4$ or hemin showed increased yields of active proteins [44].

A further study demonstrated that the supplementation of 100 µM ferric ions ($FeCl_3$) in culture media enhanced the expression of the recombinant porcine lactoferrin, an iron binding protein [45].

4.3.2. Evaluation of different promoters and N-terminal modifications for the recombinant production of β-carotene isomerases in *P. pastoris*

Gene expression driven by the *AOX1* promoter is strong and can overwhelm the cellular machinery, especially if the target protein is complex. Thus, another approach was to test promoters of different strength and regulatory profile. The idea was to use another inducible promoter, the *CAT* promoter and to test two constitutive promoters, the *ADH2* and the *TPI* promoter. To easily assess the expression levels of the different isomerase expression constructs via fluorescence measurements, the gene coding for eGFP was C-terminally fused to the isomerase gene. Cultivation of the resulting strains was then performed in deep-well plates, measuring GFP fluorescence at 488/507 nm after defined time points.

Furthermore two different N-terminal modifications were tested. Both strategies based on the replacement of the chloroplast signal sequence with either the maltose binding protein sequence (MBP) or with the mitochondrial targeting sequence of alternative oxidase (AOD). The expectations of these strategies are to improve the isomerase expression and hence to increase the activity.

MBP is a fusion partner from *E. coli* and improves expression for foreign proteins. It increases the solubility and yield, and enhances the stability [46] [47]. In this study the fusion of MBP to the isomerases was performed, to investigate if the MBP has the same effect in *P. pastoris*.

The AOD is present in mitochondria of plants, fungi and yeasts and plays a key role in the respiratory pathway. It is a non-energy conserving oxidase and reduces the ATP yield of respiration. It contains a signal sequence for mitochondria and it is able to influence the gene expression by controlling the level of potential mitochondrial signalling molecules such as superoxide, nitric oxide and important redox couples. AOD is also important during stress including abiotic stress situations such as low temperature, drought, nutrient deficiency and biotic stress such as bacterial infection [48] [49]. The main focus for fusion of the AOD signal sequence to the isomerases is, to target the isomerase into the mitochondria, an organelle which exists in *P. pastoris* in contrast to the chloroplasts. In Figure 14 the screening results are displayed.

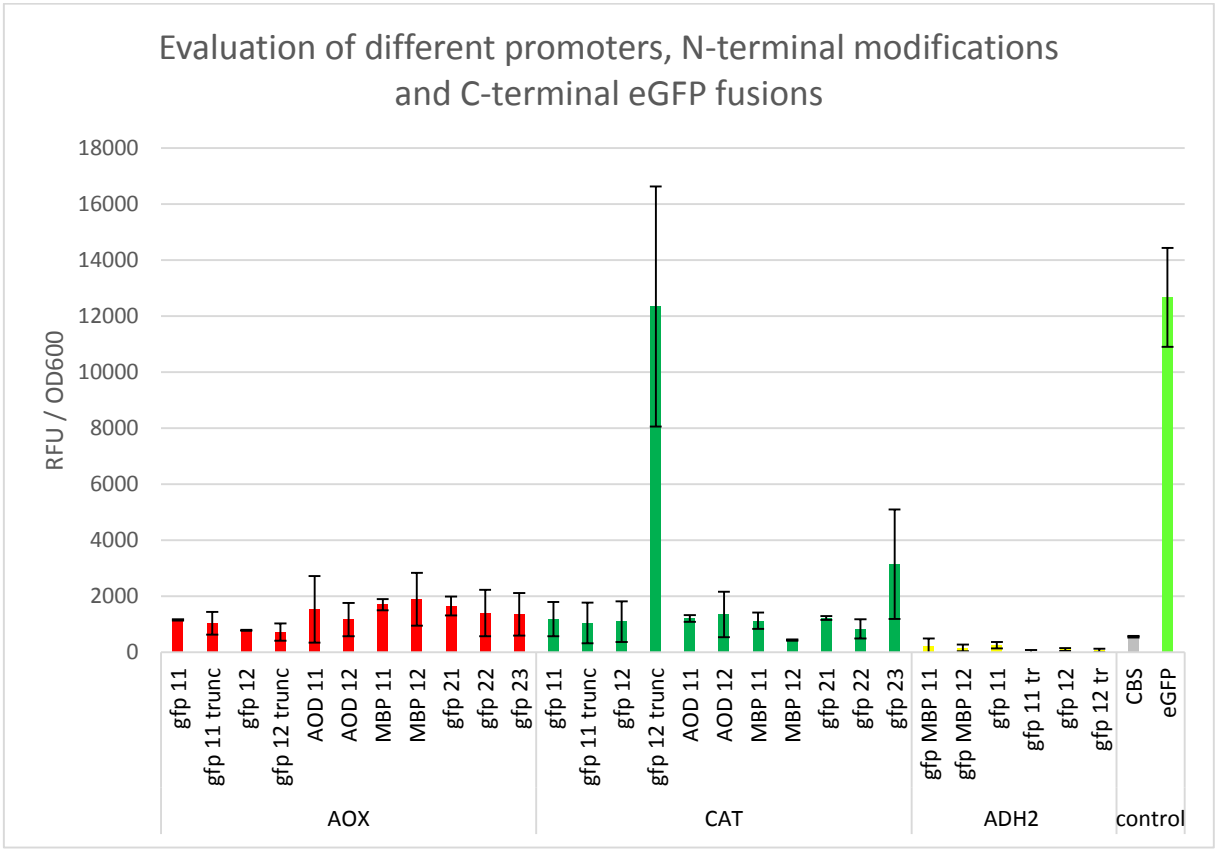

Figure 14: Evaluation of different promoters and N-terminal modifications in *Pichia pastoris* CBS7435. The red bars indicates the inducible *AOX* promoter, green bars show the inducible *CAT* promoter and the yellow bars show the constitutive *ADH2* promoter. Measurement was done after 60h of methanol induction for inducible promoters and after 60h of cultivation start for constitutive promoter. The controls are displayed in grey and light green. 11: *O. sativa*, 12: *A. thaliana*, 21: *B. rapa*, 22: *C. sativa*, 23: *M. pusilla*, AOD: alternative oxidase, MBP: maltose binding protein, CBS: wildtype strain CBS7435, trunc: chloroplast signal sequence removed. The average and standard deviation were calculated by measuring each expression construct three times.

The expression constructs with the *AOX1* promoter showed weak expression that is in accordance with the results described above.

The truncated isomerase of *A. thaliana* with the *CAT* promoter showed the highest and nearly the same fluorescence level as the eGFP control strain. All other P_{CAT} based expression constructs displayed similar expression as the P_{AOX1} ones.

In this study the truncated isomerases were created by removing the chloroplast signal sequence.

In addition, the MBP and AOD fusion constructs did not increase the expression as expected. *Dälken et al.* demonstrated the increase of expression level in *P. pastoris* after fusion the human GrB to bacterial MBP. These results indicate that the bacterial MBP is also active in yeast. The biological activity of the protein of interest could be interfered with the MBP fusion, so the separation of the proteins after expression is required. This can be done by an exogenously added protease that recognizes a site between the fusion proteins [47].

The constructs with the *ADH2* promoter showed hardly any eGFP fluorescence and also the *TPI* promoter constructs were not expressed and did not produce eGFP (data not shown). Because of this really weak expression, the remaining N-terminal modifications with these promoters were not constructed.

The next step was the analysis of the truncated isomerase of *A. thaliana* under the control of P_{CAT} with HPLC to verify the results. The P_{CAT} is active without methanol induction and when the glucose concentration in the medium is low. This construct were tested in the carotene producing yeast strain with once the *AOX1* promoter and once the *GAP* promoter. Figure 15 shows the obtained HPLC chromatograms.

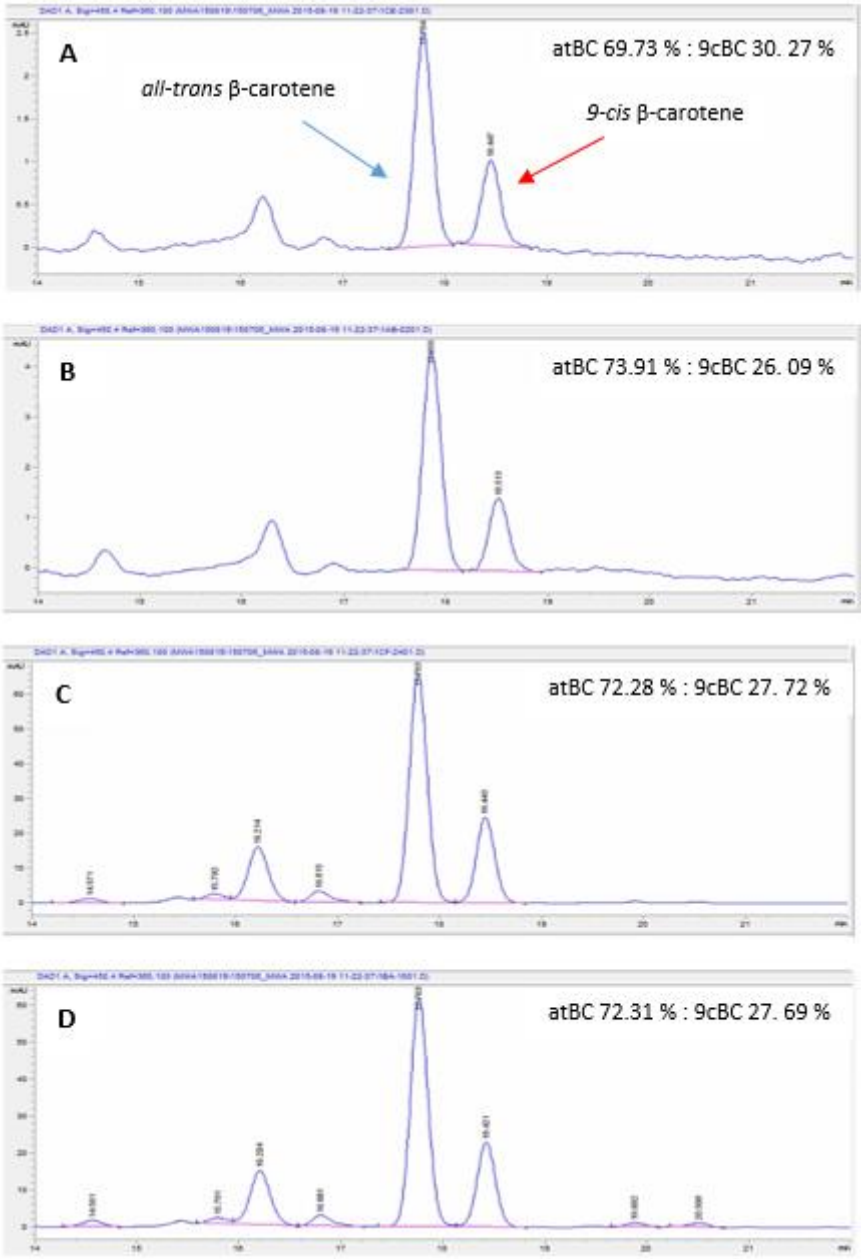

Figure 15: HPLC analysis to assess the *in vivo* activity of the *A. thaliana* truncated isomerases in the carotene producing *P. pastoris* strain. UV-chromatogram at 450 nm. Peaks were detected at 17.8 and 18.5. A) GAP crtEBIY *P. pastoris* strain B) *A. thaliana* truncated isomerase with GAP promoter C) AOX crtEBIY *P. pastoris* strain D) *A. thaliana* truncated isomerase with AOX promoter. The ratios of the *all-trans* and *9-cis* β-carotene detected in analysed samples and wildtypes are indicated. atBC: *all-trans* β-carotene, 9cBC: *9-cis* β-carotene

Also the strains harbouring the isomerase expression construct with the *CAT* promoter did not produce the *9-cis* β-carotene. The high fluorescence observed for this construct could also be explained by the high expression of eGFP and no expression of the isomerase. Maybe the eGFP was cleaved off and was active and the enzyme was degraded. A SDS-PAGE can be done for analysis.

4.3.3. Evaluation of methanol and oleic acid induction

Another strategy was tested to improve the expression level of the isomerases. Therefore the isomerase of *A. thaliana* fused with eGFP and under the control of the *CAT* promoter was induced with 0.01 % (w/v) oleic acid instead of methanol. The results are shown in Figure 16.

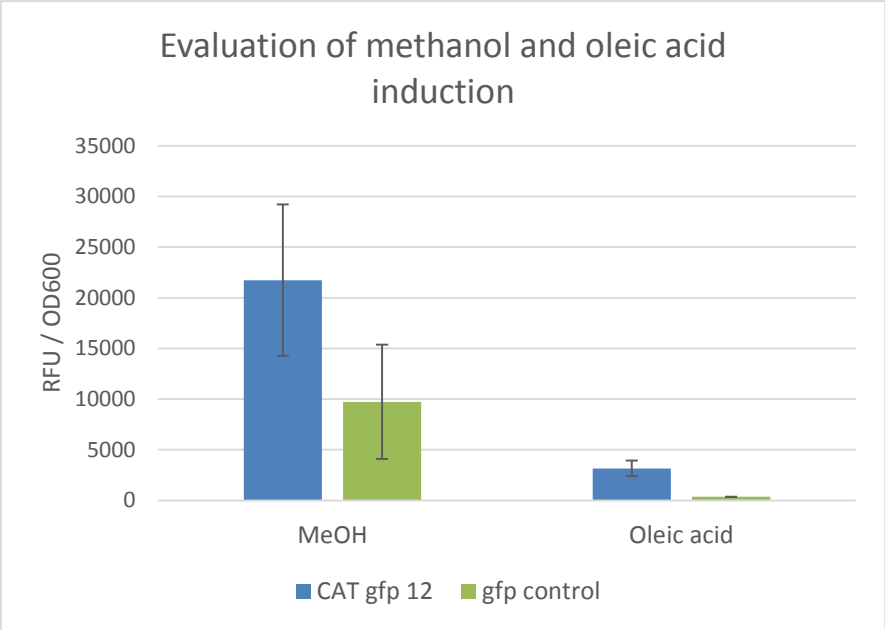

Figure 16: Evaluation of methanol and oleic acid induction for *A. thaliana* isomerase fused with eGFP with *CAT* promoter. The average and the standard deviation were calculated with 42 values of each sample.

The induction with methanol was about 6-fold higher compared to oleic acid induction. Therefore methanol was used for further studies. The gfp control was only the eGFP and was less expressed than the fusion protein. A reason for this could be that the average of the control was calculated only with three values.

Kobayashi et al. increased the protein expression by oleic acid under the control of *AOX2* promoter. They added 0.01 % (w/v) of oleic acid to the culture medium [50]. In our study the oleic acid was added to the induction medium (500x B, 10x YNB, 1 M Pi buffer pH 6, H_2O). This might be a reason that the induction with oleic acid showed hardly activity. Also this strategy for production of *9-cis* β-carotene didn't work very well in the case of the isomerases.

Vogl et al. described expression levels of proteins under the control of P_{CAT} and induced with oleic acid that are similar as with methanol induction. They used 0.2 % (w/v) oleic acid supplemented with 0.02 % (v/v) Tween40 [12].

In conclusion, all tested expression constructs didn't result in isomerases with *in vivo* activity for β-carotene. All obtained chromatograms looked very similar: the *all-trans* β-carotene peak is always higher than the *9-cis* β-carotene peak and the ratios between them are nearly the same as in the control strain. In literature there is not much known about the β-carotene isomerases. *Alder et al* describes the isomerase from *O. sativa*, which catalyses the isomerization of *all-trans* β-carotene. The so produced *9-cis* β-carotene undergoes further reactions for producing carlactone in the plant [51] [52]. The isomerization step is reversible (Figure 17), therefore *all-trans* β-carotene can also be produced from *9-cis* β-carotene and this substrate seems to be preferred [51].

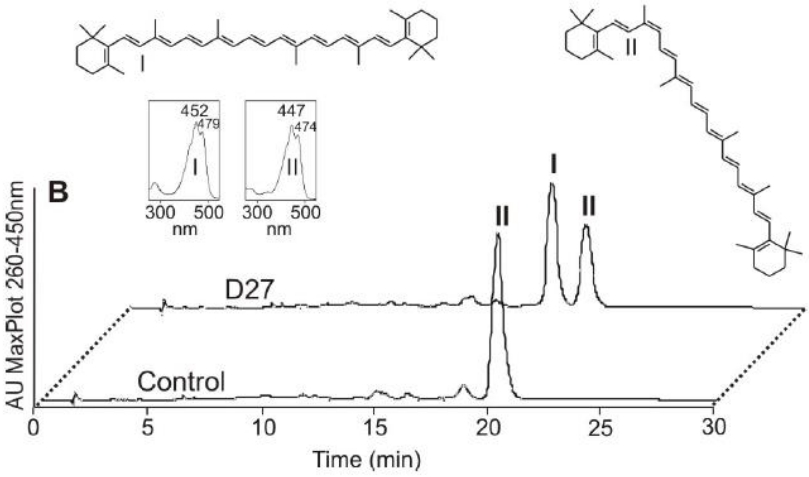

Figure 17: Isomerization of *9-cis* β-carotene to *all-trans* β-carotene synthesized by β-carotene isomerase from *O. sativa* [51a]. I = *all-trans* β-carotene. II = *9-cis* β-carotene.

They also described that the isomerization step could occur in a nonspecific way by photo-60 isomerization (induced by light) or thermos-isomerization (induced by heat) [51] [52].

Another possibility for no production of *9-cis* β-carotene could be the extraction step. The samples were incubated at 60° C and it is possible that these conditions are too harsh *9-cis* β-carotene and causes the re-isomerisation back to *all-trans* β-carotene. To exclude this assumption, the *9-cis* β-carotene reference was also incubated at 60° C twice. However, the HPLC chromatograms looked the same as in Figure 6 A.

It is possible that the *9-cis* β-carotene was produced but a re-isomerisation step converted it back to the starting material. One solution could be to bring imbalance between *all-trans* and

9-cis β-carotene by associate the isomerisation step to further reactions. By doing so, the *9-cis* β-carotene cannot re-isomerase back as it undergoes the next reaction. An idea was to combine the isomerases with the β-carotene monooxygenases and retinal dehydrogenase. The results are displayed in 4.6.

A further possible strategy was the isomerization of *all-trans* retinoic acid into *9-cis* retinoic acid with the β-carotene isomerase. However, it is not known if the isomerases accepts the retinoic acid as substrate or not. Nevertheless this strategy was also tested and results are shown in 4.6.

4.3.4. Localisation study

Due to the fact that *P. pastoris* doesn't have chloroplasts and the signal sequence of the isomerase, thus, isn't relevant, the AOD signal sequence was fused to the isomerases, instead of the natural one. To determine if the isomerases fused with eGFP were correctly localized to the mitochondria, a fluorescent microscopy analysis was done. The samples were stained with the Mitotracker® Red CMXRos from Invitrogen for detection of the mitochondria. This dye is visible at 579/599 nm.

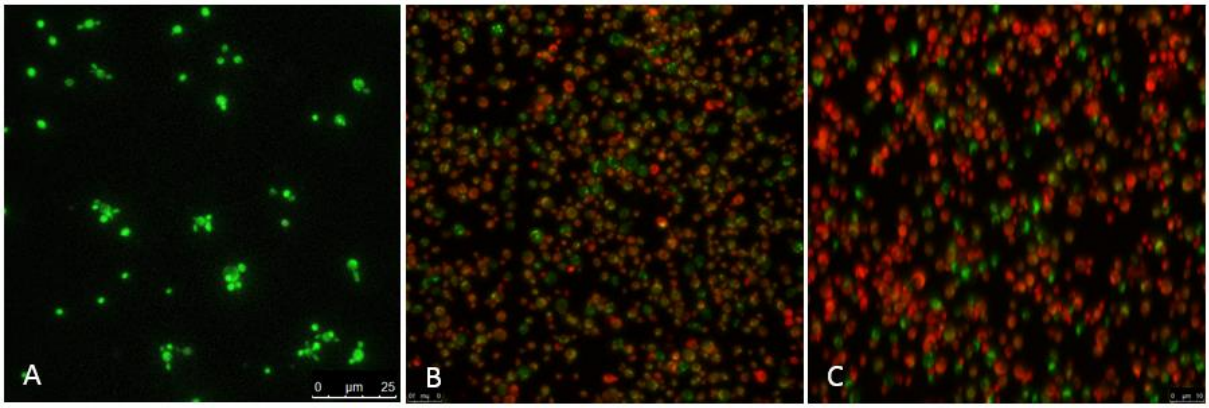

Figure 18: Fluorescent microscopy for analysis of isomerases fused with eGFP and AOD. A) *P. pastoris* cells expressing the isomerase-GFP fusion from *A. thaliana* under the control of P_{CAT}. B) *P. pastoris* strain containing the expression construct of the truncated isomerase-GFP fusion from *O. sativa* fused with AOD under the control of P_{CAT}. C) *P. pastoris* strain containing the expression construct of the truncated isomerase from *O. sativa* fused with AOD under the control of P_{AOX1}. Green: fluorescence at 488/507 nm, red: fluorescence at 579/599 nm.

In Figure 18 panel A *P. pastoris* cells expressing the isomerase from *A. thaliana* under the control of the *CAT* promoter is shown. The whole cells appeared green indicating that the eGFP aggregated in the cells. The *P. pastoris* strains with the expression construct of the

truncated isomerase from *O. sativa* fused with the AOD signal sequence either under the control of *CAT* or *AOX* promoter are shown in panel B and C. There are no single red points per cell observed, which should indicate the mitochondria. Instead of that the whole cells shined red. In addition, there are no single green points per cell visible (panel B and C) which might indicate that the isomerases were not targeted to the mitochondria, but this is hardly to see with this magnification. So it's impossible to say if the AOD signal sequence is working or not. It seems to be that there were problems with the staining. A reason for that might be that the Mitotracker® Red CMXRos was too old.

4.4. Recombinant production of β-carotene 15, 15´-monooxygenases in *P. pastoris*

The β-carotene 15, 15´- monooxygenase catalyses the cleavage of *all-trans* β-carotene into two molecules of retinal. For this study, four monooxygenases from different organism have been tested. The monooxygenase from marine bacterium, mouse, chicken and rat were recombinantly produced in the carotene producing *P. pastoris* strain, and the retinal production *in vivo* was analysed by HPLC. Figure 19 displays the obtained chromatograms for all tested monooxygenases.

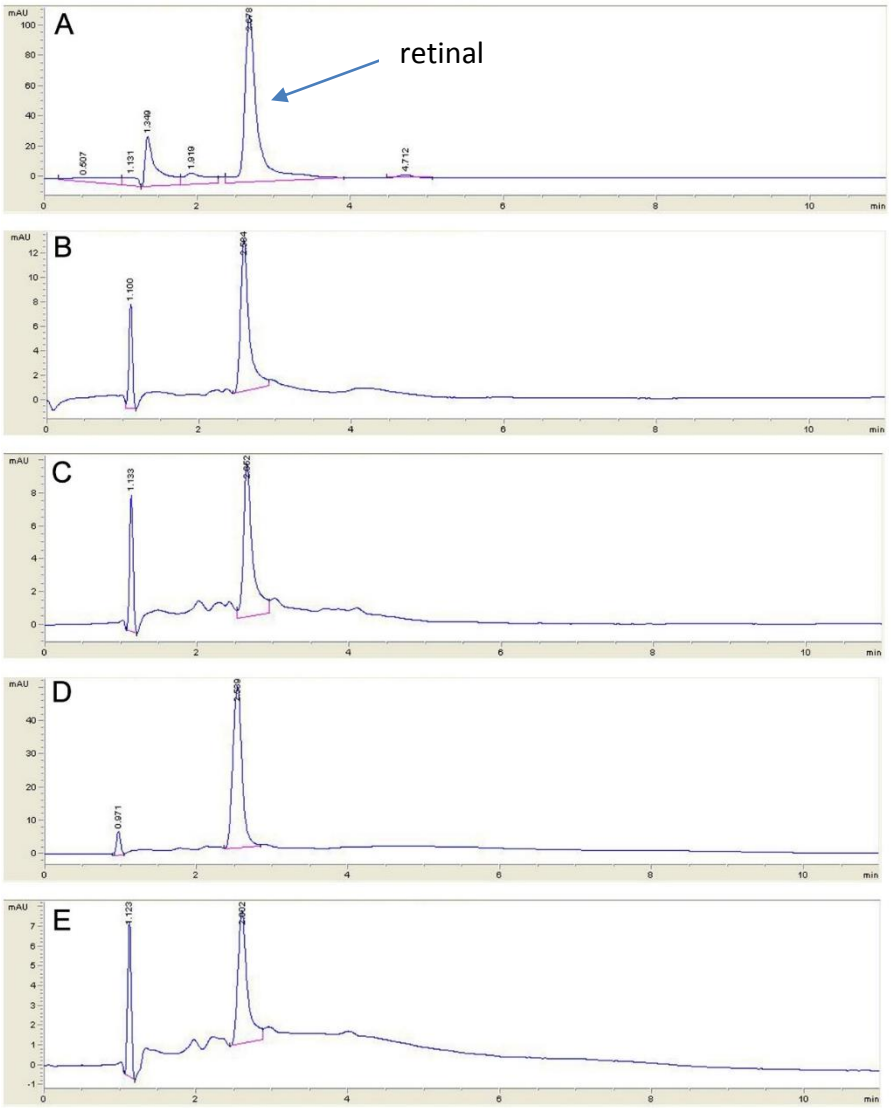

Figure 19: HPLC analysis to assess the *in vivo* activity of the 15‘-15‘ carotene monooxygenase in the β-carotene producing *P. pastoris* strain (B, C, D, E). UV-chromatogram at 380 nm. Retinal peak detected at minute 2.5. A) *all-trans* retinal reference B) murine bacterium monooxygenase C) mouse monooxygenase D) chicken monooxygenase E) rat monooxygenase. The peak at minute one is unknown and has to be identified.

All four monooxygenases were active and produced *all-trans* retinal, however to a different extent. This was a first functional check and no further quantitative analysis was performed. Therefore, the monooxygenase from mouse and chicken were selected for further studies because they showed the highest product peaks.

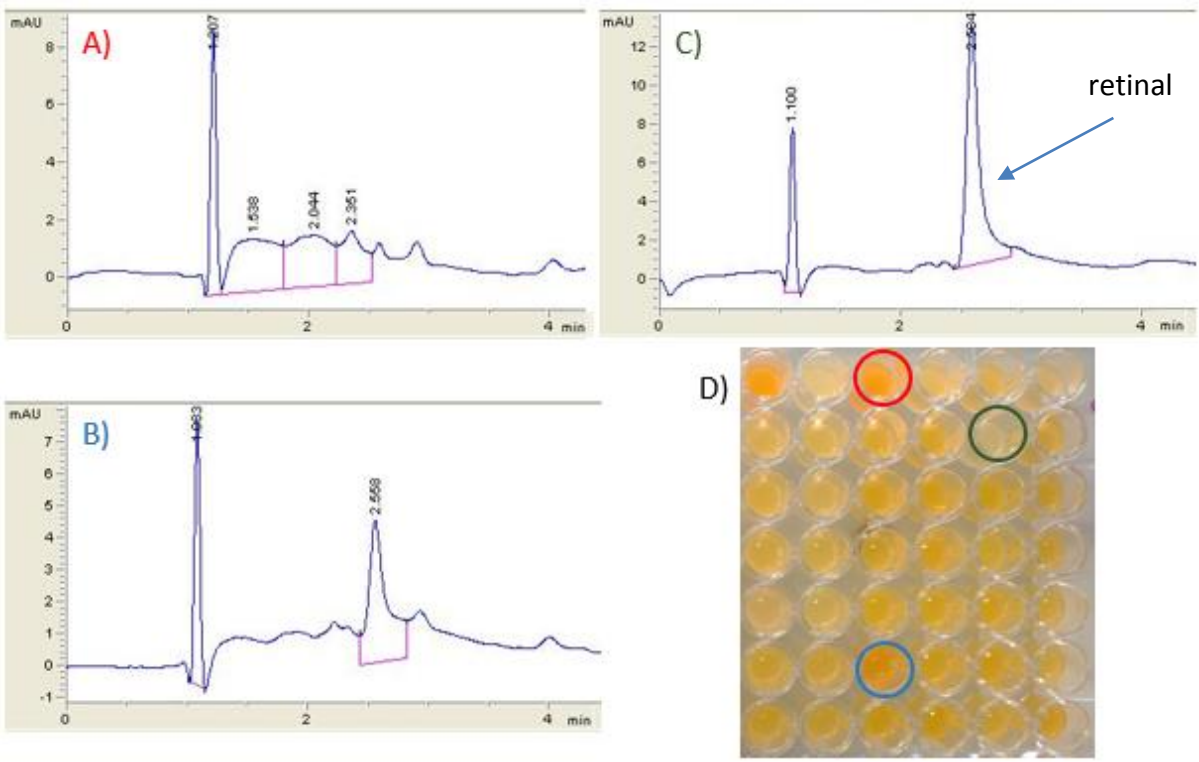

Figure 20: HPLC analysis to assess the *in vivo* activity of the 15'-15' carotene monooxygenase from murine bacterium in the β-carotene producing *P. pastoris* strain. UV-chromatogram at 380 nm. The retinal was detected at minute 2.6. The peak at the first minute could either be the injectionpeak or a by-product. A) murine bacterium sample A9 B) murine bacterium sample F9 C) murine bacterium sample B11 D) microtiterplate of cultures of murine bacterium monooxygenase in β-carotene producing *P. pastoris* strain. Red, green and blue circles indicate the analysed samples in A, B and C, respectively.

The circles in Figure 20 panel D indicate the cultures of monooxygenase from murine bacterium expressed in β-carotene producing *P. pastoris* strain which were analysed by HPLC and shown in panel A, B and C. It might be that the expression level is linked to the colour of the cells. The lighter the colour, the more retinal is produced. The retinal peak was detected after 2.6 minutes. The peak at minute 1 is either a by-product or the injection peak. The sample of the monooxygenase in panel A showed no activity and the colour in panel D was dark orange. In contrast, the sample in panel C showed expression and the colour is light orange.

4.5. Combining β-carotene 15, 15´- monooxygenases and retinal dehydrogenases for retinoic acid production

For the first trial to produce *all-trans* retinoic acid, monooxygenases were combined with retinal dehydrogenases. Two constructs were created and used to transform the β-carotene

producing yeast strain: The first construct harboured the genes for the β-carotene monooxygenase and the retinal dehydrogenase from mouse, the second construct comprised the genes of the monooxygenase from chicken in combination with the dehydrogenase gene from rat. To reduce the size of the plasmid and for better transformation efficiency, the genes for monooxygenase and dehydrogenase were linked with a 2A sequence. The cultivation was the same as described before.

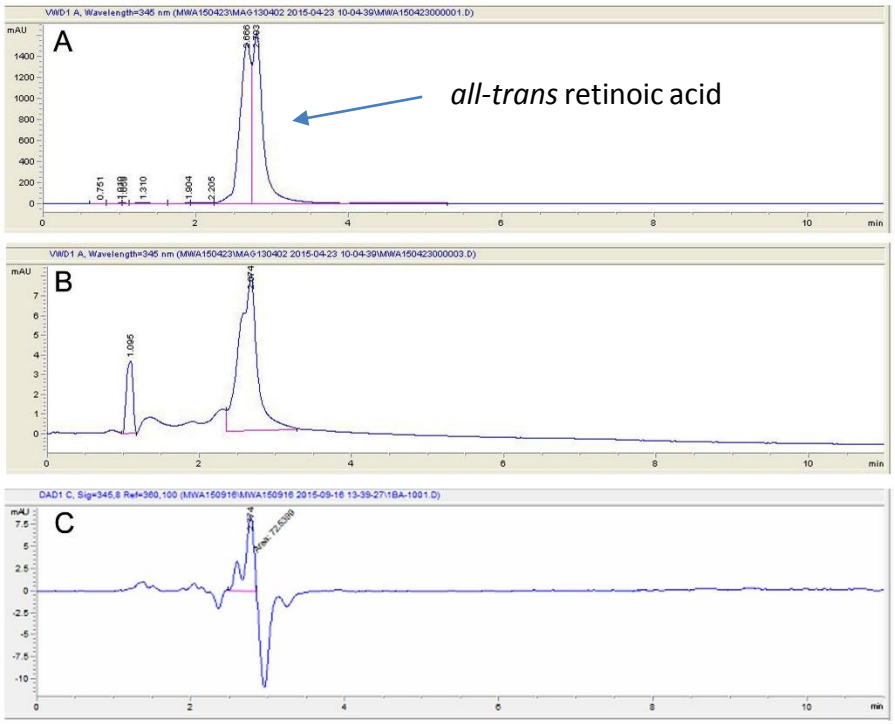

Figure 21: HPLC analysis to assess the *in vivo* activity of the 15'-15' carotene monooxygenase combined with retinal dehydrogenase with the 2A sequence in the β-carotene producing *P. pastoris* strain. UV-chromatogram at 345 nm. The *all-trans* retinoic acid peak is detected at minute 2.8. A) *all-trans* retinoic acid reference B) combination of mouse monooxygenase and mouse dehydrogenase C) combination of chicken monooxygenase and rat dehydrogenase

Figure 21 illustrates the chromatograms of the analysed samples and it can be seen that both combinations were active and were producing *all-trans* retinoic acid. To optimize the *all-trans* retinoic acid production the bidirectional promoters BZ6 was used instead of the 2A sequence. Benefit for using bidirectional promoters is the regulation of the expression level of the two genes. It is possible to regulate the time frame by fusion of a constitutive and an inducible promoter. For example a helper protein (chaperone) is under the control of the constitutive promoter and the gene of interest under an inducible promoter. Hence, the helper protein is available when the GOI is expressed. In addition, weak and strong promoters can be combined

to influence the expression level of the genes and therefore the ratios of the expressed proteins [35]. The results are shown in Figure 22.

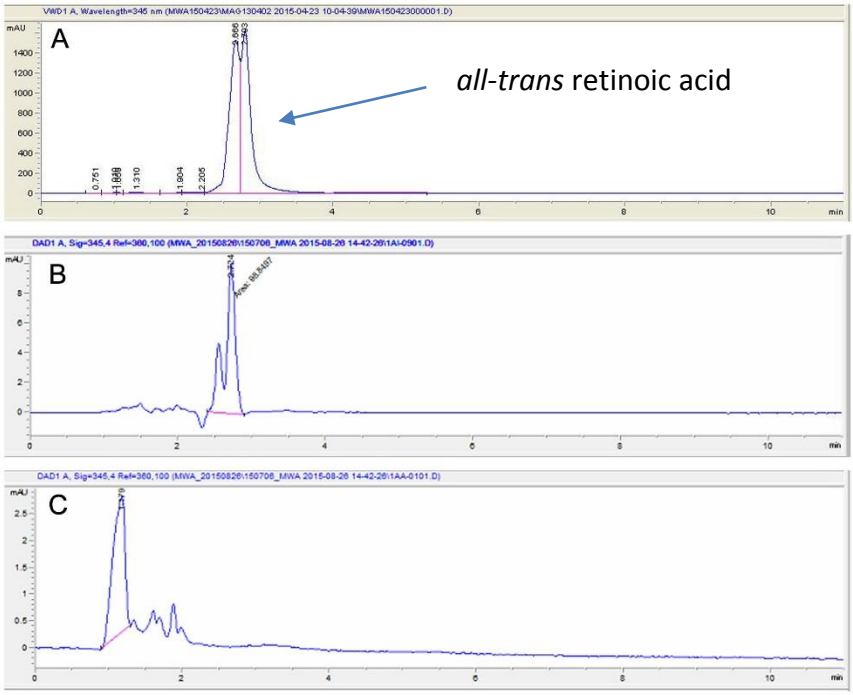

Figure 22: HPLC analysis to assess the *in vivo* activity of the 15'-15' carotene monooxygenase combined with retinal dehydrogenase in the β-carotene producing *P. pastoris* strain under the control of the bidirectional promoter BZ6. UV-chromatogram at 345 nm. The *all-trans* retinoic acid peak is detected at minute 2.8. A) *all-trans* retinoic acid reference. There is a double peak visible because the concentration (w/v) of the reference was too high B) bidirectional combination of chicken monooxygenase and rat dehydrogenase C) bidirectional combination of mouse monooxygenase and mouse dehydrogenase. This combination does not produce *all-trans* retinoic acid.

It can be seen that the combination of mouse monooxygenase and mouse retinal dehydrogenase with the bidirectional promoter did not produce *all-trans* retinoic acid (Figure 22 C), but β-carotene only (data not shown). A possible reason for this may be that the enzymes were not active due to the folding was incorrect. The peak in Figure 22 C) could be the injection peak. In contrast, the chicken/rat bidirectional construct was active. The double peak in Figure 22 B) could be a result from the extraction step.

To quantify the amount of retinoic acid produced by the recombinant *P. pastoris* strains a shake flask cultivation was performed as described before. Cultivations were performed in biological triplicates.

For the calculation of the produced *all-trans* β-carotene, the peak areas of the *all-trans* and *9-cis* β-carotene were summed up. The standard curves of *all-trans* retinoic acid and β-carotene were employed to determine the produced product in mg. Table 15 shows the results of the

quantitative analysis of the chicken/rat combination with 2A sequence and with bidirectional promoter.

Table 15: Analysis of the produced *all-trans* retinoic acid by chicken and rat combination. The 2A construct indicates the monooxygenase from chicken and dehydrogenase from rat linked with a T2A sequence. The bidirectional construct is under the control of the bidirectional promoter BZ6. The crtEBIY is the β-carotene producing yeast strain and indicates the control. The product yield for the bidirectional construct was calculated by using the average and standard deviation of biological triplicates. The product yield for the 2A construct was calculated from one cultivation.

Sample:	β-carotene [mg/g]	*All-trans* retinoic acid [mg/g]
crtEBIY	0.64	-
bidirectional construct	0.10	0.10
2A sequence	0.42	0.18

The construct with the 2A sequence shows a higher product yield for *all-trans* retinoic acid than the bidirectional construct. A low amount of β-carotene and retinoic acid is produced by the bidirectional combination. A possible reason might be that there occurred side reactions. Additionally, the incubation room had a power failure for about 8 hours, so the cultivations were not shaken and also the temperature was not correct. This might influence the cultivations and also the product yields.

The product yield for the 2A construct was calculated from one cultivation. Due to another extraction buffer for the second and third cultivation, the HPLC chromatograms looked different and were not included.

Retinal was not observed during the analysis. This finding may indicate that the used dehydrogenases are not the limiting enzymes. Another possibly reason can be that enzymes from *P. pastoris* are able to consume the retinal. A by-product was formed that has to be identified in ongoing studies.

A further combination with monooxygenase from mouse and dehydrogenase from rat has been tested. This construct also produced *all-trans* retinoic acid but 2-fold less than the chicken/rat combination (3.05 µg/mL : 7.91 µg/mL). The cultivation was performed in a 96-deep well plate.

For the first time, the production of *all-trans* retinoic acid in a biologically way was shown. Different strategies for pathway expression were employed either with 2A sequences or with bidirectional promoters. In this study only three enzyme combinations with 2A sequences and two constructs with bidirectional promoter were tested. For optimizing the retinoic acid production different enzyme combinations and also different promoters can be tested in ongoing studies.

The monooxygenase from human could be tested. Although this enzyme doesn't accept the *9-cis* β-carotene, maybe it shows affinity for the *all-trans* β-carotene [53]. This could be a possibility to increase the amount of *all-trans* retinoic acid.

In literature there is a lot known about the β-carotene 15, 15´- monooxygenases and retinal dehydrogenases from different organism. The monooxygenase from mouse accepts the *9-cis* β-carotene and yield *all-trans* retinal and 9-*cis*-retinal in a molar ratio of 3:1 [17]. An option would be to engineer the protein for increased substrate specificity.

As already described in chapter 1, the mouse retinal dehydrogenase shows two order of magnitude more activity in vitro with *9-cis* retinal than with *all-trans* retinal [26]. This could also be a reason why the product yield of retinoic acid for the mouse combination is very low.

Also the retinal dehydrogenases from rat and human have high efficiency for *9-cis* retinal [15] [25]. Perhaps a high yield of retinoic acid could be reached by engineering these enzymes for *all-trans* retinal specificity.

4.6. Analysis of combined β-carotene isomerases, monooxygenases and retinal dehydrogenases

At the end of this study all three enzymes were combined for producing the final product, *9-cis* retinoic acid. Therefore, the truncated isomerase of *A. thaliana* under the control of the *CAT* promoter was cloned into the bidirectional chicken/rat construct. The final combination was cultivated in deep-well plates and some wells were analysed as shown in Figure 23.

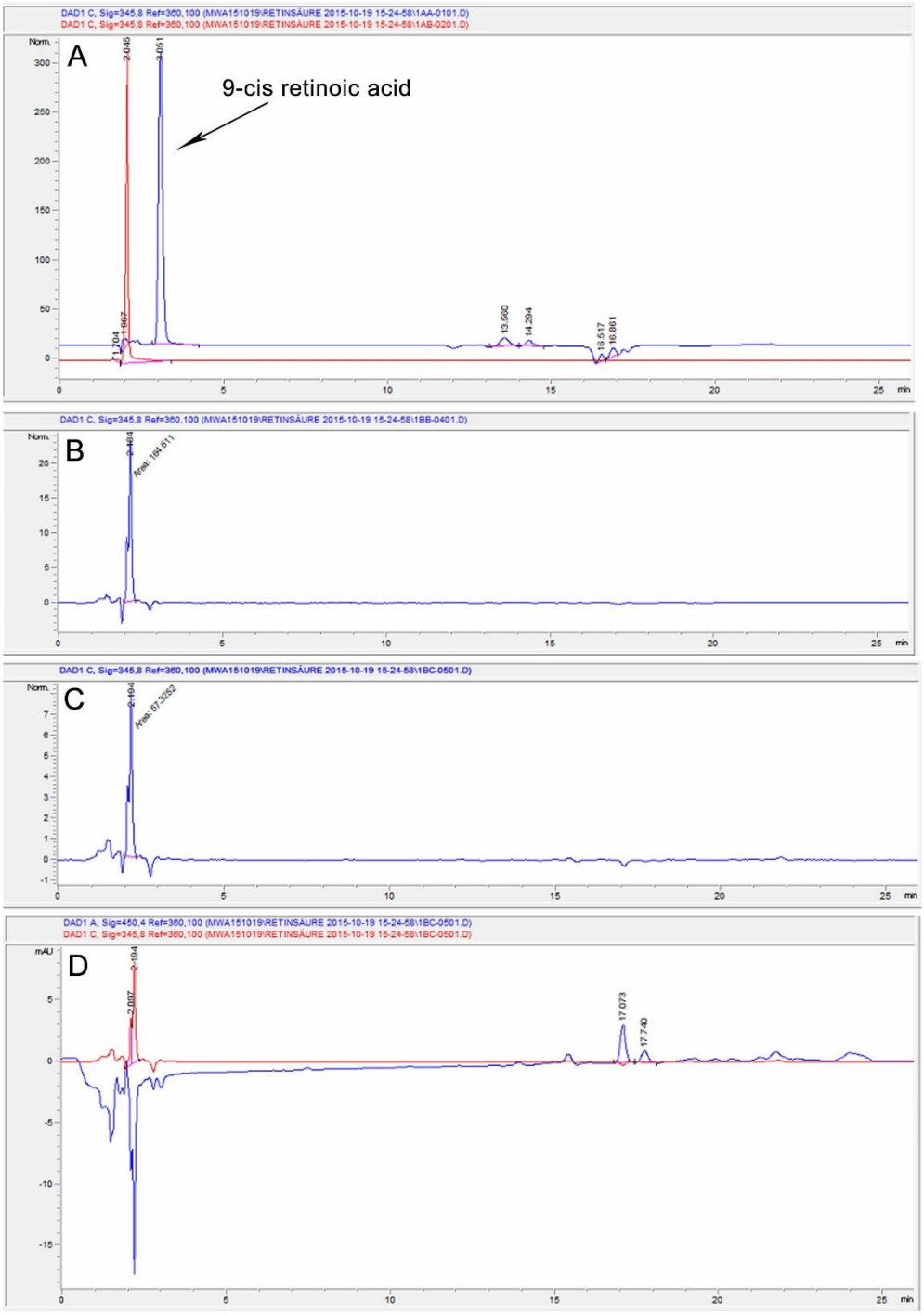

Figure 23: HPLC analysis to assess the *in vivo* activity of the combined β-carotene isomerase, 15'-15' carotene monooxygenase and retinal dehydrogenase in the β-carotene producing *P. pastoris* strain under the control of a bidirectional promoter BZ6. UV-chromatogram at 345 nm. The *all-trans* retinoic acid peak is detected at minute 2 and the *9-cis* retinoic acid peak is detected at minute 3. A) *all-trans* retinoic acid (red) *9-cis* retinoic acid (blue) B) sample d5 producing *all-trans* retinoic acid C) sample e2 producing *all-trans* retinoic acid D) Sample e2 converting β-carotene, shown in blue (UV chromatogram at 450 nm) into *all-trans* retinoic acid, shown in red (UV chromatogram at 345 nm). The blue peak downward is the opposite of retinoic acid and was measured at 450 nm.

This enzyme combination did not produce the *9-cis* retinoic acid. Table 16 indicates the calculated product yield.

Table 16: Analysis of the produced *all-trans* retinoic acid by combined β-carotene isomerase, 15'-15' carotene monooxygenase and retinal dehydrogenase in the β-carotene producing *P. pastoris* strain under the control of the bidirectional promoter BZ6. C3, f6, e2, d5 are samples from the 96-well plate.

Sample:	β-carotene [µg/mL]	*All-trans* retinoic acid [µg/mL]
c3	276.2	-
f6	189.6	-
e2	9.4	4.0
d5	6.4	11.47

The sample d5 showed the highest product yield with 11.47 µg/mL *all-trans* retinoic acid and 6.40 µg/mL β-carotene. It seems that the sample e2 converted less β-carotene into retinoic acid in comparison to d5. No retinoic acid is produced by the samples c3 and f6.

Retinal production was not observed but an unknown by-product as described before.

The production of retinoic acid could be related to the colour of the cultivation as displayed in Figure 24.

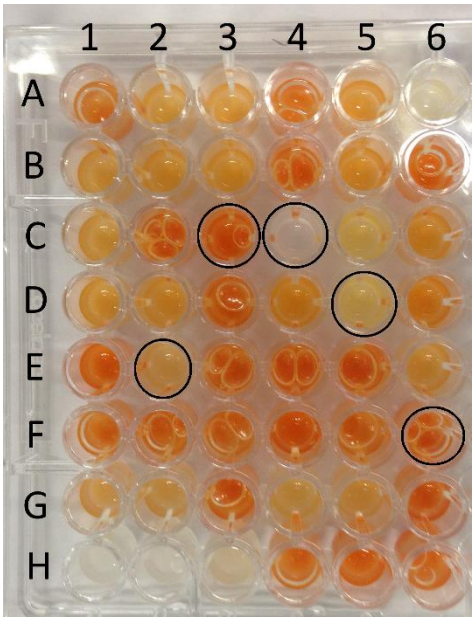

Figure 24: Microtiterplate with cultures of the combined β-carotene isomerase, 15'-15' carotene monooxygenase and retinal dehydrogenase in the β-carotene producing *P. pastoris* strain under the control of a bidirectional promoter BZ6.

The wells d5 and e2 shows a bright orange yellow colour in contrast to c3 and f6 which display a really dark orange colouring. Data in Table 16 and Figure 24 suggest that the brighter the

colour, the less β-carotene is produced. Analysis of c3 and f6 shows that these samples did not produce retinoic acid but they show a peak at minute 15 (Figure 25), so they convert the carotene into another unknown product. Sample c4 was also analysed but didn't show any product and even the carotene peaks weren't visible in the chromatogram. Maybe the white colour indicates no product.

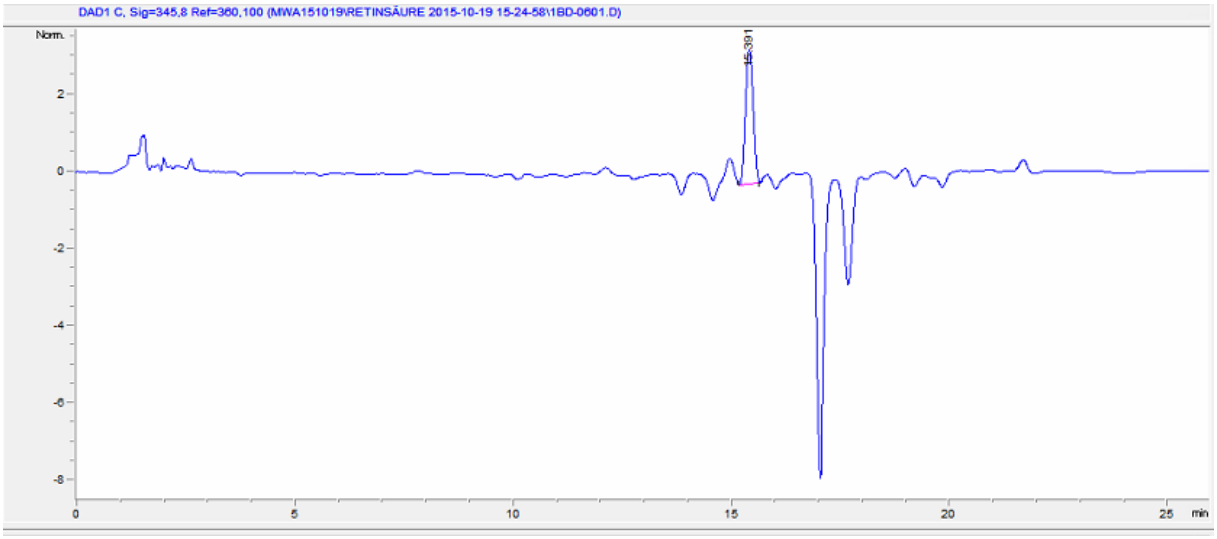

Figure 25: HPLC chromatogram to assess the *in vivo* activity of combined isomerase, monooxygenase and retinal dehydrogenase. UV-chromatogram at 345 nm. No retinoic acid was detected but an unknown by-product at minute 15 was formed.

In this study the highest concentration of β-carotene was 0.64 mg/g CDW. However, *Vogl et al* reported up to 5 mg/g CDW of β-carotene and 2 mg/g CDW of lycopene using strong inducible promoters (P_{AOX1}, P_{DAS1}, P_{DAS2}, P_{PMP20}) for the best carotenoid producing *P. pastoris* strain so far [12]. However, this strain was not yet available at the time this study was started.

The high concentration of β-carotene and the low concentration of the *all-trans* retinoic acid indicates that there occurs side reactions and no 100 % product yield is reached. If the obtained β-carotene of 0.64 mg would be fully converted into retinoic acid without any by-products, 0.36 mg *all-trans* retinoic acid are expected. In comparison to 0.18 mg that are obtained with no 100 % conversion by the 2A sequence, by-products have to be formed which agrees with the peak in Figure 25.

Retinal was produced by the tested bidirectional and 2A constructs and by the combination of all enzymes but it was fully converted into retinoic acid. Reasons for that are described previously (chapter 4.5.).

The combined constructs produced *all-trans* retinoic acid and showed an unknown peak. Hence, a by-product is formed by the monooxygenase and dehydrogenase. The higher the retinoic acid peak, the lower is the peak of the by-product.

Although, retinoic acid is produced, not all β-carotene is used up. So, a point to consider is the monooxygenase catalysed step. This reaction step can be a bottleneck in the pathway. An idea is to optimize this enzyme by engineering.

5. Conclusion & Outlook

In this study *all-trans* retinoic acid was produced in *P. pastoris* for the first time. To establish and implement the retinoic acid pathway in β-carotene producing yeast strain, the 15'-15' carotene monooxygenase and retinal dehydrogenase were analysed by HPLC.

The β-carotene isomerases encoded by the *P. pastoris* optimized genes did not show activity and were not detectable on SDS-Page, even with different N-terminal tags and promoters. Also the *E. coli* genes with optimized codon usage for β-carotene isomerase were not expressed.

All tested monooxygenases were expressed and showed activity for *all-trans* β-carotene. In this study the focus was on the monooxygenase from mouse and chicken.

For the production of *all-trans* retinoic acid the monooxygenase was combined with a retinal dehydrogenase. These enzymes were either under the control of the bidirectional promoter BZ6 or linked together with a 2A sequence. Two constructs have been tested. The first construct was the mouse monooxygenase and the mouse retinal dehydrogenase. It produced *all-trans* retinoic acid by using the 2A sequence but not with the bidirectional promoter. For the second construct the monooxygenase from chicken was combined with the dehydrogenase from rat. This combination obtained 0.18 mg/g (CDW) retinoic acid with the 2A sequence.

Although, the isomerases did not show activity, a combination with all three enzymes required for the retinoic acid pathway was created. Therefore, the chicken/rat combination under the control of the bidirectional promoter was combined with the truncated isomerase from *A. thaliana* via a 2A sequence. The product yield was 11.47 µg/mL *all-trans* retinoic acid for cultivation in a deep-well plate.

In general a pathway which produces *all-trans* retinoic acid from *all-trans* β-carotene in *P. pastoris* is established and can be improved by strain and protein engineering to increase the product yield.

At present time, there is only less known about how *9-cis* retinoic acid is formed or regulated within cells [54].

For ongoing studies the production of *9-cis* retinoic acid could be realised by different strategies: a) isomerization through non-enzymatic processes, b) enzymatic two step oxidation of *9-cis* retinol to *9-cis* retinal by *9-cis* retinol dehydrogenase and further to *9-cis* retinoic acid by retinal dehydrogenase or c) cleavage of *9-cis* β-carotene either directly into *9-cis* retinoic acid or via 9-cis retinol/9-cis retinal [55].

Krinsky et al. described the *in vivo* biosynthesis of *9-cis* retinoic acid by intestinal cleavage of *9-cis* β-carotene in ferrets (strategy c). They demonstrate that the intestinal mucosa and portal blood concentrations of detectable *9-cis* retinoic acid was higher if it undergoes perfusion with *9-cis* β-carotene. In contrast, a perfusion with *all-trans* β-carotene or control micellular solution did not reach a high concentration. It is not determined, how the retinoic acid was formed but a series of 9-cis β-apocarotenals were involved. However, this pathway for production of *9-cis* retinoic acid is not significant due to the rate of cleavage of *9-cis* β-carotene is only 6-7 % of that of *all-trans* β-carotene [55] [56].

Another strategy was reported by *Urbach and Rando*. The isomerization from *all-trans* retinoic acid to *9-cis* retinoic acid is catalysed by sulfhydryl groups inside of microsomes from bovine liver without any enzyme activity [54]. Thus chemical steps might be combined with biochemical conversion and perhaps also selective extraction methods.

6. References

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Supplementary Data

S1: Primer

Primer #	Primer name	Primer sequence (5'-3')
C27	eGFP_seq_rev	GATAACGGGAAAAGCATTGAACAC
C43	AOXTT_seq_rev	TCCCAAACCCCTACCACAAG
C372	5AOX_seq	GACTGGTTCCAATTGACAAGC
C435	Pp_AOD_NotI_rev	TATTGCGGCCGCTTATAAAACGAGCTCATCTCTTTCCCATC CGAC
C624	monox_chicken_seq1_fw	CAC TTG GAA GTT GTC TGC AG
C625	monox_chicken_seq2_fw	CAA GTC CTG ATG CTA GAG AAG
C626	monox_mouse_seq1_fw	GGT AAA TCT CCA GTT AAG CAC G
C627	monox_mouse_seq2_fw	GTC CTT CAC AGA ATT GGC TAG
C632	GSK3_EcoRI_fwd	taatGAATTCcgaaacgATGGGTCTGATGCTGATTGATTGGTG C
C633	GSK3_NotI_rev	ttaaGCG GCC GCT TAA TTT TTG ATT TTA ATG CGC GAT GAG TGC GGA C
C634	GSK4_EcoRI_fwd	taatGAATTCcgaaacgATGGAAATTATCTTTGGTCAGAACAA GAAAGAACAAC
C635	GSK4_NotI_rev	ataaGCG GCC GCT TAC AGG GAA CTA CCG CCA TGG CC
C636	GSK5_EcoRI_fwd	aataATTCcgaaacgATGGAAACCATCTTCAACCGTAACAAA GAAGAAC
C637	GSK5_NotI_rev	tattGCG GCC GCT TAT TCG GTT TCG GCA CCC AGG TC
C638	GSK6_EcoRI_fwd	aataGAATTCcgaaacgATGGAAATTATCTTTGGTCGTAACAA GAAAGAACAGC
C639	GSK6_NotI_rev	tatt GCG GCC GCT TAC AGG GAT TTA CCG CCA TGG CC
C640	GSK7_EcoRI_fwd	aata GAATTCcgaaacgATGGCCGGCAAACGCGAAC
C641	GSK7_NotI_rev	tatt GCG GCC GCT TAG TAT TTG ATC GTG ATG GTT TTG ATT TCG GTG
C642	GSK8_EcoRI_fwd	aata GAATTCcgaaacgATGAGCAGCAGCGGTACCCC
C643	GSK8_NotI_rev	ttaa GCG GCC GCT TAT GAA TTT TTC TGG GAG ATT TTA ACC GTC ACG G
C644	GSK9_EcoRI_fwd	aataGAATTCcgaaacgATGAAAAAACAGGGTAGCCCGTCTA ATCC
C645	GSK9_NotI_rev	ttaa GCG GCC GCT TAG CTG TTT TTC TGC GGG ATT TTA ATC GTA ACG G
C646	GSK10_EcoRI_fwd	aata GAATTCcgaaacgATGAGCTCTCCGGCACAGCC
C647	GSK10_NotI_rev	ttaa GCG GCC GCT TAG GAA TTT TTC TGA CTA ATT TTC ATG GCC ACC G
C648	pAOX_fw	AAC ATC CAA AGA CGA AAG GTT GAA TGA AAC C
C649	AOX_TT_rev	GCA CAA ACG AAG GTC TCA CTT AAT CTT CTG TAC
C677	Pp_AOD_SpeI_fw	TAT AAC TAG TCG AAA CGA TGT TAA AAC TGT ACG CAA TAA GGC CAA TTA GGC
C707	MBP_Pp_seq_fw	CATGAACGCTGACACTGACTAC

C708	tr_Iso_Arab_PP_seq_fw	GAT TGG TCG ATA CAG CTA CCA GAG
C709	tr_Iso_Rice_PP_seq_fw	CTG CAT CAG CTC CAG CCA AG
C710	MBP_Ec_seq	CAC ATG AAT GCA GAC ACC GAT TAC
C711	Iso_Arab_Ec_seq_fw	GCT GTG TTC TAA ACC GGT CTA TAG
C712	Iso_Rice_Ec_seq_fw	GTT ATG CGT CGC TGC TGT TCC
C739	crtE_FWD_Gibson	ACGACAACTTGAGAAGATCAAAAAACAACTAATTATTGAAAGAATTCCGAAACGATGAC
C740	crtY_REV_Gibson	GAAGCCTGCATCTCTCAGGCAAATGGCATTCTGACATCCTCTTGAGCGGCCGCTTAACG
C741	pCAT_MBP fw	TCTAGTCAAGACTTACAATTAAAGAATTCACTAGTcgaaacgATGAAGATCGAGGAAGGTAAGTTAGTGATTTGGATC
C742	pCAT_gfp_rev	CTC AGG CAA ATG GCA TTC TGA CAT CCT CTT GAG CGG CCG CTT ACT TGT ACA ATT CAT CCA TGC CAT GTG TAA TCC CAG C
C743	pCAT_rice_fw	AACACTTGCTCTAGTCAAGACTTACAATTAAAACTAGTCGAAACGATGGAAACTACAACCTTGGTCTTGC
C744	pCAT_rice_trunc_fw	CACTTGCTCTAGTCAAGACTTACAATTAAAACTAGTCGAAACGATGGCAGTCATGGCTAGACC
C752	crtE_FWD_Gibson	ATACCTCTATACTTTAACGTCAAGGAGATTTAAATGAGCTCGAATTCCGAAACGATGAC
C753	crtY_REV_Gibson	GGGGGGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGACGCGGCCGCTTAACG
C760	DHmouse1_fw	TCAAAAAACAACTAATTATTGAAAGAATTCcgaaacgATGGCTGGTAAAAGAGAATTGCTTATGTTGGAGAAC
C761	DHmouse1_rev	GTA AGG AAC CAC GGC CCT CTG CAC GAT ATT TGA TAG TAA TGG TCT TAA TCT CAG TGA AAA AGT CGT AAG AAT CTT TAG C
C762	moxymouse2_fw	TGATGTGGAAGAAAATCCAGGACCCGAAATCATCTTCGGTCAAAACAAGAAAGAACAGTTGGAGC
C763	moxymouse2_rev	AAT GGC ATT CTG ACA TCC TCT TGA GCG GCC GCT TAC AAG GAA GAT CCA CCA TGA CCT GCA GTG AAA TC
C776	BZ6 seq_fw	CTT GTA TCC TGA GTG ACC GTT G
C777	BZ6 seq_rev	CAT TTT TTC ACT TCA ACT TTT GGG G
C781	Dhhuman_fw	CCTGCGGTGATGTGGAAGAAAATCCAGGACCCTCTTCCTCAGGTACACCAGACCTTCCTGTTTTGC
C782	Dhhuman_rev	CAT TCT GAC ATC CTC TTG AGC GGC CGC TTA AGA GTT CTT TTG GGA AAT CTT AAC AGT GAC GGT TTT AAC
C783	Dhchick_fw	ACCTGCGGTGATGTGGAAGAAAATCCAGGACCCAAGAAACAAGGTTCTCCATCCAACCCAGC
C784	Dhchick_rev	CAA ATG GCA TTC TGA CAT CCT CTT GAG CGG CCG CTT AAG AGT TTT TCT GAG GGA TCT TAA TGG TAA CTG TTT TGA CCT C
P15210	DAS1TTseq	GATGGAGTCTATACTAGGCTAACATAAAC
	pBAD_fw	ATGCCATAGCATTTTTATCC

S2:

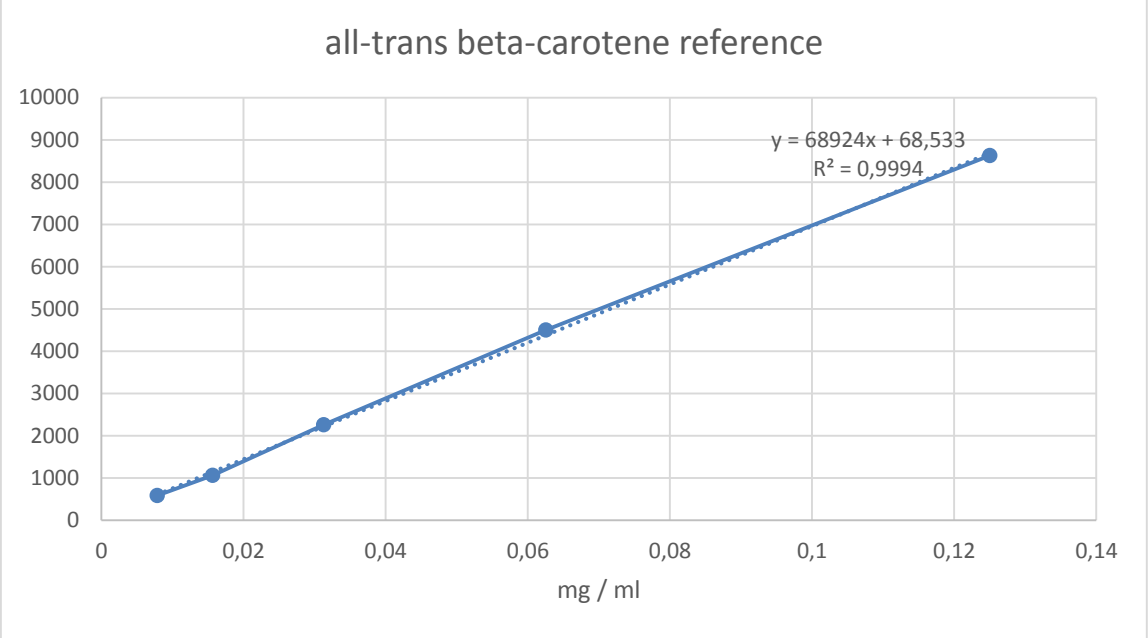
NuPAGE® MOPS SDS Running Buffer (20X), pH 7.7
50 mM MOPS
50 mM Tris Base
0.1% SDS
1 mM EDTA

S3: Cultivation media

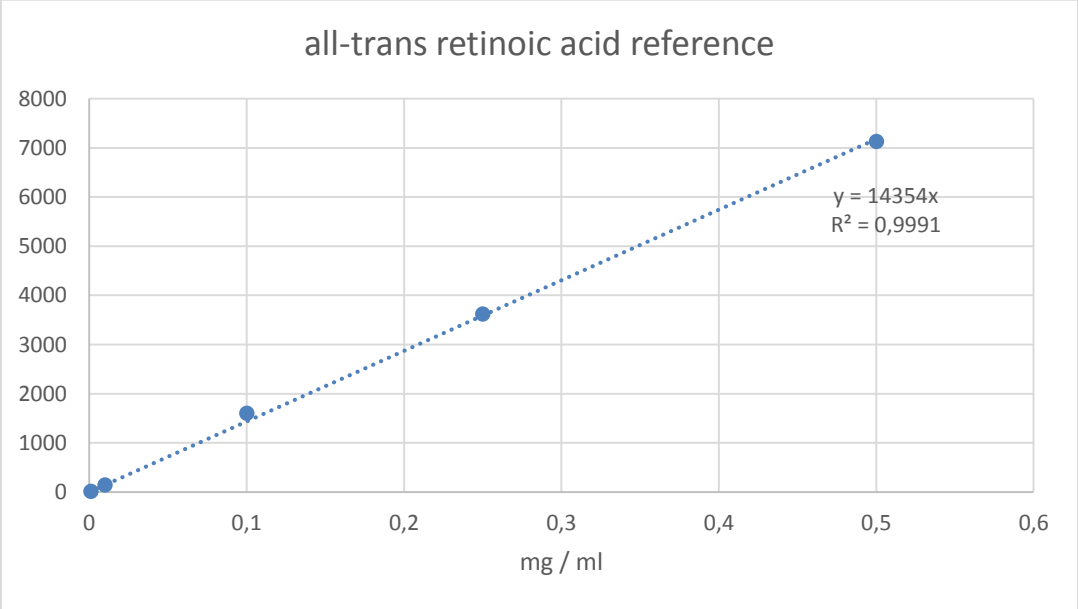
BMD1 %		**BMM2**		**BMM 10**	
Component	200 mL	Component	50 ml	Component	50 ml
500x B	0.4 ml	500x B	0.1 ml	500x B	0.1 ml
10x YNB	20 ml	10x YNB	5 ml	10x YNB	5 ml
10x D (20%)	10 ml	Methanol	0.5 ml	Methanol	2.5 ml
1 M Pi buffer pH 5.5	40 ml	1 M Pi buffer pH 6	10 ml	1 M Pi buffer pH 6	10 ml
H_2O	130 ml	H_2O	35 ml	H_2O	32.5 ml

Stock for media	Components
500x Biotin (filter sterilized)	200 mg Biotin
10x D (glucose)	220 g glucose monohydrate
10x YNB	134 g yeast nitrogen base
1 M Pi buffer (potassium phosphate buffer)	30 g K_2HPO^4, 118 g KH_2PO^4

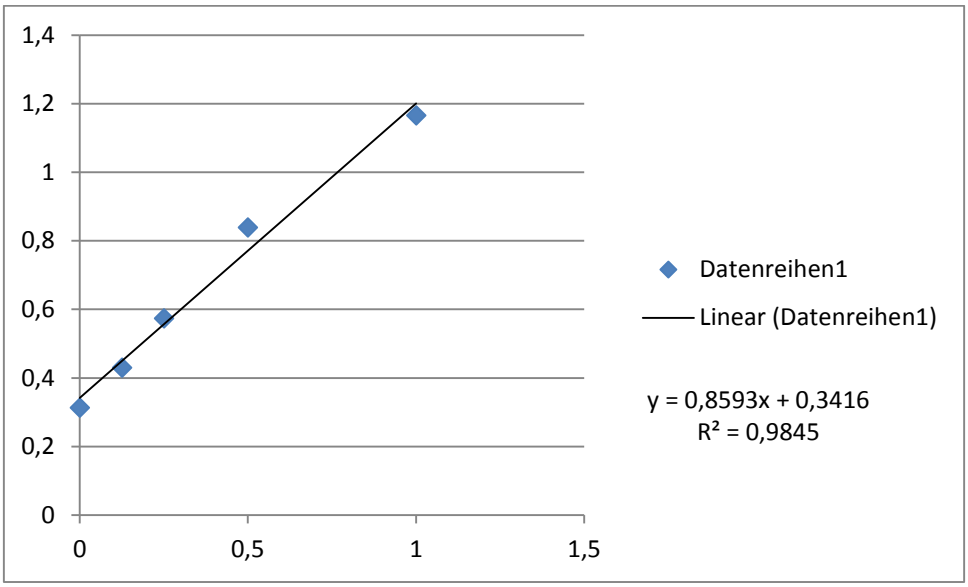
S4: calibration curve *all-trans* β-carotene



Calibration curve *all-trans* retinoic acid



S5: Bradford GSK samples



	A	conc	conc corr	µL für 15 µg	
11b	1,2835	1,09612475	10,9612475	1,4	
11ta	1,129	0,91632724	9,16327243	1,6	
12t	0,748	0,47294309	4,72943093	3,2	
12tc	1,1145	0,89945304	8,99453043	1,7	
13a	1,141	0,9302921	9,30292098	1,6	
14a	1,0755	0,85406726	8,54067264	1,8	
15a	1,166	0,95938555	9,59385546	1,6	
16a	1,1165	0,90178052	9,01780519	1,7	
17b	1,1445	0,93436518	9,34365181	1,6	
18a	1,1525	0,94367508	9,43675084	1,6	
19a	0,688	0,40311882	4,03118818	3,7	
20a	0,469	0,14826021	1,48260212	5,1	nur 7.5µg
wt	0,9865	0,75049459	7,50494589	2,0	

Appendix

Gene name: Beta-C-iso_Rice_E.coli_opt

GATATCATGGAAACCACCACCCTGGTGCTGCTGCTGCCGCATGGCGGTGCCGGCGGTGTTCGTCCGGCGGCGGCGGCAAC
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AGCTCCGGCGAAGAAAACCGAAACCGCGGCCATGATGTCGACGGTCCAGACCGAAACCGCAGCTGCGCCGCCGGCAACC
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GAAAAAGATGGTTACGAAAGCCTGATTGACGCCGCACTGGCGATTAGTCGTATCTTTTCCCTGGATAAACAGAGCGAAAT
CGTTACCCAAGCTCTGGAACGCGCGCTGCCGTCTTATATTCTGACCATGATCAAAGTCATGATGCCGCCAAGCCGTTTCTCT
CGCGAATACTTTGCTGCGTTCACGACCATTTTCTTTCCGTGGCTGGTTGGCCCGTGCGAAGTCATGGAAAGTGAAGTGGAA
GGTCGTAAAGAGAAAAACGTGGTTTATATCCCGAAATGTCGCTTTCTGGAATCCACGAACTGCGTTGGCATGTGTACCAAT
CTGTGCAAAATTCCGTGTCAGAAATTCATCCAAGATAGTCTGGGTATGAAAGTGTACATGAGCCCGAATTTTGAAGACATG
TCTTGCGAAATGATTTTTGGTCAGCAACCGCCGGAAGATGACCCGGCCCTGAAACAGCCGTGCTTCCGTACCAAATGTGTT
GCAAAACAAAACCACGGCGTCAATTGTAGCATCTAAGATATC

Gene name: Beta-C-iso_Arabidopsis-E.coli-opt

GATATCATGAACACCAAACTGTCACTGTCGCAGACCAAAATCTTTACGTTCACCACGTGGTTTAATGATACCCGTTCTGGCC
TGGACCGTCGCAGCTCTATCAGCCCGACGCTGTGTTCTAAACCGGTCTATAGTGGTAAACTGAAAGCGGCCAAAGAAACC
GCACGCATTGAAACCAGCAACACGAAAAATGCTTCAATCGAAGATAGCTTTTTCTCTAAAATCGCAATCAACTACCTGAGC
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GTGAGTCGCAACTTTGACACCAAACAGCAACATGAATTTGTTCTGAGCTCTCTGGATCGTGCGCTGCCGACGGTCATTAGT
TCCCTGATCAAAATGGCCTTTCCGCCGAGTAAAGTGTCCCGCGAACTGTTTGCGCTGTTCACCACGATTTCTTTTGCATGGC
TGGTCGGCCCGAGTGAAGTGCGTGAAACCGAAGTTAACGGTCGCAAAGAAAAATCCGTGGTTTACATCGAAAAATGTCGT
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Gene name: Beta-C-iso_Rice-Pichia-opt

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AGCTCCAGCCAAGAAAACCGAAACTGCCGCAATGATGAGTACCGTCCAGACAGAGACCGCTGCCGCACCACCTGCTACTG
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GAAGGATGGATACGAATCTCTTATCGACGCTGCCTTGGCTATTTCAAGAATCTTTAGTCTTGATAAGCAATCTGAGATCGTC
ACACAGGCCCTTGAAAGAGCATTGCCTTCCTACATCTTGACTATGATCAAAGTTATGATGCCACCTTCTAGATTCTCCAGAG
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TGAAATGATTTTCGGTCAACAGCCACCTGAAGATGACCCTGCTTTGAAACAACCATGTTTCAGAACTAAGTGCGTCGCTAA
ACAGAACCACGGTGTTAATTGTTCTATCTAAGATATC

Gene name: Beta-C-iso_Arabidopsis-Pichia-_opt

GATATCATGAACACTAAGTTGTCACTTAGTCAAACCAAAATTTTTACTTTCACTACATGGTTCAACGATACAAGATCCGGTT
TGGACAGAAGATCCTCCATCTCACCAACCCTTTGTAGTAAGCCTGTTTACTCTGGAAAGTTGAAAGCTGCCAAAGAAACAG
CTAGAATTGAGACTTCCAACACAAAGAATGCCTCAATCGAAGATTCTTTCTTTTCTAAGATCGCCATCAACTACTTGTCTAA
AAATCTTCAGGACGCAGCTGGAATTTCAAGTTCTTCCAAGTCAACTGATTATGACAGATTGGTCGATACAGCTACCAGAGT
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Gene name: Beta-C-mono_-ox_Marine_bac-_Pichia_-opt

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ACAGCTTTCTTTTTGATTTGGTATCAACTTCCTGCTTTTTCCCTTTTGATTTTCCTTTTGATCTCAATCATCCACTTCGGTATGG
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AATTAAGATATC

Gene name: Beta-C-mono_-ox_Mouse-_Pichia_-opt

GATATCATGGAAATCATCTTCGGTCAAAACAAGAAAGAACAGTTGGAGCCTGTTCAAGCAAAAGTCACAGGTTCTATTCCA
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TTTTGAAGTACGACATCCTTACTAAGTCAAGTTTGAAGTGGTCCGAAGAGTCATGCTGGCCAGCTGAACCTTTGTTCGTTCC
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TTATTCCAGATGCTGACTGGAACGCCGTTAAGCAGACTCCTGCTGAGACACAAGAAGTCGAAAACTCTGATCACCCAACTG
ACCCTACAGCCCCAGAATTGTCACACAGTGAGAATGATTTCACTGCAGGTCATGGTGGATCTTCCTTGTAAGATATC

Gene name: Beta-C-mono_-ox_chicken-Pichia_-opt

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CACTTGGAAGTTGTCTGCAGTATTCCATCAAGAAGTTTGCTTCAACCTTCTTACTATCATTCCTTCGGAATCACCGAAAACTA
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GTCCTGATGCTAGAGAAGAGGACGAAGGTGTTGTCTTGACTTGCGTTGTCGTTTCTGAGCCAAATAAGGCACCATTTTTGC
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Gene name: Beta-C-mono_-ox_rat-_Pichia_-opt

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TGTTTATTCCTGATGCAGGTTGGAACGCTGTTAAACAAACCCCAGCTAAGACTCAGGAAGATGAAAACTCTGACCATCCAA
CCGGACTTACTGCACCTGGTTTGGGACACGGAGAGAATGATTTCACTGCTGGTCATGGTGGAAAGTCTTTGTAAGATATC

Gene name: retinalDH_mouse-Pichia-_opt

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CAGCTAGAGAGGCTTTTCCAGCCTGGTCTTCCAGAAGTCCTCAAGAGAGATCCTTGGTTCTTAATAGATTGGCTGATGTCCT
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AGAGTTGCTAAGAAATTGCAATCAGGTCTTGTCTGGACCAACTGTTGGTTGATTAGAGAATTGAATCTTCCATTTGGTGGA
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TATTAAGATATC

Gene name: retinalDH_human-Pichia-_opt

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Gene name: retinalDH_chicken-Pichia-_opt_

GATATCATGAAGAAACAAGGTTCTCCATCCAACCCAGCACCTGTTTTGCCTGCTCTTCCAGAACCTTTGAAGGACCTTAAGA
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CAAACCTTAAAAGAGTTACCTTGGAACTTGGTGGAAAGTCCCCTAATATTATCTTCGCCGATGCAGACTTGGATGAAGCTG
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C

Gene name: retinalDH_rat_liver-Pichia-_opt

GATATCATGTCTTCCCCAGCACAACCTGCTGTTCCAGCCCCTCTTGCAAACTTGAAGATCCAGCATACCAAAATTTTTATCAA
CAACGAGTGGCACGACTCAGTTAGTGGTAAAAAGTTCCCAGTCTTGAATCCTGCTACTGAAGAGGTCATCTGTCATGTTGA
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CTGTTCAACAGATCATGAAGTTCAAAAGTATCGATGACGTTATCAAGAGAGCTAACAATACTACATACGGACTTGCAGCTG
GTGTTTTCACTAAAGACTTGGATAGAGCCATCACAGTCTCTTCCGCATTGCAAGCTGGTGTCGTTTGGGTTAACTGTTATAT
GATTTTGTCTGCTCAGTGCCCTTTTGGAGGTTTCAAGATGTCCGGAAATGGTAGAGAACTTGGAGAGCATGGTTTGTACGA
ATATACCGAGTTGAAAACTGTTGCTATGAAGATTTCTCAAAAGAACTCTTAAGATATC

Gene name: Beta_C_iso_Brassica_rapa_Pichia

GATATCATGATGAATACAAAATTGTCCCTTTCACAAACCAAGATCATCGGTTTTACAATCAGATTGAACGATACAAGAACTA
GATACGGAAGATCCTCTATTTCTGCCACCTTGTCAAGTAAACCAGCATATTCAGGTGAACTTAAAGTTGTCAAGGAAACTTC
TGGAACCGAGTTGCTTAAGAAAAAGAACGCATCCAATGAGGATAACTTCTTGTCAAAGATCGCCATCAATTACTTGTCTAA
GACACTTCAGGACACTGCTGGAATGTCTTCCTCAAGTTCTAAAAGTACTGGATACGATTCTTTGGTTGATACCGCAACAAG
AGTTGCTGGAAACCTTGATATTAAGCAACAGCATGAATTGGTTCTTGTCGCTTTGGACAGAGCTTTTCCAGCCGTTATTTTG
TCTCTTATCAAAATGTTGCTTCCACCTAGTAAGTTGTCTAGAGAGATGTTTGCTCTTTTCACTACCGTTTTCTTTGCCTGGTTG
GTTGGTCCTTCTGAAGTCAGAGAAACTGAGGTTAATGGTAAAAAGGAGAAATCCATGGTCTTTATTAAAAAGTGCAGATT
CTTGGAACAAACTAATTGTGTTGGAATGTGCACCCATTTGTGTAAGATCCCTAGTCAGATTTTCATCAAGAACTCTTTGGGA
ATGCCAATCTACATGATGCCTGACTTTGATGACTTGTCTTGTAAGATGATGTTCGGTCAAGAACCACCTGAGATTAAAGAT
GACCCAGTCATGAAGCAACCTTGCTTTCAGTTCTGTAAATCCAACAGATCCTACGGTGTTAAGCACTAAGATATC

Gene name: Beta_C_iso_Camelina_sativa_Pichia

GATATCATGATGAATACCAAGTTGTCACTTAGTCATACAAAAATTTTGACTTTTACTGCTAGATTCAACGATACCAGAACAA
GATCCGGTTTGGACAGAAGATCCTCTGTTTCTCCAACTCTTTGTAGTAAGCCTATCTACTCTGGAGAATTGAAGGCTACAAA
AGAAACTGCCGTTACCGAGATGTCCAACACCAAAAATGCCTCAAACATCGAGGATTCTTTCTTTTCTAAGTTGGCTATCAAT
TACTTGTCTAAAAACCTTCAAGACGCTGCCGGAATGTCAAGTTCTTCCACAAAGTCTACTGATTATGACAGATTGGTCGATA
CTGCCACCAGAGTTGCAAGAAATTTTGACACTAAACAACAGCACGAATTGGTCCTTTCAAGTTTGGATAGAGCACTTCCAG
CTGAGATTTCTTCCTTGATCAAGGTTCTTTTTCCACCTTCAAAATTGAGTAGAGAATTGTTCGCACTTTTCACTACCATTTCCT
TCGCTTGGTTGGTTGGTCCTTCAGAAGTCAGAGAAACTGAGGTTAATGGAAGAAAGGAGAAAAGTGTTGTCTTTATTGAA
AAGTGCAGATTCTTGGAGCAATCCAACTGTGTTGGAATGTGCACTCATATTTGTAAGATCCCAAGTCAAATTTTTATGAAAA
ATTCTCTTGGAATGCCAGTTTACATGGAACCTGATTTCAACGACTTGTCTTGTAAGATGATGTTCGGTAGAGAACCACCTGA
GATTCAAGATGACCCAGTCATGAAGCAGCCTTGCTTTGAGTTCTGTAAGTCTAACAAATCCTATGGTGTTAAACACTAAGA
TATC

Gene name: Beta_C_mono_ox_Micromonas_pusilla_Pichia

GATATCATGTCTTACTCCTTTTTGGGTCAATCTACTGTTAGATACCCAGCTGCCGTCCATGGAACACCTGAAGCAAAAGCTA
CTTACGTTGATTCTCCAGCAGACAAGTTGGCTATTGAAATGAACGTTAGAATGTTGTCTGAGCTTGCTAGAGTCAAACCAA
GAGGAGATCCAACTTCCTATGACGCTTTGGTTGAAGCAGCTTTGATGCTTAGAGATGCTACCCCACCTGAGAAACTTAGAT
CCGACATTGGAAACTTGATGAGAAAGCAAATCACATCCGGTATGCCACCTTTTGCCTTGGGAGCAATGCAGGCTCTTGTTC
CATCAGAAATCTTGAGAGAGATGAATGCCACTGTTGCAAGTGAAGCCGCAGAGTGGATGTTCGGTCCTACTACCAGAGAA
ACCTTGGCTGGACAAGGTGGAAAAAGAGTTACAGTTGTCAACGTCAAGAAATGCAGATACCTTGAGGCCTCAGGTTGTGC
AAGTGTTTGCGTCAATCAGTGTAAGTTGCCAGCTCAGGATGTTATGAGATCAGAATTTGGTACCCCAGTCTATATGCAACC
TAACTTCAATGACTGTTCTTGCAGAATGTTTTTCGGACAGGAACCACTTCCTGAGTCTATTGATCCTGCTATCAAGGCCTCCT
GCTTGGAAGGTTGTGCTAGAGGAGACGCCATGGCTGCCATGTCAGCAGCTGCCGAAGCTGAGGCAGCTGCCAGTGAGGC
AGCTGCCAGAGCAGGTACCTTCCCAGATCCTCAGGCCTGGGTTGACGGTGTTGTCGGACCTAGAGCTAAGAAAAAGAACC
CACCTGAAAGAGCTAATGAGACATGTGATTTGCTTAGAGGATAAGATATC

eGFP from Pia Fauland

ATGGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAA
TTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAATTTATTTGCACTACTGGAAAACTA
CCTGTTCCATGGCCAACACTTGTCACTACTTTGACTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCA
TGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGAC
GCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAA
CATTCTCGGACACAAACTTGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAA
AGCTAACTTCAAAATTCGTCACAACATTGAAGATGGTTCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGC
GATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACC
ACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAATTGTACAAGTAA

Linker sequence

GCTGCTGCTGTTGATCCAAAGTTGTTGGGTGCT

AOD sequence

ATGTTAAAACTGTACGCAATAAGGCCAATTAGGCCAATCAAGCCATGCCTTGTGGCTTCCACTTTCAGGCACTAT

MBP sequence with linker

ATGAAGATCGAGGAAGGTAAGTTAGTGATTTGGATCAACGGAGACAAAGGTTACAATGGTTTGGCTGAAGTTGGTAAGA
AATTCGAAAAGGACACTGGTATCAAGGTTACCGTCGAGCACCCTGACAAGTTGGAGGAAAAGTTCCCACAAGTTGCTGCA
ACCGGTGATGGACCAGATATTATCTTTTGGGCCCATGACAGATTCGGTGGTTACGCACAGTCCGGTCTTTTGGCTGAGATT
ACTCCAGATAAAGCATTCCAAGACAAGTTGTATCCTTTCACTTGGGATGCTGTTCGTTACAACGGTAAGCTGATTGCCTATC
CTATTGCTGTCGAAGCCTTATCTTTGATCTACAACAAGGACTTGTTGCCAAATCCACCTAAGACCTGGGAGGAAATTCCTGC
ACTTGACAAGGAGTTGAAAGCCAAGGGAAAGTCTGCTCTGATGTTCAACCTTCAAGAACCATACTTTACTTGGCCATTGAT
TGCCGCTGACGGAGGTTATGCTTTCAAGTACGAGAACGGTAAATACGATATCAAGGACGTGGGTGTCGATAATGCTGGTG
CTAAGGCTGGATTGACCTTTCTTGTTGACTTAATCAAGAACAAGCACATGAACGCTGACACTGACTACTCTATTGCTGAAGC
TGCATTCAACAAAGGTGAAACCGCCATGACTATTAACGGTCCTTGGGCTTGGTCCAACATTGACACTTCTAAGGTCAACTA
CGGTGTTACTGTCCTTCCAACCTTCAAGGGACAACCTTCTAAGCCATTCGTTGGTGTCTTATCAGCTGGAATCAATGCCGCT
TCTCCAAACAAAGAACTTGCAAAGGAGTTCCTGGAAAACTACTTGCTGACCGACGAGGGTTTGGAGGCTGTTAACAAGGA
CAAGCCTCTGGGAGCTGTTGCATTGAAGTCATACGAGGAAGAGTTGGCTAAAGATCCAAGAATTGCCGCTACTATGGAGA
ACGCACAAAAGGGAGAAATCATGCCAAACATCCCTCAAATGTCCGCTTTCTGGTACGCCGTTAGAACTGCTGTGATTAACG
CCGCATCTGGTAGACAGACCGTCGACGAGGCTTTGAAGGATGCACAAACCTTGATCAACGGTGACGGTGCTGGACTTGAA
GTTTTGTTCCAGGGTCCTGAGAACCTTTACTTTCAAGCT

ADH2 promoter sequence

ATTTAAATCGCAGCGTTTTCTGACGGTACTAGAGGACTCTTAGGGGAAGGTAGAATCAATAAAGATCATATTAGGTAAGC
AAATTTTGGATGGAATAGGAGACTAGGTGTGGATGCGCGATCTCGCCAAATTGCACGACCAGAGTGGATGCCGGATGGT
GGTAAACCGTTTCTTCCTTTTTACCACCCAAGTGCGAGTGAAACACCCCATGGCTGCTCTCCGATTGCCCCTCTACAGGCAT
AAGGGTGTGACTTTGTGGGCTTGAATTTTACACCCCCTCCAACTTTTCTCGCATCAATTGATCCTGTTACCAATATTGCATGC
CCGGAGGAGACTTGCCCCCTAATTTCGCGGCGTCGTCCCGGATCGCAGGGTGAGACTGTAGAGACCCCACATAGTGACAA
TGATTATGTAAGAAGAGGGGGGTGATTCGGCCGGCTATCGAACTCTAACAACTAGGGGGGTGAACAATGCCCAGCAGTC
CTCCCCACTCTTTGACAAATCAGTATCACCGATTAACACCCCAAATCTTATTCTCAACGGTCCCTCATCCTTGCACCCCTCTTT
GGACAAATGGCAGTTAGCATTGGTGCACTGACTGACTGCCCAACCTTAAACCCAAATTTCTTAGAAGGGGCCCATCTAGTT
AGCGAGGGGTGAAAAATTCCTCCATCGGAGATGTATTGACCGTAAGTTGCTGCTTAAAAAAAATCAGTTCAGATAGCGAG
ACTTTTTTGATTTCGCAACGGGAGTGCCTGTTCCATTCGATTGCAATTCTCACCCCTTCTGCCCAGTCCTGCCAATTGCCCAT
GAATCTGCTAATTTCGTTGATTCCCACCCCCCTTTCCAACTCCACAAATTGTCCAATCTCGTTTTCCATTTGGGAGAATCTGC
ATGTCGACTACATAAAGCGACCGGTGTCCGAAAAGATCTGTGTAGTTTTCAACATTTTGTGCTCCCCCCGCTGTTTGAAAAC
GGGGGTGAGCGCTCTCCGGGGTGCGAATTCGTGCCCAATTCCTTTCACCCTGCCTATTGTAGACGTCAACCCGCATCTGGT
GCGAATATAGCGCACCCCCAATGATCACACCAACAATTGGTCCACCCCTCCCCAATCTCTAATATTCACAATTCACCTCACTA
TAAATACCCCTGTCCTGCTCCCAAATTCTTTTTTCCTTCTTCCATCAGCTACTAGCTTTTATCTTATTTACTTTACGAAAACTAG
T

TPI promoter sequence

ATTTAAATTCAACGAGACACTCTTCCGTCAGTTCCAAAACCATAAGTTTGCCGATGTGTTGGTCCTTGTAACGCATGGAATT
TGGGCCAGGGTATTTTTGATGAAATGGTTCAGATGGTCTGTGGAGGAGTTTGAAGGCTTACGAAATATACCACATTGCCA
GTTTATACAGATGGTTAAGGGTGAAAATCAACGTTACACCTTGACGACCCCATTATTACGATGGCGTGAAGGAGATGAAG
ACCGGGTAGAAGAAATAAGAAAAGCGGTACAGTTTAGGTCCGGAGATCTAGGGAAGGAGGCCTTAGCTTATATTGTAGC
TGCTGAGAGAGAGGCAGCTGCTGGAAGATCTGAAGGCCCTATCACGTATGATGATGGTGATGACCATTAGAGAACGCCC
AGAGATTGATAGCCAGTTCTTGGACAACAATTCGGAACTTTATTCACGGTGCAAACATGATTTGTGTGGATAGCTTCAAGT
CAGACATTTCATCTCATCCCCCCTTTTACTGCTGCTAATCACCGTTAGTCCGACAGTTACTCTAATCAATATTTATTAGTGTTT
TAGTTGCGCAAAACTCGAGCCTCTTTTCCTTATCTCTTGACACTTCCTGGAGTCGAAGTTTTTCAGCGCAAATTCACTCTACA
ATGTCTACCGATACTAGACCGCCTATCTTCCCCCTCTAAATAGCCTATTGGAAGGGTGCAATAAGGTATATAAATCTGGCGC
GATTCCCCCGGACTTTTATGATCCACATCACCTCATCTTACTGCCCTCACTCTCTTTCCTGATCCTCCCAGGTCCACCGATTTC
CTCACTATCGTCGGATTTCTCCTTCCAGCGCCCTAGAGAATTCCGTAACCACCGCAAAAATAGCAGCCCCCCCCTCACCCAT
TTTTTTATTTAAAAGAACACCTTACTGGCCCGTTTTCGTTTCTCCTTTACTACAATTGATTTTTAATTTTCAGTTTTTTTCATTG
ATATACAAGATCTATCACAAACACAACTAGT

CAT promoter sequence

TAATCGAACTCCGAATGCGGTTCTCCTGTAACCTTAATTGTAGCATAGATCACTTAAATAAACTCATGGCCTGACATCTGTA
CACGTTCTTATTGGTCTTTTAGCAATCTTGAAGTCTTTCTATTGTTCCGGTCGGCATTACCTAATAAATTCGAATCGAGATTG
CTAGTACCTGATATCATATGAAGTAATCATCACATGCAAGTTCCATGATACCCTCTACTAATGGAATTGAACAAAGTTTAAG
CTTCTCGCACGAGACCGAATCCATACTATGCACCCCTCAAAGTTGGGATTAGTCAGGAAAGCTGAGCAATTAACTTCCCTC
GATTGGCCTGGACTTTTCGCTTAGCCTGCCGCAATCGGTAAGTTTCATTATCCCAGCGGGGTGATAGCCTCTGTTGCTCATC
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CAATTAAA

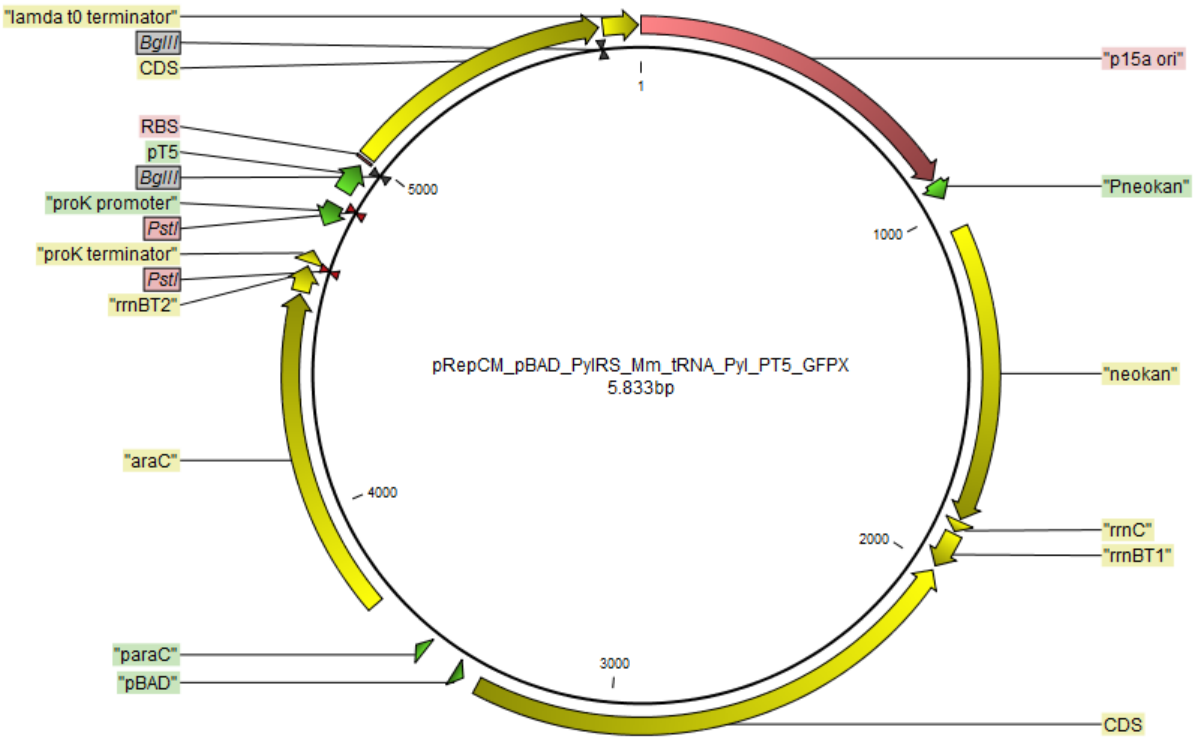

Figure 26: pREP_pBAD strain from Patrik Fladischer

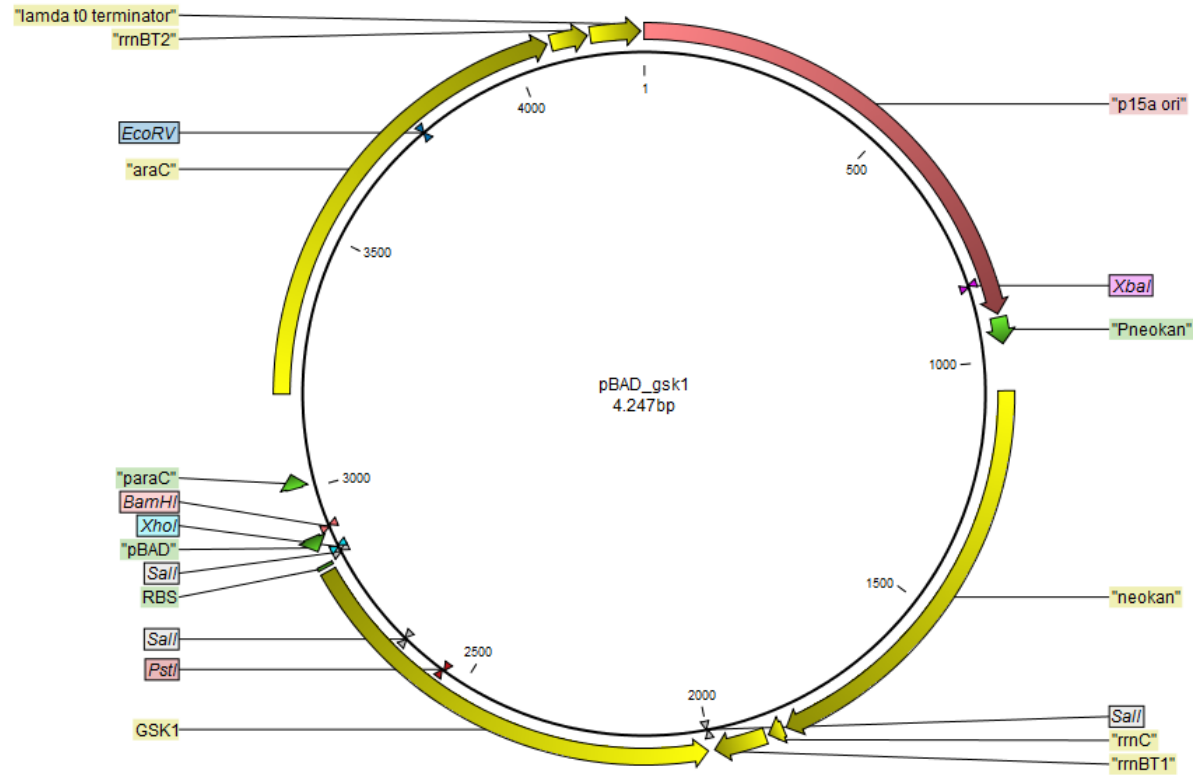

Figure 27: Example for *E. Coli* optimized isomerase from rice in prepared pBAD vector

Glycerol stocks:

P1	GSK 1	E.coli Top10F´ PpT4-S Iso_rice_E.coli_opt
P2	GSK 2	E.coli Top10F´ PpT4-S Iso_ara_E.coli_opt
P3	GSK 1 trunc	E.coli Top10F´ PpT4-S Iso_rice_trunc_E.coli_opt
P4	GSK 2 trunc	E.coli Top10F´ PpT4-S Iso_ara_trunc_E.coli_opt
P5	GSK 11	E.coli Top10F´ PpT4-S Iso_rice_Pp_opt
P6	GSK 11 trunc	E.coli Top10F´ PpT4-S Iso_rice_trunc_Pp_opt
P7	GSK 12	E.coli Top10F´ PpT4-S Iso_ara_Pp_opt
P8	GSK 12 trunc	E.coli Top10F´ PpT4-S Iso_ara_trunc_Pp_opt
P9	GSK 13	E.coli Top10F´ PpT4-S MO_marinebact_Pp_opt
P10	GSK 14	E.coli Top10F´ PpT4-S MO_mouse_Pp_opt
P11	GSK 15	E.coli Top10F´ PpT4-S MO_chic_Pp_opt
P12	GSK 16	E.coli Top10F´ PpT4-S MO_rat_Pp_opt
P13	GSK 17	E.coli Top10F´ PpT4-S retinalDH_mouse_Pp_opt
P14	GSK 18	E.coli Top10F´ PpT4-S retinalDH_human_Pp_opt
P15	GSK 19	E.coli Top10F´ PpT4-S retinalDH_chic_Pp_opt
P16	GSK 20	E.coli Top10F´ PpT4-S retinalDH_rat_Pp_opt
P17	chic/rat	E.coli Top10F´ PpT4-S MOchic_DHrat_Pp_opt
P18	chic/human	E.coli Top10F´ PpT4-S MOchic_DHhuman_Pp_opt
P19	chic/mouse	E.coli Top10F´ PpT4-S MOchic_DHmouse_Pp_opt
P20	chic/chic	E.coli Top10F´ PpT4-S MOchic_DHchic_Pp_opt
P21	mouse/rat	E.coli Top10F´ PpT4-S MOmouse_DHrat_Pp_opt
P22	mouse/human	E.coli Top10F´ PpT4-S MOmouse_DHhuman_Pp_opt
P23	mouse/chic	E.coli Top10F´ PpT4-S MOmouse_DHchic_Pp_opt
P24	mouse/mouse	E.coli Top10F´ PpT4-S MOmouse_DHmouse_Pp_opt
P25	bidi mouse	E.coli Top10F´ PpT4-S bidi mouse_Pp_opt
P26	bidi chic/rat	E.coli Top10F´ PpT4-S bidi chic/rat_Pp_opt
P27	3er combi	E.coli Top10F´ PpT4-S CATIsoAra_bidichic/rat_Pp_opt
P28	GSK 21	E.coli Top10F´ PpT4-S Iso_Brassica_rapa_Pp_opt
P29	GSK 22	E.coli Top10F´ PpT4-S Iso_Camelina_sativa_Pp_opt
P30	GSK 23	E.coli Top10F´ PpT4-S Iso_Micro_pusilla_Pp_opt
P31	AOXgfp11	E.coli Top10F´ PpT4-S Iso_rice_gfp_Pp_opt
P32	AOXgfp11trunc	E.coli Top10F´ PpT4-S Iso_rice_trunc_gfp_Pp_opt
P33	AOXgfp12	E.coli Top10F´ PpT4-S Iso_ara_gfp_Pp_opt
P34	AOXgfp12trunc	E.coli Top10F´ PpT4-S Iso_ara_trunc_gfp_Pp_opt
P35	AOX11AOD	E.coli Top10F´ PpT4-S Iso_rice_AOD_gfp_Pp_opt
P36	AOX12AOD	E.coli Top10F´ PpT4-S Iso_ara_AOD_gfp_Pp_opt
P37	AOXMBP11	E.coli Top10F´ PpT4-S Iso_rice_MBP_gfp_Pp_opt
P38	AOXMBP12	E.coli Top10F´ PpT4-S Iso_ara_MBP_gfp_Pp_opt
P39	GSK21gfp	E.coli Top10F´ PpT4-S Iso_Brassica_rapa_gfp_Pp_opt
P40	GSK22gfp	E.coli Top10F´ PpT4-S Iso_Camelina_sativa_Pp_opt
P41	GSK23gfp	E.coli Top10F´ PpT4-S Iso_Micro_pusilla_Pp_opt
P42	CAT11	E.coli Top10F´ PpT4-S CAT_Iso_rice_Pp_opt
P43	CAT11trunc	E.coli Top10F´ PpT4-S CAT_Iso_rice_trunc_Pp_opt

P44	CAT12	E.coli Top10F´ PpT4-S CAT_Iso_ara_Pp_opt
P45	CAT12trunc	E.coli Top10F´ PpT4-S CAT_Iso_ara_trunc_Pp_opt
P46	CAT21	E.coli Top10F´ PpT4-S CAT_Iso_Brassica_rapa_Pp_opt
P47	CAT22	E.coli Top10F´ PpT4-S CAT_Iso_Camelina_sativa_Pp_opt
P48	CAT23	E.coli Top10F´ PpT4-S CAT_Iso_Micro_pusilla_Pp_opt
P49	CATgfp11	E.coli Top10F´ PpT4-S CAT_Iso_rice_gfp_Pp_opt
P50	CATgfp11trunc	E.coli Top10F´ PpT4-S CAT_Iso_rice_trunc_gfp_Pp_opt
P51	CATgfp12	E.coli Top10F´ PpT4-S CAT_Iso_ara_gfp_Pp_opt
P52	CATgfp12trunc	E.coli Top10F´ PpT4-S CAT_Iso_ara_trunc_gfp_Pp_opt
P53	CAT11AOD	E.coli Top10F´ PpT4-S CAT_Iso_rice_AOD_gfp_Pp_opt
P54	CAT12AOD	E.coli Top10F´ PpT4-S CAT_Iso_ara_AOD_gfp_Pp_opt
P55	CATMBP11	E.coli Top10F´ PpT4-S CAT_Iso_rice_MBP_gfp_Pp_opt
P56	CATMBP12	E.coli Top10F´ PpT4-S CAT_Iso_ara_MBP_gfp_Pp_opt
P57	CAT GSK21gfp	E.coli Top10F´ PpT4-S CAT_Iso_Brassica_rapa_gfp_Pp_opt
P58	CAT GSK22gfp	E.coli Top10F´ PpT4-S CAT_Iso_Camelina_sativa_Pp_opt
P59	CAT GSK23gfp	E.coli Top10F´ PpT4-S CAT_Iso_Micro_pusilla_Pp_opt
P60	BAD GSK1	E.coli BL21 pBAD-S Iso_rice_E.coli_op
P61	BAD GSK1trunc	E.coli BL21 pBAD-S Iso_rice_trunc_E.coli_op
P62	BAD GSK2	E.coli BL21 pBAD-S Iso_ara_E.coli_opt
P63	BAD GSK2trunc	E.coli BL21 pBAD-S Iso_ara_trunc_E.coli_opt
P64	BAD GSK1MBP	E.coli BL21 pBAD-S Iso_rice_trunc_MBP_E.coli_opt
P65	BAD GSK2MBP	E.coli BL21 pBAD-S Iso_ara_trunc_MBP_E.coli_opt
P66	pP GSK1 crt	Pichia P. crtEBIY PpT4-S Iso_rice_E.coli_opt
P67	pP GSK1tr crt	Pichia P. crtEBIY PpT4-S Iso_rice_trunc_E.coli_opt
P68	pP GSK2 crt	Pichia P. crtEBIY PpT4-S Iso_ara_E.coli_opt
P69	pP GSK2tr crt	Pichia P. crtEBIY PpT4-S Iso_ara_trunc_E.coli_opt
P70	pP GSK11 CBS	Pichia P. CBS7435 PpT4-S Iso_rice_Pp_opt
P71	pP GSK11tr CBS	Pichia P. CBS7435 PpT4-S Iso_rice_trunc_Pp_opt
P72	pP GSK12 CBS	Pichia P. CBS7435 PpT4-S Iso_ara_Pp_opt
P73	pP GSK12 tr CBS	Pichia P. CBS7435 PpT4-S Iso_ara_trunc_Pp_opt
P74	pP GSK13 CBS	Pichia P. CBS7435 PpT4-S MO_marinebact_Pp_opt
P75	pP GSK14 CBS	Pichia P. CBS7435 PpT4-S MO_mouse_Pp_opt
P76	pP GSK15 CBS	Pichia P. CBS7435 PpT4-S MO_chic_Pp_opt
P77	pP GSK16 CBS	Pichia P. CBS7435 PpT4-S MO_rat_Pp_opt
P78	pP GSK17 CBS	Pichia P. CBS7435 PpT4-S retinalDH_mouse_Pp_opt
P79	pP GSK18 CBS	Pichia P. CBS7435 PpT4-S retinalDH_human_Pp_opt
P80	pP GSK19 CBS	Pichia P. CBS7435 PpT4-S retinalDH_chic_Pp_opt
P81	pP GSK20 CBS	Pichia P. CBS7435 PpT4-S retinalDH_rat_Pp_opt
P82	pP GSK21 CBS	Pichia P. CBS7435 PpT4-S Iso_Brassica_rapa_Pp_opt
P83	pP GSK22 CBS	Pichia P. CBS7435 PpT4-S Iso_Camelina_sativa_Pp_opt
P84	pP GSK23 CBS	Pichia P. CBS7435 PpT4-S Iso_Micro_pusilla_Pp_opt
P85	pP GSK11 crt	Pichia P. crtEBIY PpT4-S Iso_rice_Pp_opt
P86	pP GSK11tr crt	Pichia P. crtEBIY PpT4-S Iso_rice_trunc_Pp_opt
P87	pP GSK12 crt	Pichia P. crtEBIY PpT4-S Iso_ara_Pp_opt
P88	pP GSK12 tr crt	Pichia P. crtEBIY PpT4-S Iso_ara_trunc_Pp_opt

P89	pP GSK13 crt	Pichia P. crtEBIY PpT4-S MO_marinebact_Pp_opt
P90	pP GSK14 crt	Pichia P. crtEBIY PpT4-S MO_mouse_Pp_opt
P91	pP GSK15 crt	Pichia P. crtEBIY PpT4-S MO_chic_Pp_opt
P92	pP GSK16 crt	Pichia P. crtEBIY PpT4-S MO_rat_Pp_opt
P93	pP chic/rat crt	Pichia P. crtEBIY PpT4-S MOchic_DHrat_Pp_opt
P94	pP chic/hum crt	Pichia P. crtEBIYPpT4-S MOchic_DHhuman_Pp_opt
P95	pP chic/mou crt	Pichia P. crtEBIY PpT4-S MOchic_DHmouse_Pp_opt
P96	pP chic/chic crt	Pichia P. crtEBIY PpT4-S MOchic_DHchic_Pp_opt
P97	pP mou/rat crt	Pichia P. crtEBIY PpT4-S MOmouse_DHrat_Pp_opt
P98	pP mou/hum crt	Pichia P. crtEBIY PpT4-S MOmouse_DHhuman_Pp_opt
P99	pP mou/chic crt	Pichia P. crtEBIY PpT4-S MOmouse_DHchic_Pp_opt
P100	pP mou/mou crt	Pichia P. crtEBIY PpT4-S MOmouse_DHmouse_Pp_opt
P101	PP bidi mouse crt	Pichia P. crtEBIY PpT4-S bidi mouse_Pp_opt
P102	pP bidi chic/rat crt	Pichia P. crtEBIY PpT4-S bidi chic/rat_Pp_opt
P103	pP 3er kombi crt	Pichia P. crtEBIY PpT4-S CATIsoAra_bidichic/rat_Pp_opt
P104	pP CAT GSK11 crt	Pichia P. crtebiy PpT4-S CAT_Iso_rice_Pp_opt
P105	pP CAT GSK11tr crt	Pichia P. crtebiy PpT4-S CAT_Iso_rice_trunc_Pp_opt
P106	pP CAT GSK12 CBS	Pichia P. CBS7435 PpT4-S CAT_Iso_ara_Pp_opt
P107	pP CAT GSK12 tr crt	Pichia P. crtebiy PpT4-S CAT_Iso_ara_trunc_Pp_opt
P108	pP CAT GSK21 CBS	Pichia P. CBS7435 PpT4-S CAT_Iso_Brassica_rapa_Pp_opt
P109	pP CAT GSK22 CBS	Pichia P. CBS7435 PpT4-S CAT_Iso_Camelina_sativa_Pp_opt
P110	pP CAT GSK23 CBS	Pichia P. CBS7435 PpT4-S CAT_Iso_Micro_pusilla_Pp_opt
P111	pP CAT GSK12 tr crt GAP	Pichia P. GAP crtebiy PpT4-S CAT_Iso_ara_trunc_Pp_opt
P112	pP CAT GSK22 crt	Pichia P. crtEBIY PpT4-S CAT_Iso_Camelina_sativa_Pp_opt
P113	pP CAT GSK22 crt GAP	Pichia P. GAP crtEBIY PpT4-S CAT_Iso_Camelina_sativa_Pp_opt
P114	pP CAT GSK11 crt GAP	Pichia P. GAP crtebiy PpT4-S CAT_Iso_rice_Pp_opt
P115	pP CAT GSK11 tr crt GAP	Pichia P. GAP crtebiy PpT4-S CAT_Iso_rice_trunc_Pp_opt
P116	pP AOX GSK23 crt	Pichia P. crtEBIY PpT4-S Iso_Micro_pusilla_Pp_opt
P117	pP AOX GSK22 crt	Pichia P. crtEBIY PpT4-S Iso_Camelina_sativa_Pp_opt
P118	pP AOX GSK21 crt	Pichia P. crtEBIY PpT4-S Iso_Brassica_rapa_Pp_opt
P119	BAD GSK1 crt	E.coli crtEBIY pBAD-S Iso_rice_E.coli_opt
P120	BAD GSK1trunc crt	E.coli crtEBIY pBAD-S Iso_rice_trunc_E.coli_opt
P121	BAD GSK2 crt	E.coli crtEBIY pBAD-S Iso_ara_E.coli_opt
P122	BAD GSK2trunc crt	E.coli crtEBIY pBAD-S Iso_ara_trunc_E.coli_opt
P123	BAD GSK1MBP crt	E.coli crtEBIY pBAD-S Iso_rice_trunc_MBP_E.coli_opt
P124	BAD GSK2MBP crt	E.coli crtEBIY pBAD-S Iso_ara_trunc_MBP_E.coli_opt

Bidirectional promoter BZ6: pDAS2-699+pCore1-HTA1-81

Media	Components
BEDS 1 L	1.63 g Bicin in 920 ml H_2O; pH 8.3 with 5 M NaOH; add 30 ml ethylene glycol, 50 ml DMSO (dimethylsulfoxid) and 1 M sorbitol; sterile filtration and aliquot in 12 ml
SOC 1 L	3.46 g glucose, 20 g tryprone, 5 g yeast extract, 0.56 g NaCl, 2 g $MgCl_2$, 0.16 g KCl, 2.46 g $MgSO_4$
YPD (Agar) 1 L	10 g yeast extract, 20 g peptone, 100 ml 10x D (15 g agar)
Buffer A	50 mM KPi pH 7.9, 1 mM EDTA, 5% glycerol, 2 mM DTT, 1 mM PMSF (polymethylsulfonyfluorid)