PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
SCHOOL OF ENGINEERING

HETEROLOGOUS PRODUCTION OF THE EPOXYCAROTENOID VIOLAXANTHIN IN *SACCHAROMYCES CEREVISIAE*

VICENTE FRANCISCO CATALDO VON BOHLEN

Thesis submitted to the Office of Graduate Studies in partial fulfillment of the requirements for the Degree Doctor in Engineering

Advisor:
EDUARDO AGOSIN TRUMPER

Santiago de Chile, April, 2020

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ABSTRACT

Microbial production of carotenoids has mainly focused towards a few products, such as β-carotene, lycopene and astaxanthin. However, other less explored carotenoids, like violaxanthin, have also shown unique properties and promising applications. Violaxanthin is a plant-derived epoxidated carotenoid with strong antioxidant activity and a key precursor of valuable compounds, such as fucoxanthin and β-damascenone. In this thesis, we report for the first time the heterologous production of epoxycarotenoids in yeast. We engineered the yeast *Saccharomyces cerevisiae* following multi-level strategies for the efficient accumulation of violaxanthin. Starting from a β-carotenogenic yeast strain, we first evaluated the performance of several β-carotene hydroxylases (CrtZ), and zeaxanthin epoxidases (ZEP) from different species, together with their respective N-terminal truncated variants. The combined expression of CrtZ from *Pantoea ananatis* and truncated ZEP of *Haematococcus lacustris* showed the best performance and led to a yield of 1.6 mg/g_{DCW} of violaxanthin. Further improvement of the epoxidase activity was achieved by promoting the transfer of reducing equivalents to ZEP by expressing several redox partner systems. The co-expression of the plant truncated ferredoxin-3, and truncated root ferredoxin oxidoreductase-1 resulted in a 2.2-fold increase in violaxanthin yield (3.5 mg/g_{DCW}). Finally, increasing gene copy number of carotenogenic genes enabled reaching a final production of 7.3 mg/g_{DCW} in shake flask cultures and batch bioreactors, which is the highest yield of microbially produced violaxanthin reported to date.

Keywords: Carotenoid, xanthophyll, epoxycarotenoid, violaxanthin, metabolic engineering, *saccharomyces cerevisiae*.

RESUMEN

La producción de carotenoides en microorganismos se ha centrado en moléculas clásicas como β-caroteno, licopeno y astaxantina. Sin embargo, existen carotenoides menos estudiados que también poseen interesantes propiedades y aplicaciones. Este es el caso de la violaxantina, un epoxicarotenoide presente en plantas, que posee una potente actividad antioxidante y es precursor de otras moléculas de interés comercial como la β-damascenona y la fucoxantina. En este trabajo se reportan por primera vez cepas de levadura productoras de epoxicarotenoides. Para esto, utilizando herramientas de ingeniería metabólica a varios niveles, se construyeron cepas de la levadura *Saccharomyces cerevisiae* capaces de producir violaxantina. En primer lugar, partiendo de una cepa β-carotenogénica, se evaluaron enzimas β-caroteno hidroxilasas (CrtZ/CrtR-b2) y zeaxantina epoxidasas (ZEP) de diferentes especies, en conjunto con sus versiones truncadas en diversas posiciones aminoterminales. El mejor desempeño se logró con la expresión combinada de una CrtZ de *Pantoea ananatis* y una ZEP truncada de *Haematococcus lacustris*, llevando a un rendimiento de violaxantina de 1.6 mg/g_{DCW}. Posteriormente, con el objetivo de incrementar la actividad epoxidasa, se co-expresaron diferentes sistemas accesorios de oxidorreducción, que promueven la transferencia de equivalentes reductores hacia la enzima ZEP. La co-expresión de una ferredoxina-3 truncada y una ferredoxina oxidorreductasa-1 truncada incrementó el rendimiento de biosíntesis de violaxantina en 2.2 veces (3.5 mg/g_{DCW}). Finalmente, un aumento en el número de copias de los genes carotenogénicos permitió incrementar el rendimiento de este carotenoide a 7.3 mg/g_{DCW}, que corresponde al mejor rendimiento de violaxantina producida en microrganismos reportado hasta la fecha.

Palabras clave: Carotenoide, xantófila, epoxycarotenoide, violaxantina, ingeniería metabólica, *Saccharomyces cerevisiae*.

1. INTRODUCTION

1.1 Microbial cell factories

The discovery, characterization, and fine control of microorganisms have favorably impacted our quality of life. From the production of ancestral fermented beverages and foods to the current biosynthesis of complex fine chemicals and therapeutic proteins, microbial fermentations have shown extraordinary potential and versatility in many productive applications.

The increasing concerns on climate change and the eventual depletion of fossil reserves have driven the development of new renewable methods to produce bulk and fine chemicals. Industrial biotechnology faces this challenge using living cells and/or enzymes to produce compounds of industrial interest. The application of microorganisms such as bacteria, yeasts, and algae as microbial cell factories enable the conversion of cheap and renewable feedstocks into value-added chemicals (H. Zhao & Tan, 2015). Currently, several companies are commercializing chemical commodities produced by non-genetically modified microorganisms. Examples of these biobased processes are ethanol and acetone/butanol production by *Saccharomyces cerevisiae* and *Clostridium acetobutylicum*, respectively (Gustavsson & Lee, 2016). However, this approach is restricted to a few chemicals that have enough productivity in wild-type microorganisms.

The increasing knowledge in enzymes and metabolic pathways, together with the significant advances in synthetic biology tools have facilitated engineering the metabolism of

microorganisms. The main application of metabolic engineering is the insertion and optimization of heterologous or non-natural pathways to produce target molecules in a selected microbial host (e.g., biosynthesis of plant metabolites in bacteria; Stephanopoulos, 2012). Additionally, metabolic engineering can be useful to enhance the natural production of endogenous compounds in some microorganisms. Moreover, *de novo* pathway design and rational enzyme engineering have made possible the biosynthesis of non-natural compounds (e.g., 1,4-butanediol and adipic acid; Biz et al., 2019).

1.2 *Saccharomyces cerevisiae* as a cell factory

S. cerevisiae is currently considered a model organism for diverse biotechnological applications, including the production of a wide range of high-value chemicals (Lian, Mishra, & Zhao, 2018). Among the advantages of this yeast as cell factory are the following: an extensively studied biology, a broad spectrum of genetic engineering tools available, its suitable for large-scale operations, and it is generally regarded as safe (GRAS). The latter is particularly advantageous in the production of food ingredients and nutraceuticals (J. Wang, Guleria, Koffas, & Yan, 2016).

Many research groups have demonstrated the excellent performance as *S. cerevisiae* as host to produce heterologous compounds as diverse as isoprenoids, alkaloids, and phenylpropanoids (R. Chen, Yang, Zhang, & Zhou, 2020). However, the use of this yeast has not been restricted to the laboratory scale proof of concepts and prototyping. In the last years, some compounds produced in metabolically-engineered yeast have reached the market. Probably, one of the most iconic cases is the antimalarial drug artemisinin, which went into

commercial production in 2013. In this semi-synthetic approach, the late-stage precursor artemisinic acid is produced by metabolically-engineered yeast in titers up to 25 g/L by fermentation, using glucose and ethanol as carbon sources. Then, the artemisinic acid is converted to artemisinin by multistep chemical synthesis (Kung, Lund, Murarka, McPhee, & Paddon, 2018). Other successful examples of chemicals industrially produced in yeast are the flavors nootkatone, valencene, and vanillin (by Evolva), as well as the multifunctional sesquiterpene farnesene (by Amyris).

1.3 Construction of gene expression cassettes in yeast

Metabolic engineering of new pathways requires the expression of several genes. In yeast, heterologous genes can be extrachromosomally expressed in episomal or centromeric plasmids, or integrated and expressed from the chromosomes using integrative plasmids (Gnügge & Rudolf, 2017). Both kinds of plasmids are commonly used as the so-called shuttle vectors. These plasmids have genetic sequences that enable their maintenance in both *Escherichia coli* and *S. cerevisiae*. This feature facilitates the assembling, analysis, and amplification of the plasmid in *E. coli*, for subsequent transformation and gene expression in yeast.

1.3.1 *In vitro* DNA assembly methods

To construct any expression vector, it is necessary to assemble the target heterologous genes to the backbone of a vector. In spite of the technological advances in chemical DNA synthesis, assembly methods are still required for the construction of large DNA fragments, i.e., > 200 bp (Hughes & Ellington, 2017; Kosuri & Church, 2014). For these tasks, there are

currently several commercial and in-house *in vitro* DNA assembly technologies available. Depending on the scientific principle underpinning the assembly method, two types of techniques can be distinguished: restriction/ligation-based (e.g., biobrick (Shetty, Endy, & Knight, 2008) and Golden Gate (Engler, Gruetzner, Kandzia, & Marillonnet, 2009; Engler, Kandzia, & Marillonnet, 2008)), and sequence homology-based methods. Due to its high versatility and assembly efficiency, the latter has gained increasing acceptance in the field (Chao, Yuan, & Zhao, 2015).

The first assembly method based on sequence homology was OE-PCR (Overlap Extension PCR; Horton, Hunt, Ho, Pullen, & Pease, 1989). This ligase-free approach assembles DNA fragments in two rounds of PCR. First, DNA templates are separately amplified using primers to yield overlapping regions. The products are then mixed in a second round of PCR where overlapping regions act as primers. Finally, DNA polymerase extends and produces the sought spliced product. Although this technique is still widely used, it is laborious and has been gradually replaced by more efficient assembly methods like SLIC (M. Z. Li & Elledge, 2007), USER (Bitinaite et al., 2007; Vaisvila & Bitinaite, 2013), and Gibson assembly (Gibson et al., 2009). Particularly, the latter has been widely adopted in the community because of its simplicity for joining multiple DNA parts in a single isothermal reaction. As in OE-PCR, Gibson assembly employs overlapping PCR products, but in this case, a T5 exonuclease is used to hydrolyze 5' ends of DNA, thereby generating complementary overhangs for specific annealing. Lastly, DNA polymerase and Taq ligase sequentially repair the double-strand yielding a covalently joined seamless product.

1.3.2 DNA assembly by homologous recombination in yeast

Another group of efficient assembly methods exploits the inherent Homologous Recombination (HR) machinery of *S. cerevisiae*. HR assembly in *S. cerevisiae* was first reported for the construction of yeast extrachromosomal expression vectors (Juhas & Ajioka, 2017; H. Ma, Kunes, Schatz, & Botstein, 1987; Oldenburg, Vo, Michaelis, & Paddon, 1997; Raymond, Pownder, & Sexson, 1999), but has also been extended for the construction of expression vectors for other model organisms (Dudley et al., 2009; Joska, Mashruwala, Boyd, & Belden, 2014; Kilaru & Steinberg, 2015). This method requires the insertion of homology regions by PCR to both the target DNA parts (e.g., gene, markers, etc.) and the linearized backbone vector. The PCR products are then directly transformed in yeast, where the circular vector is assembled by HR. Using this approach, an assembly of up to nine fragments in a 21-kb vector was carried out using 60-bp overlap regions (Kuijpers et al., 2013). Furthermore, this method has been shown to be much more effective for dealing with large DNA fragments than traditional cloning in *E. coli* (Kouprina & Larionov, 2016). For instance, due to instability issues of large DNA constructs in *E. coli*, *in vivo* DNA assembly in yeast was critical for assembling the first synthetic bacterial genomes (Gibson, Benders, Axelrod, et al., 2008; Gibson, Benders, Andrews-Pfannkoch, et al., 2008; Gibson et al., 2010).

1.3.3 Unclonable genes and their expression in yeast

Many gene products, either non-coding RNA or proteins, can be toxic in *E. coli* (Kimelman et al., 2012). Furthermore, some genes are toxic in *E. coli* even in the absence of a bacterial

promoter, pointing to DNA toxicity and/or genetic instability. Examples of known genes that cannot be cloned in *E. coli* include Vssc1 sodium (Lee & Soderlundb, 2009) and Cch1 calcium channels (Vu, Bautos, Hong, & Gelli, 2013), to name a few. These phenomena have been related to intrinsic DNA toxicity (Kouprina & Larionov, 2016).

Most DNA assembly methods require bacterial amplification steps, which restrict its application to genes that can be cloned in the bacterial host without significant toxic effects. In yeast, extrachromosomal expression vectors can be directly assembled by *in vivo* recombination, thereby bypassing bacterial transformation. However, this approach is not applicable to integrative vectors. In this way, the development of cloning-free DNA assembly methods specially designed for the construction of integrative yeast expression cassettes that cannot be cloned remains unexplored.

1.4 Carotenoids

Carotenoids are yellow, orange, or red pigments extensively distributed in nature with highly diversified structures and functions. Carotenoids are present in all plants and algae but also can be found in several archaea, bacteria, and fungi (Alcaíno, Baeza, & Cifuentes, 2016). Chemically, carotenoids share a hydrocarbon backbone composed of 40 carbon atoms formed from eight C_5 isoprenoid units joined head-to-tail. The most characteristic structural feature of carotenoids is the long polyene chain that alternates double and single bonds. This conjugated double bond system imparts the visible-light absorbing properties to carotenoids that give their color and many of their functions. Numerous modifications over this basic structure generate the great diversity of carotenoids observed in nature. Such modifications

include saturation of double bonds, electronic rearrangements, chain shortening or elongation, and introduction of oxygen functional groups (Rodriguez-Amaya, 2016).

Carotenoids can be classified according to their atomic composition. Hydrocarbon carotenoids such as lycopene and β-carotene are called carotenes, while oxygenated carotenoids are denominated xanthophylls. Oxygen in xanthophylls can be presented as hydroxyl (e.g., zeaxanthin), carbonyl (e.g., astaxanthin), and epoxide (e.g., violaxanthin) groups, plus their combinations and substituted derivatives (Bhosale & Bernstein, 2005; Rodriguez-Amaya, 2016).

1.4.1 Biological functions of carotenoids

Carotenoids have a diverse array of biological functions depending on their structure and the organism of origin. In photosynthetic bacteria, algae, and plants, carotenoids are essential in photo-protection and light-harvesting. In their photo-protective role, carotenoids can absorb excess energy from chlorophylls and dissipate it as heat. This mechanism prevents the generation of highly reactive singlet oxygen, impeding harmful oxidation of chlorophylls, lipids, and proteins. Additionally, carotenoids also contribute with energy to power photosynthesis. They act as accessory light-harvesting pigments, absorbing light in 450-550 nm spectral range, and transferring this excitation energy to the chlorophylls. (Hashimoto, Uragami, & Cogdell, 2016).

Carotenoids also play an important role in non-photosynthetic tissues of plants, such as flowers, fruits, and roots. These molecules provide fruits and flowers with attractive colors that promote seed dispersal and pollination by enhancing plant-animal interactions.

Furthermore, the enzymatic cleavage of carotenoids produces odor-active molecules called C_{13}-apocarotenoids, that promote even more fruit and flower attractant properties (Rosas-Saavedra & Stange, 2016). In root and vascular tissues, carotenoids are biosynthetic precursors of the phytohormones abscisic acid and strigolactones, which are involved in abiotic stress response and adapting plant architecture to nutrition availability, respectively (Felemban, Braguy, Zurbriggen, & Al-Babili, 2019).

Carotenoids are also essential for animals. Unlike photosynthetic organisms, animals cannot biosynthesize carotenoids and hence are either directly obtained from food or partly modified by metabolic reactions after the ingest (Maoka, 2020). Carotenoids that contain unsubstituted β-ionone rings such as β-carotene are precursors of retinoids (vitamin A), which are essential to establish and sustain vision in animals (von Lintig, 2012). Interestingly, the pigmentation of some animals as consequence of carotenoid consumption has been related to communicative functions like sex recognition (e.g., birds), masking coloration (e.g., chameleon), or warning coloration (e.g., coral aspid) (Vershinin, 1999). Many marine animals also accumulate and convert dietary carotenoids. For example, crustaceans convert β-carotene into astaxanthin and accumulate it in multiple tissues, providing camouflage and protection against the harmful effects of light (Maoka, 2011, 2020).

1.4.2 Carotenoid biosynthesis

Like other isoprenoids, the universal building block of carotenoids is the 5-carbon compound isopentenyl diphosphate (IPP). Depending on the organism and specific cellular compartment, this precursor can be generated by two different pathways. The mevalonate

(MVA) pathway provides IPP by a condensation of 3 molecules of Acetyl-CoA followed by a reduction, two phosphorylations, and final decarboxylation. MVA pathway operates in most eukaryotes and archaea but has also been found in few bacteria (Miziorko, 2011). By contrast, the alternative methylerythritol 4-phosphate (MEP) pathway employs the glycolytic intermediates D-glyceraldehyde 3-phosphate and pyruvate to generate IPP in seven reactions. MEP pathway is the most ubiquitous route to biosynthesize 5-carbon isoprene units in the bacteria domain (L. Zhao, Chang, Xiao, Liu, & Liu, 2013). Plants and some algae have both MVA and MEP pathways, even though they are confined in different compartments and supplying C_5 units for different biosynthetic routes. Cytosolic MVA pathway in plants sustains biosynthesis of sterols, heme groups, ubiquinone, and dolichols, among others. Conversely, the MEP pathway is localized in plastids, and it is responsible for feeding with isoprene units the biosynthesis of carotenoids, chlorophylls, plastoquinone, and tocopherols (Andrade et al., 2017).

Although the number of enzymes required and the stereochemistry of the intermediates could differ, all carotenogenic organisms have the same universal carotenogenic pathway up to lycopene. In order to generate C_{40} lycopene, it is necessary to condensate eight C_5 isoprene units. First, IPP is isomerized to dimethylallyl diphosphate (DMAPP) in a reaction catalyzed by IPP-isomerase. Then, isoprenyl diphosphate synthases catalyze a head-to-tail sequential condensation of DMAPP with three molecules of IPP, forming geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and finally, geranylgeranyl diphosphate (GGPP, C_{20}). Phytoene, the first C_{40} compound of the pathway, is formed by the condensation of two GGPP molecules by the enzyme phytoene synthase. Finally, after four consecutive desaturations

introduced by phytoene desaturase, phytoene is transformed into lycopene (Rosas-Saavedra & Stange, 2016).

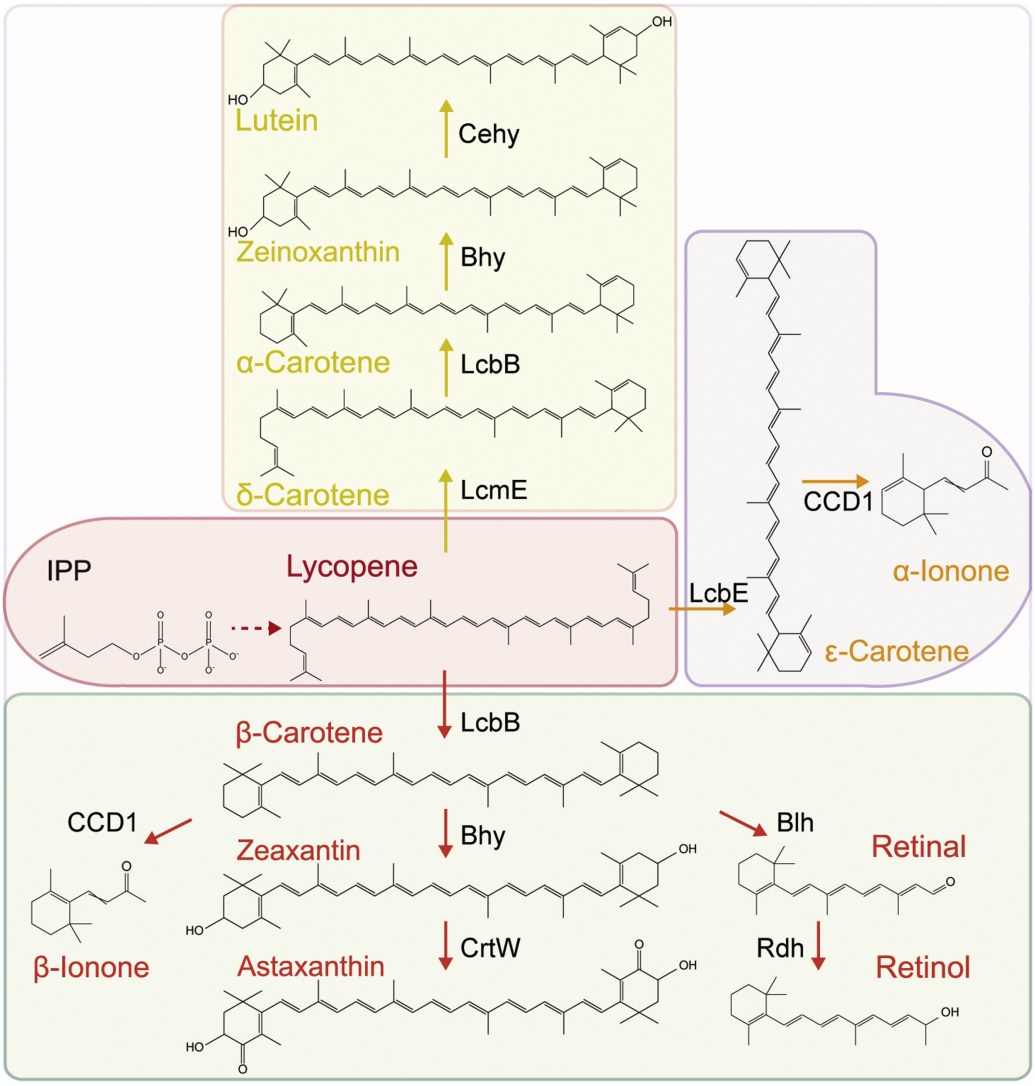

Figure 1. Carotenoid biosynthetic pathway. Lycopene acts as node for the biosynthesis of diverse carotenoids and apocarotenoids. LcbB: lycopene β-cyclase, Bhy: β-carotene hydroxylase, CrtW: β-carotene 4-ketolase, CCD1: carotenoid cleavage dioxygenase 1, Blh: 15, 15 cleavage dioxygenase, Rdh: retinol dehydrogenase, LcmE: lycopene monofunctional ε-cyclase, Cehy: α-hydroxylase, LcbE: lycopene bifunctional ε-cyclase. The dashed arrows indicate multiple steps. (Reprinted from C. Li, Swofford, & Sinskey, 2020).

Lycopene is considered a central node in the carotenogenic pathway because it can be converted into a large variety of carotenoids by subsequent modifications. Figure 1 illustrates lycopene as a precursor and the reactions that yield the most common carotenoids in nature. The cyclization reactions represent an essential branching point in the carotenoid pathway. Each lycopene end can be independently cyclized into cyclohexenyl β- or ε-rings by β- and ε-cyclases, respectively. If both ends of lycopene are β-cyclized, the resulting product is β-carotene. However, if one of the lycopene ends suffers a β-cyclization while the another an ε-cyclization, α-carotene is obtained. The introduction of oxygen groups to these cyclized carotenes yield xanthophylls such as lutein and zeaxanthin (Nisar, Li, Lu, Khin, & Pogson, 2015).

1.4.3 Applications of carotenoids

The attractive color, antioxidative properties, and health benefits of carotenoids have enabled a wide range of applications of these molecules in animal feed, food, cosmetic, and nutraceutical industries.

The development of animal nutrition technologies has optimized feed formulations to offer the required nutrients at the lowest cost. In this scenario, carotenoids are included in feeds to support animal health and improve the appetitive appearance of some products (Langi, Kiokias, Varzakas, & Proestos, 2018). For example, zeaxanthin, lutein, and capsanthin are applied to enhance egg yolks and broiler's skin color. At the same time, astaxanthin is a common additive in farmed salmons to obtain the characteristic pink-red color.

In humans, in addition to their already mentioned function in eye health, retinoids derived from β-carotene are essential to maintain physiological processes such as reproduction, immunity, brain function, and embryonic growth (Meléndez-Martínez, 2019). The xanthophylls zeaxanthin and lutein protect the retina from the photochemical injury of blue light. Their consumption reduces the risk of macular degeneration and improves visual performance (Rodriguez-Concepcion et al., 2018). Additionally, the ingest of several other carotenoids has been associated with numerous health benefits such as improved cognitive performance, reduced risk of cardiovascular disease, skin protection against UV-light, immune modulation, weight management, and cancer prevention (Eggersdorfer & Wyss, 2018). These positive effects on human health have prompted the commercialization of carotenoids as nutraceutical formulations, functional colorants in processed foods, and skin protectors in cosmetics.

1.4.4 Microbial production of carotenoids

Although the manufacturing of carotenoids is still dominated by chemical synthesis, naturally-produced carotenoids are gaining attention and market demand (Barreiro & Barredo, 2018). Despite the wide distribution of these pigments in plants, the low concentration of some carotenoids has hampered the development of economically feasible extraction processes (Dias et al., 2018). In this scenario, microbial production emerges as an efficient alternative to produce natural carotenoids. For instance, several companies are currently producing β-carotene and astaxanthin from the microalgae *Dunaliella salina* and *Haematococcus lacustris*, respectively (Cezare-Gomes et al., 2019). However, carotenoid productivity in microalgae is largely limited by the low specific growth rates of these

photosynthetic organisms (μ = 0.2-1.0 day^{-1}, Cheng et al., 2016; Xu et al., 2018). To overcome these challenges, numerous studies have shown that metabolic engineered bacteria and yeasts are promising carotenoid-producing cell factories (Niu et al., 2017; C. Wang et al., 2019).

As described in the previous section, *S. cerevisiae* has been widely used as a metabolic chassis to produce diverse compounds. Carotenoid production in yeast has been tackled using different metabolic and evolutionary engineering strategies. Yamano, Ishii, Nakagawa, Ikenaga, & Misawa (1994) first demonstrated the capability of *S. cerevisiae* to accumulate lycopene and β-carotene by expressing the biosynthetic genes CrtE, CrtB, CrtI and CrtY from *Pantoea ananatis* (formerly *Erwinia uredovora*). Later, β-carotene-accumulating yeast strains were constructed using the carotenogenic genes CrtE, CrtI, and CrtYB from *Xanthophyllomyces dendrorhous,* together with the overexpression of truncated HMG1 (Verwaal et al., 2007). Then, lycopene, as well as β-carotene production, was improved using controllable promoters (Y. Chen et al., 2016; Xie et al., 2014), enzyme-directed evolution (Xie, Lv, Ye, Zhou, & Yu, 2015), strain adaptative evolution (Reyes, Gomez, & Kao, 2014), lipid-droplets induction (Ma et al., 2019), and strain performance comparison in bioreactors (López et al., 2019). Shi et al. (2019) constructed lycopene-producing strains of *S. cerevisiae* using a systematic optimization strategy. In that work, screening of carotenogenic genes, adjusting of gene copy number, knocking down endogenous genes, and regulating an inducible galactose system. This strategy enabled to reach up to 75 mg/g_{DCW} of lycopene, which is the highest specific yield for carotenoid production in *S. cerevisiae* reported to date. Aside from carotenes, *S. cerevisiae* has also been engineered to accumulate xanthophylls

(oxygen-containing carotenoids), such as astaxanthin (Jin et al., 2018; Ukibe, Hashida, Yoshida, & Takagi, 2009) and zeaxanthin (Shao, Zhao, & Zhao (2009); J. Sun et al., 2012)

Heterologous production of carotenoids has not been restricted to *S. cerevisiae*; other microorganisms such as *E. coli* have also been evaluated as carotenoid production hosts. Early works on carotenoids production in *E. coli* focused on the engineering of the native MEP pathway (S. Kim & Keasling, 2001) and the discovery of knock-out target for increased lycopene production (Alper, Jin, Moxley, & Stephanopoulos, 2005; Alper, Miyaoku, & Stephanopoulos, 2005). More recently, several strategies have been applied to increase carotenogenesis in *E. coli*, including co-expression of the native MEP pathway together with the heterologous MVA pathway (Rad et al., 2012; Yang & Guo, 2014), the introduction of a dynamically controlled MVA pathway (Shen et al., 2016), and increased NADPH and ATP supplies (J. Zhao et al., 2013). The latter work achieved the highest specific yield reported for carotenoid production in *E. coli* (60 mg/g_{DCW} of lycopene).

Another attractive microorganism for heterologous carotenoid production is the oleaginous yeast *Yarrowia lipolytica*. Despite that the use of this yeast in metabolic engineering applications is more recent than *E. coli* and *S. cerevisiae*, it has shown to be a promissory chassis for natural lycopene (Zhang, Nie, Chen, Wei, & Hua, 2019), β-carotene (Gao et al., 2017) and astaxanthin biosynthesis (Kildegaard et al., 2017). Moreover, the randomized combinatorial assembly of each carotenogenic gene with different promoters enabled the accumulation of 90 mg/g_{DCW} of β-carotene in this yeast, corresponding to the highest microbial producer of carotenoids described to date (Larroude et al., 2017).

1.5 Violaxanthin: applications and biosynthesis

Regardless of the successful experiences and knowledge accumulated in the microbial biosynthesis of few carotenoids, many others with interesting potential applications remain unexplored. This is the case of violaxanthin, an epoxidated xanthophyll present in chloroplasts of all vascular plants and some algae (Alcaíno, Baeza, & Cifuentes, 2016b). In these photosynthetic organisms, violaxanthin participates in the so-called violaxanthin cycle, a photoprotective mechanism essential for the dissipation of excess light energy in photosystem II (Goss & Jakob, 2010; Jahns, Latowski, & Strzalka, 2009). Besides the important physiological role of violaxanthin, this epoxycarotenoid has several promising applications. Fu et al. (2011) reported that purified violaxanthin has stronger antioxidative capacity than β-carotene and lutein in three out of four performed assays. In that work, violaxanthin showed the best performance on ABTS radicals scavenging, inhibition of hemolysis induced by H_2O_2, and inhibition of lipid peroxidation. More recent studies confirmed the potent antioxidant capacity of this carotenoid (Araki et al., 2016; F. Wang, Huang, Gao, & Zhang, 2018). Additionally, violaxanthin displayed an antiproliferative effect on human cancer cell lines (Pasquet et al., 2011), anti-inflammatory activity in macrophage cells (Soontornchaiboon, Joo, & Kim, 2012) and photoprotective effects against UVB-irradiated human dermal fibroblasts (H. M. Kim et al., 2019).

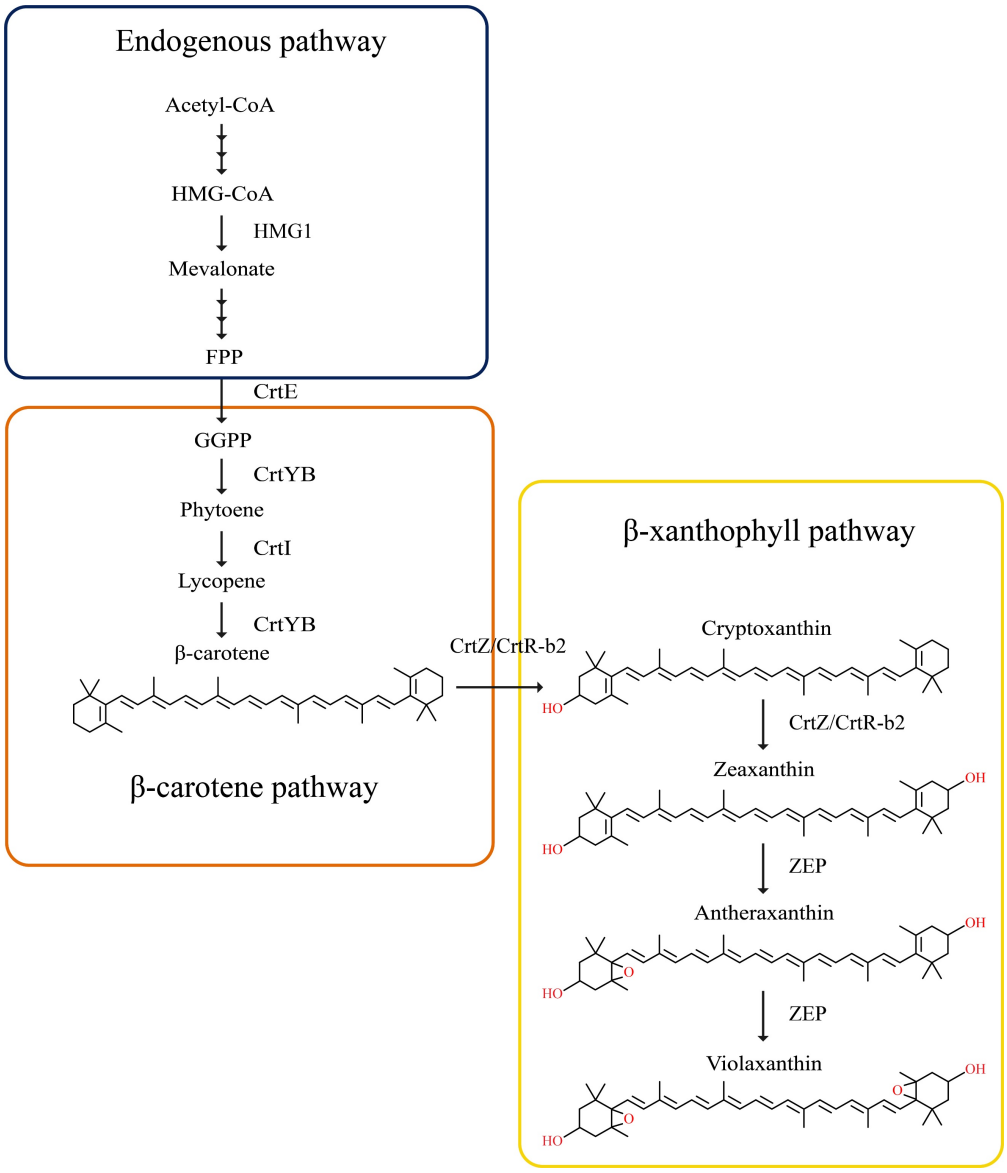

Figure 2. Engineered violaxanthin biosynthetic pathway expressed in *S. cerevisiae*. The endogenous yeast metabolism provides FPP precursors through the mevalonate pathway. Expression of the heterologous genes CrtE, CrtYB, and CrtI yield β-carotene from FPP. The additional integration of the β-xanthophyll pathway genes enables conversion of β-carotene to violaxanthin. The sequential hydroxylation of both rings of β-carotene by CrtZ/CrtR-b2 yields zeaxanthin, which is then further epoxidated in both sides by ZEP to generate violaxanthin. FPP: Farnesyl diphosphate, GGPP: Geranylgeranyl diphosphate, CrtE: Geranylgeranyl diphosphate synthase, CrtYB: Bifunctional lycopene cyclase/phytoene synthase, CrtI: Phytoene desaturase, CrtZ/CrtR-b2: β-carotene hydroxylase, ZEP: Zeaxanthin epoxidase.

Violaxanthin is also the precursor of the valuable natural compounds β-damascenone and fucoxanthin. β-damascenone is a high-value apocarotenoid widely applied in flavors and fragrances industry. Currently, the bulk of β-damascenone is chemically synthesized, and its concentration in natural products is extremely low (Sefton, Skouroumounis, Elsey, & Taylor, 2011). Since the labeling of flavors as natural products are becoming more and more important, the production of β-damascenone by fermentation seems promising (Cataldo, López, Cárcamo, & Agosin, 2016). Natural β-damascenone is currently offered only in 1% and 5% formulations, and the price of a pure product should be around USD 50,000/kg (J. Mulligan, personal communication, August 9, 2017). On the other hand, fucoxanthin is a marine carotenoid with several therapeutic properties, including anti-obesity, anti-diabetes, and anticancer activities (Mikami and Hosokawa, 2013; Z. Wang et al., 2019). This carotenoid is currently extracted from brown algae and commercialized as a nutraceutical. Pure fucoxanthin prices range from USD 40,000 to USD 80,000 per kilogram (AlgaHealth, 2019). Considering that the concentration of fucoxanthin in brown algae is low (172-720 mg/kg_{DW}; Kraan, 2013), microbial biosynthesis could offer a competitive production method.

Violaxanthin is biosynthesized through the β-xanthophyll pathway, which consists of four sequential oxidation steps, starting from β-carotene (Figure 2). First, β-carotene hydroxylases (CrtZ or CrtR-b2) introduce hydroxyl groups in both cyclohexenyl rings of β-carotene to yield zeaxanthin. Further epoxidation of both rings catalyzed by zeaxanthin epoxidases (ZEP) lead to the formation of violaxanthin. Although the pathway is well known, efficient heterologous production of violaxanthin remains elusive. Zhu, Yamamura, Nishihara, Koiwa, & Sandmann (2003) first reported violaxanthin biosynthesis in *E. coli*, even though

no quantification of the xanthophyll was performed. Several other attempts to produce violaxanthin in *E. coli* were unsuccessful (Dambek et al., 2012; Marin et al., 1996; Misawa, 2013). Recently, Takemura et al. (2019) evaluated the expression of several ZEP genes in a zeaxanthin-producing *E. coli* strain, reaching up to 0.23 mg/g_{DCW} of violaxanthin.

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The incorporation of the β-xanthophyll pathway, together with several enzyme and pathway engineering steps, result in an efficient biosynthesis of violaxanthin in the yeast *S. cerevisiae*.

2.2 General objective

The general objective of this thesis is to design, construct, and characterize the first violaxanthin-producing yeast.

2.3 Specific objectives

1. To develop reliable methods for cloning-free assembly and genomic integration of unclonable gene expression cassettes in *S. cerevisiae*.
2. To evaluate β-carotene hydroxylases from different species and their truncated variants for zeaxanthin biosynthesis in yeast.
3. To construct the basal violaxanthin-producing yeast strain by evaluation of zeaxanthin epoxidases from different species and their truncated variants.
4. To assess the impact of different hydroxylase-epoxidase fusion proteins on violaxanthin production.
5. To improve violaxanthin accumulation by co-expression of redox partners and adjustment of gene copy number of β-carotene and β-xanthophyll pathways.
6. To characterize the kinetics of β-xanthophylls accumulation in batch bioreactor fermentations.

3. MATERIALS AND METHODS

3.1 Genes and plasmids

All the heterologous genes used in this study (PaCrtZ, HlCrtZ, CrtR-b2, SlZEP, HlZEP, AtZEP, FD3, RFNR1, CrtE, CrtI and CrtYB) were codon-optimized and synthesized by Genscript (Nanjing, China). Genes coding for *S. cerevisiae* YAH1 and ARH1 were amplified by PCR from genomic DNA of BY4742 strain. Gene sequences can be found in Table A1 (Appendix A).

Yeast integrative expression vectors were constructed by Gibson Assembly (Gibson et al., 2009) using the plasmid library developed by Mikkelsen et al. (2012). Genes and backbone vectors were PCR-amplified by Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, USA). PCR reactions were carried out in 100 µL containing 0.5 pmol/µL of each primer, HF buffer 5x, and 0.02 U/µL of Phusion DNA polymerase. The PCR protocol consisted of an initial denaturation at 98°C for 2 min, then 35 cycles of amplification (98°C for 10 s, 60-65°C for 30 s and 72°C for 3-6 min), followed by a final extension of 72°C for 10 min. The resulting PCR products were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, USA), according to the manufacturer's instructions. Then, purified DNA fragments were mixed with 1.33x Gibson master mix (isothermal buffer, T5 exonuclease 0.005U/µL, Phusion DNA polymerase 0.03U/µL, and Taq DNA ligase 5.3 U/µL) in 10 µL of final volume and incubated for 60 min at 50ºC. The reaction products were transformed in *E. coli* Top10 cells (Thermo Fisher Scientific, USA). Finally, the assembled plasmids were purified using E.Z.N.A plasmid mini Kit (Omega Bio-tek, USA) and verified

by sequencing (Macrogen, South Korea). An example of integrative vector map are shown in Figure 3, and all primers used in this study are listed in Table A2 (Appendix A).

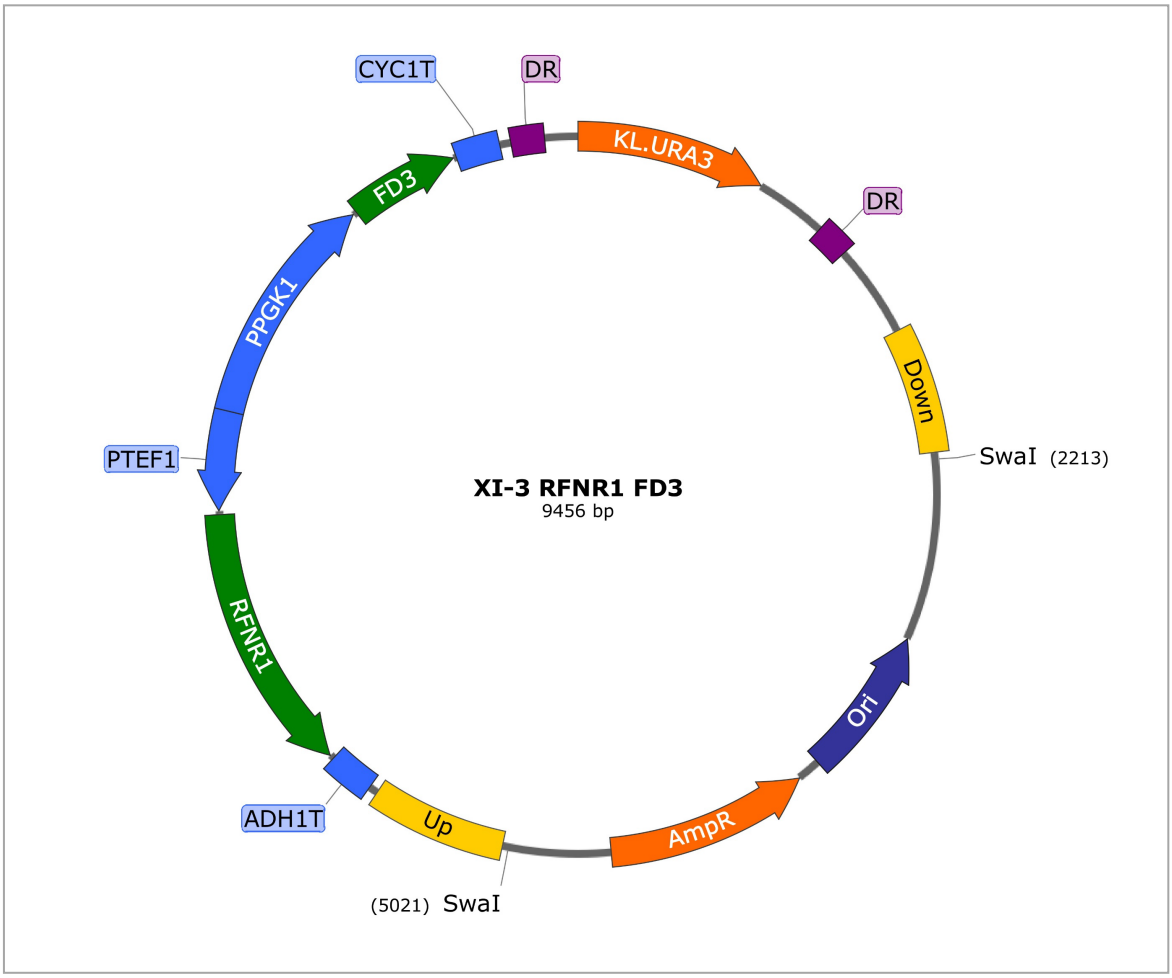

Figure 3. Example of the integrative vectors used in this study. The plasmids have a replication origin (Ori) and ampicillin resistance gene (AmpR) for amplification and analysis in *E. coli*. The yeast expression cassettes of the plasmids consist of a bidirectional constitutive promoter (PTEF1-PPGK1) and two terminators (ADH1T and CYC1T) for the combined expression of two genes. UP and DOWN design the homology regions for the integration in specific chromosomic sites (in the example, site 3 of chromosome XI). KL.URA3 marker enables the selection of the transformants in SC-URA medium, while direct repeats sequences (DR) enables marker recycling for multiple transformation rounds. The plasmids were digested with SwaI restriction enzyme prior to yeast transformation.

3.2 Strain construction

The β-carotenogenic strain SM14-ΔURA3 was used as parent strain for construction of β-xanthophyll-producing strains. The former strain was obtained by targeted deletion of URA3 marker of SM14 strain (López et al., 2019; Reyes et al., 2014). Integrative plasmids were linearized by SwaI digestion (New England Biolabs, USA), and transformed using the LiAc/SS Carrier DNA/PEG method (Gietz, 2014).

Yeast transformants were plated on synthetic complete medium without uracil (SC-URA; 1.8 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 0.8 g/L CSM-Ura mixture, 20 g/L of glucose, and 20 g/L of agar) and incubated for three days at 30ºC. The integrations of plasmids and expression cassettes were verified by genomic PCR. For subsequent strain transformations, URA3 marker was excised by counter-selection in SC plates containing 100 µg/mL of 5-Fluoroorotic acid (5-FOA). All the strains constructed in this study are listed in Table 1.

Table 1. *S. cerevisiae strains used in this thesis.*

Strain	Parent strain	Genotype description	Reference
SM14	FY2	MATα, P_{TDH3}_CrtYB_T_{CYC1} P_{TDH3}_CrtI_T_{CYC1} P_{TDH3}_CrtE_T_{CYC1} ΔCTT1	Reyes et al. (2014)
SM14-ΔURA3	SM14	ΔURA3	López et al. (2019)
PaCrtZ	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1}	This study
HlCrtZ	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_HlCrtZ_T_{ADH1}	This study
tr-HlCrtZ	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_tr69-HlCrtZ_T_{ADH1}	This study
CrtR-b2	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_CrtR-b2_T_{ADH1}	This study
tr-CrtR-b2	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_tr57-CrtR-b2_T_{ADH1}	This study
SlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} $_{PGK1}$_SlZEP_T_{CYC1}	This study
tr25-SlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr25-SlZEP_T_{CYC1}	This study
tr49-SlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr49-SlZEP_T_{CYC1}	This study
tr75-SlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr75-SlZEP_T_{CYC1}	This study
tr100-SlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr100-SlZEP_T_{CYC1}	This study
HlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_HlZEP_T_{CYC1}	This study

tr30-HlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr30-HlZEP_T_{CYC1}	This study
tr59-HlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr59-HlZEP_T_{CYC1}	This study
tr80-HlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr80-HlZEP_T_{CYC1}	This study
tr100-HlZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr100-HlZEP_T_{CYC1}	This study
AtZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_AtZEP_T_{CYC1}	This study
tr30-AtZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr30-AtZEP_T_{CYC1}	This study
tr57-AtZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr57-AtZEP_T_{CYC1}	This study
tr80-AtZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr80-AtZEP_T_{CYC1}	This study
tr100-AtZEP	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ_T_{ADH1} P_{PGK1}_tr100-AtZEP_T_{CYC1}	This study
CrtZ-R-ZEP [a]	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ-R-tr59-HlZEP_T_{ADH1}	This study
CrtZ-F-ZEP [b]	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_PaCrtZ-F-tr59-HlZEP_T_{ADH1}	This study
ZEP-R-CrtZ [a]	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_tr59-HlZEP-R-PaCrtZ_T_{ADH1}	This study
ZEP-F-CrtZ [b]	SM14-ΔURA3	XI-5Δ:: P_{TEF1}_tr59-HlZEP-F-PaCrtZ_T_{ADH1}	This study

tr25-ARH1/tr-YAH1	tr59-HlZEP	XI-3Δ:: P_{TEF1}_tr25-ARH1_T_{ADH1} P_{PGK1}_tr32-YAH1_T_{CYC1}	This study
tr50-ARH1/tr-YAH1	tr59-HlZEP	XI-3Δ:: P_{TEF1}_tr50-ARH1_T_{ADH1} P_{PGK1}_tr32-YAH1_T_{CYC1}	This study
RFNR1/FD3	tr59-HlZEP	XI-3Δ:: P_{TEF1}_RFNR1_T_{ADH1} P_{PGK1}_FD3_T_{CYC1}	This study
RFNR1/tr-FD3	tr59-HlZEP	XI-3Δ:: P_{TEF1}_RFNR1_T_{ADH} P_{PGK1}_tr49-FD3_T_{CYC1}	This study
tr-RFNR1/tr-FD3	tr59-HlZEP	XI-3Δ:: P_{TEF1}_tr65-RFNR1_T_{ADH1} P_{PGK1}_tr49-FD3_T_{CYC1}	This study
tr-FD3	tr59-HlZEP	XI-3Δ:: P_{PGK1}_tr49-FD3_T_{CYC1}	This study
tr-RFNR1	tr59-HlZEP	XI-3Δ:: P_{TEF1}-tr65-RFNR1-T_{ADH1}	This study
+CrtE	tr-RFNR1/tr-FD3	X-2Δ:: P_{PGK1}_CrtE_T_{CYC1}	This study
+CrtYB	tr-RFNR1/tr-FD3	X-2Δ:: P_{PGK1}_CrtYB_T_{CYC1}	This study
+CrtI	tr-RFNR1/tr-FD3	X-2Δ:: P_{PGK1}_CrtI_T_{CYC1}	This study
+YB/tr59-HlZEP	tr-RFNR1/tr-FD3	X-2Δ:: P_{TEF1}_CrtYB_T_{ADH1} P_{PGK1}_tr59- HlZEP_T_{CYC1}	This study
+YB/2 tr59-HlZEP	+YB/tr59-HlZEP	XI-2Δ:: P_{PGK1}_tr59-HlZEP_T_{CYC1}	This study
SM14 + CrtI	SM14-ΔURA3	X-2Δ:: P_{PGK1}_CrtI_T_{CYC1}	This study

[a] R: rigid linker, sequence: (EAAAK)3

[b] F: flexible linker, sequence: $(GGGGS)_3$

3.3 Cloning-free methods for the assembly and integration of ZEP expression cassettes

ZEP-containing plasmids showed high toxicity in *E. coli* and were unclonable by traditional Gibson assembly. To overcome these issues, we developed two cloning-free methods for the assembly and genomic integration of ZEP expression cassettes, named Full *in vitro* Gibson assembly and direct assembly by homologous recombination.

For Full *in vitro* Gibson assembly, 4 μL of the Gibson reaction products (section 3.1) were used as PCR templates where the UP-F and DOWN-R primers were employed for the amplification of the UP-DOWN cassettes. These PCR products were digested with 5 U of DpnI for 1 h to eliminate the residual parental vector. The resulting PCR product (~1.2 pmol in 100 μL) was directly used for yeast transformation.

Direct assembly by homologous recombination was performed using three PCR-amplified fragments (F1, F2, and F3). To generate each set of fragments, six primers were designed: UP-F, UP-R, DOWN-F, DOWN-R, ZEP-F, and ZEP-R. Overlapping regions between fragments were included in the 5' sequence of the primers (exemplified in Figure 4). To evaluate the effect of the overlap length on the assembly efficiency, three sets of primers were designed for each HlZEP and SlZEP expression cassette with overlap lengths of 40, 60 and 100 bp. Transformations were performed using 3 pmol of each fragment mixed to a 100-μL final volume. Primer sequences are listed in Table A2 (Appendix A).

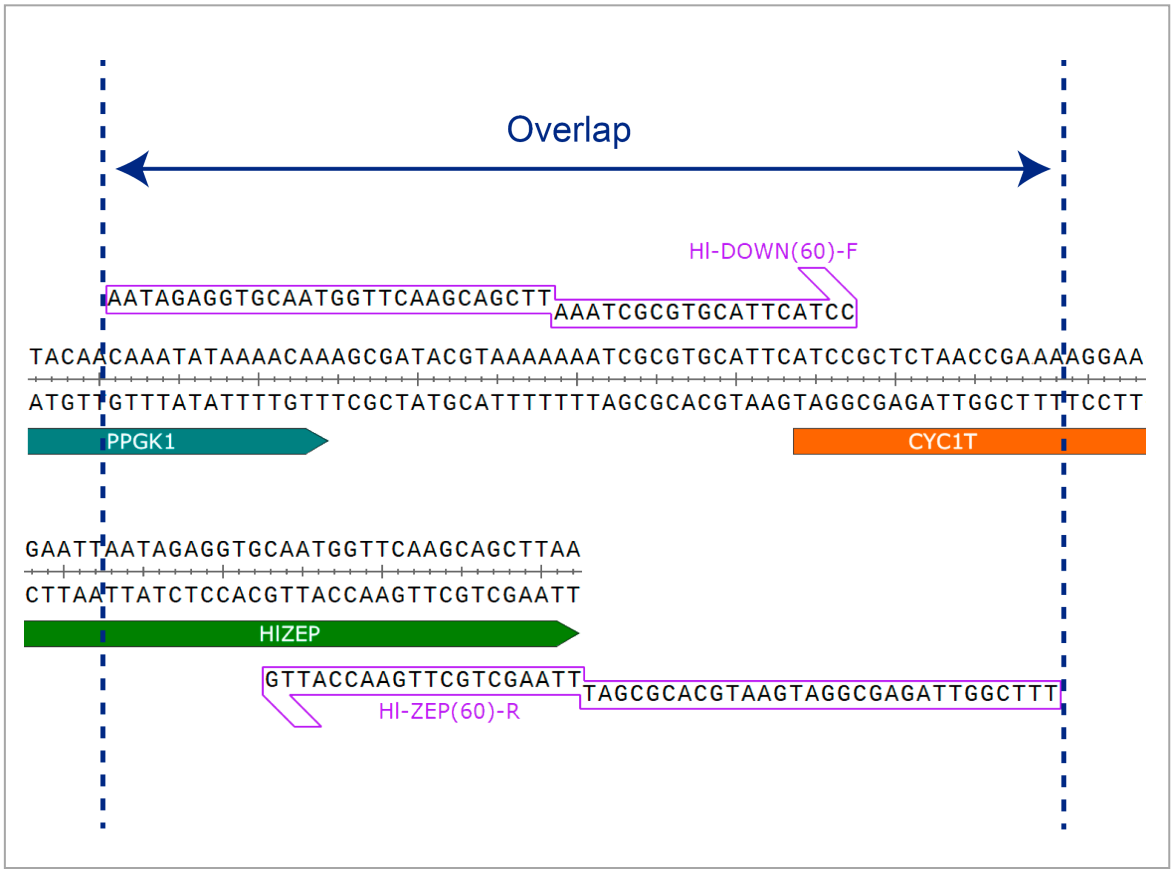

Figure 4. Illustration of primer design for generation of overlapping fragments. The homology region between fragments is included in the 5' non-priming sequences of the primers.

To evaluate the integration efficiency of each method, 20 colonies (10 for HlZEP and 10 for SlZEP) were individually picked and cultured for 16 h at 30°C in liquid YPD media. Genomic DNAs were then extracted using Wizard Genomic DNA Purification Kit (Promega, USA). Confirmation of correct chromosomal integration of the assembled expression cassettes was carried out by PCR amplification of the previously extracted DNA. Four PCR rounds were performed on each strain for efficiency analysis: one that amplified the entire assembled cassette from the UP to DOWN region, and three that amplified between the recombination regions of each fragment. PCR reactions were carried out with Phusion High-

Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) using the same cycling parameters described in section 3.1.

3.4 Shake flask cultures

Single colonies were picked from agar plates and inoculated in tubes containing 3 mL of YPD medium (1% yeast extract, 2% peptone and 2% glucose) and pre-cultured overnight at 30°C and 160 rpm in an orbital shaker incubator. For shakes flask cultures, precultures were inoculated into 100 mL baffled shake flasks containing 20 mL of YPD at an initial optical density (OD_{600}) of 0.1, and grown under the same conditions for 48 h. Biomass concentrations were determined by OD_{600} in a Genesys 20 spectrophotometer (Thermofisher, USA), using the linear relationship of 1 OD_{600} = 0.4 g_{DCW}/L determined experimentally.

3.5 Batch fermentations in bioreactors

Batch fermentations were carried out in 1 L in-house bioreactors controlled by SIMATIC PCS7 system (Siemens, Germany), and equipped with a condenser, a stirrer, and two Rushton turbines, operated with brushless DC motors (Oriental Motor, Japan). The dissolved oxygen concentration and the temperature were measured with an Oxymax COS22D electrode (Endress+Hauser, Switzerland), and the pH with a Memosens CPS76D electrode (Endress+Hauser, Switzerland). The fermentations were performed in YPD medium (2% glucose), at 30°C, pH 5.0, and dissolved oxygen concentration above 4.0 mg/L. The latter was maintained with a split-range control scheme varying the agitation, air and pure oxygen gas flows (Cárcamo et al., 2014). As the oxygen demand increases, the control system first

increases the agitation from 200 to 400 rpm, then the air flow from 0.3 to 0.6 L/min, and finally, if needed, pure oxygen gas flow from 0 to 0.5 L/min.

Culture samples were collected every 2 h for biomass, carotenoids, and extracellular metabolites quantification. Extracellular glucose and ethanol concentrations were quantified in duplicate by HPLC, as detailed in Sánchez, Pérez-Correa, & Agosin (2014).

3.6 Carotenoid extraction

For each sample, 32 mg of biomass were centrifuged, and the resulting supernatant was discarded. Carotenoid extraction was conducted by sequential homogenization of the cells in hexane and ethanol. First, the cells were mixed with 400 μL of 0.5 mm glass beads (Sigma-Aldrich, USA) and 1 mL of hexane, and lysed for 8 min, using a Genie cell disruptor (Scientific Industries, USA). The disrupted mixture was centrifuged, and the supernatant was saved in a 1.5 mL microcentrifuge tube. The resulting pellet was subjected to a second extraction with 1 mL of ethanol, using the same disruption program. Both hexane and ethanol extractions were repeated until a white pellet was obtained (usually a total of 6 extractions were needed). The supernatant fractions were evaporated in a HyperVAC-MAX centrifugal vacuum evaporator (Gyrozen, South Korea) at 65°C and 1000 rpm. Finally, all carotenoids dry fractions were resuspended and mixed in a total of 1 mL of acetone and centrifuged at 20000×*g* to eliminate insoluble material. The acetone supernatant phase was analyzed by spectrophotometry, HPLC-DAD and/or UPLC-MS.

3.7 Carotenoid analysis

Total carotenoids were quantified by absorbance at 450 nm, using a Genesys 20 spectrophotometer (Thermofisher, USA). Total carotenoid concentration was calculated using a calibration curve from 0.5 to 5 mg/L of β-carotene (for SM14 strain) or zeaxanthin (for xanthophyll-accumulating strains) standards (Carotenature, Switzerland). Individual carotenoid identification and quantification were performed in a HPLC-DAD Merck-Hitachi LaChrome L7250 system (Merck, Germany) equipped with a C30 reverse-phase column (YMC, Japan). Two mobile phases were used: mobile phase A consisted of acetonitrile:methanol:2-propanol:acetone (50:30:20:2 v/v); and mobile phase B contained 2-propanol:acetone (10:1 v/v). The elution gradient was as follows (min-%A): 0-100; 10-100; 25-45; 27-45; 30-100; 40-100, with a flow rate of 1.5 mL/min. The column temperature was 35ºC and the detection was performed at 450 nm. Each carotenoid concentration was determined using calibration curves from 5 to 50 mg/L of β-carotene, cryptoxanthin, zeaxanthin, antheraxanthin and violaxanthin standards (Carotenature, Switzerland). The concentration of unidentified carotenoids was estimated using the mean response factor from identified carotenoids.

UPLC-MS analyses were performed in a Dionex Ultimate 3000 system (Thermo Fisher Scientific, USA) coupled to an Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) equipped with electrospray ionization (ESI), using the same column and elution program described above. The mass spectra parameters were as follow: positive polarity, scan range from 400 to 1000 m/z, resolution of 140000, sheath gas flow of 60 AU,

auxiliary gas flow of 40 AU, sweep gas flow of 0 AU, spray voltage of 7 kV, capillary temperature of 350°C, auxiliary gas temperature of 300°C and S-lens RF level of 100.

3.8 Bioinformatic and statistical analysis

To design truncated variants of enzymes, putative cleavage sites of plastid transit peptides of plant and algal enzymes were determined analyzing the full protein sequences with ChloroP1.1 server (Emanuelsson, Nielsen, & Heijne, 1999). Consensus transmembrane regions of the enzymes were predicted by TOPCONS server (Tsirigos, Peters, Shu, Käll, & Elofsson, 2015).

Shake flask cultures data were obtained by three independent experiments from three different colonies and are presented as mean ± standard deviation. The data were compared by unpaired Student's t-test and were considered significantly different with values of $p<0.05$.

4. RESULTS AND DISCUSSION

4.1 Genomic integration of unclonable gene expression cassettes using rapid cloning-free workflows

ZEP genes are required for the construction of β-xanthophyll pathway and violaxanthin biosynthesis (Figure 2). Unfortunately, we could not clone HlZEP and SlZEP genes in yeast integrative vectors by traditional Gibson assembly regardless of the *E. coli* strain evaluated (TOP10, DH5α, and K12). In all cases, we obtained few colonies per plate after transforming the Gibson product, and after colony PCR verification, most of them did not contain the ZEP genes. However, we were able to detect a few colonies positive for ZEP genes, which were used for plasmid isolation and sequence analysis (Figure 5). Plasmid sequences revealed that *E. coli* transformants generated large deletions in the vectors, removing the ZEP genes while retaining the ampicillin resistance (Figure 5A). Another observed mechanism of adaptation was the introduction of multiple mutations in some portions of ZEP genes (Figure 5B). These results indicate that although some transformants can initially incorporate ZEP-containing plasmids, after liquid cultures for plasmid amplification, these transformants deleted or mutated the ZEP genes. In other words, ZEP genes showed high toxicity and cannot be maintained and amplified in *E. coli.*

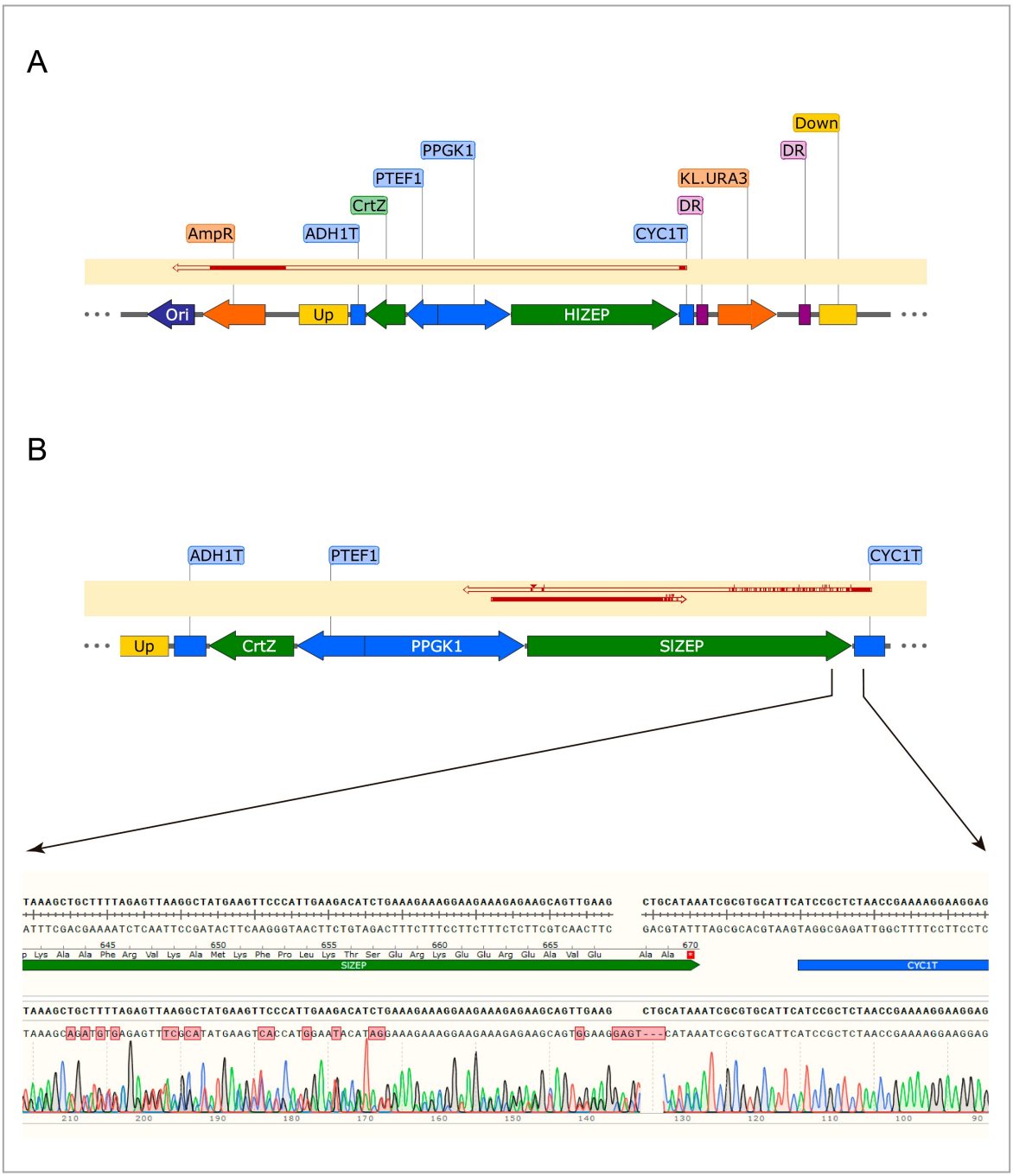

Figure 5. DNA Sequence analysis of ZEP-containing plasmids isolated from *E. coli* transformants. The plasmids were sequenced using primers that annealed in PGK1 promoter and CYC1 terminator. Red regions represent the sequenced regions that align with the reference sequence. **(A)** Large deletion of the whole expression cassette with retention of the ampicillin resistance. PGK1 did not anneal, confirming the deletion. **(B)** Multiple mutations introduced into ZEP by the transformants. The mixed peaks into the ZEP region indicate a heterogeneous population of plasmids.

Several non-coding RNA or proteins can be toxic in *E. coli* (Kimelman et al., 2012). Although in this study ZEP genes were cloned under the control of yeast PGK1 promoter, some eukaryotic promoters can still drive gene expression in *E. coli* (Antonucci, Wen, & Rutter, 1989; Gognies, Bahkali, Moslem, & Belarbi, 2012). However, we were not able to clone ZEP even in the absence of a promoter. Then, a plausible cause of ZEP toxicity is related to the toxicity of the DNA itself (Kouprina & Larionov, 2016). Unclonable non-transcribed DNA regions have been suggested to exert such effect, but the underpinning molecular mechanisms have not been fully elucidated. For instance, some cloned sequences can seemingly cause toxicity due to their high capacity to recruit and titrate essential DNA binding proteins such as replicator initiator DnaA (Kimelman et al., 2012) or RNA polymerase (Lamberte et al., 2017).

Since we cannot assemble ZEP genes into yeast integrative plasmids by traditional cloning methods, we developed two methods that enable assembly and chromosomal integration of expression constructs in yeast without the need of bacterial transformation. As illustrated in Figure 6, the ZEP genes were assembled into expression cassettes using the vector XI-5 as a backbone (i.e., integration in site 5 of chromosome XI) by two different strategies: 1) full *in vitro* Gibson assembly, and 2) direct assembly by HR. Both methods are PCR-based and do not need bacterial transformation nor plasmid isolation steps.

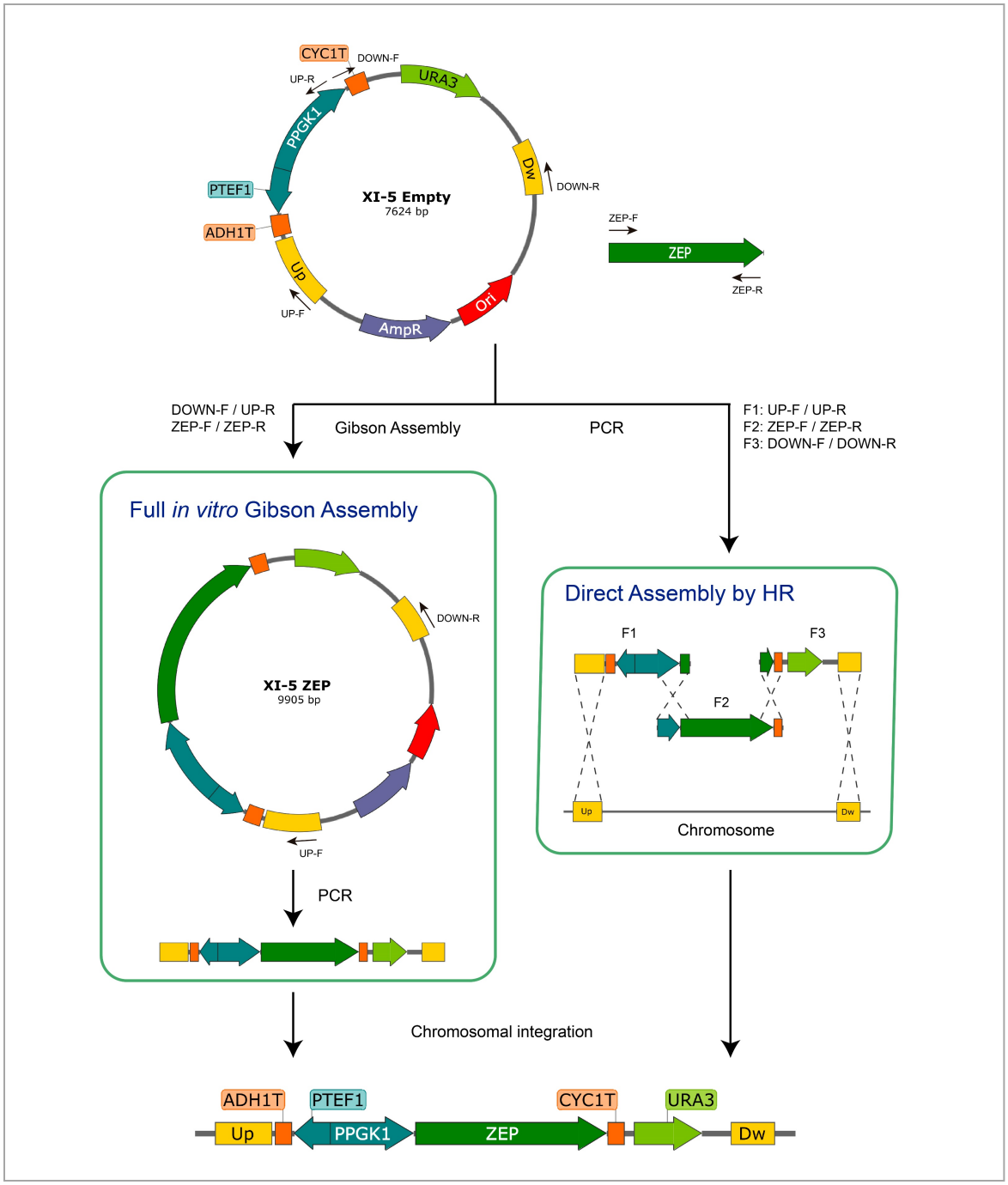

Figure 6. Schematic overview of cloning-free methods for assembly and integration of expression cassettes exemplified for ZEP expression constructs. Full *in vitro* Gibson assembly bypasses bacterial amplification using the reaction product as a template for PCR amplification of the desired integration cassette. Direct assembly by HR is based on the transformation of linear overlapping PCR products which are assembled and integrated into the genome in a single transformation event. The primers used in each method are indicated next to the arrows. F1, F2, and F3 refer to fragments 1, 2, and 3.

4.1.1 Full *in vitro* Gibson assembly

Gibson assembly requires one reaction to join DNA fragments into a vector. Typically, the reaction product is transformed and amplified in *E. coli*. For shuttle integrative vectors in yeast (exemplified in Figure 3), additional digestion with SwaI and gel purification are necessary steps for isolating the desired integrating DNA fragment and discarding bacterial elements (Ori and AmpR). Here, we propose a simple modification of this protocol, where the Gibson assembly product is used as DNA template in a PCR reaction that amplifies only the segment that will be integrated into the yeast genome (Figure 6). This PCR reaction can be transformed directly after digestion with Dpn1 (to eliminate the parental vector), without subsequent clean-up steps. Thus, both the *assembly and amplification* of the Gibson-assembly product occur *in vitro*, as opposed to the conventional Gibson method where the assembly takes place *in vitro* and the amplification occurs in *E. coli*.

This method was applied to integrate HlZEP and SlZEP expression cassettes in yeast. Resulting colonies were screened for successful integration by genomic PCR with a set of primers that amplified three segments and the whole integrated construct (Figure 7). As shown in Table 2, a 95% chromosomal integration efficiency was achieved for HlZEP and SlZEP expression cassettes, slightly less than that for SwaI digestion of the empty vector (100%). Notably, this method is likely limited by the length of the integrating fragment that can be amplified by high-fidelity polymerases. However, for integrative cassettes of one or two genes (5-8 kb), one PCR reaction using high-fidelity polymerases can easily render the required DNA amount (1 pmol) for efficient yeast transformation.

4.1.2 Direct assembly by homologous recombination (HR)

Shao et al. (2009) evaluated and demonstrated a high capacity of yeast for assembling and integrating functional expression constructs from PCR-amplified fragments in a single transformation event. Based on the recombination scheme proposed by Shao et al. (2009), we developed a direct *in vivo* DNA assembly and site-specific integration method from linear PCR products. Three overlapping fragments were generated by PCR using the backbone vector and the gene of interest as templates (Figure 6). Similar to Gibson assembly, primers were designed with a non-priming sequence at the 5' end that is homologous to the 5' end of the fragment to join (exemplified in Figure 4). All fragments were co-transformed in yeast, which assembled and ultimately integrated the construct by HR. To evaluate the effect of the overlap length on the assembly, we transformed each HlZEP and SlZEP construct with a set of fragments with 40, 60 and 100 bp of homology. Correct assembly and integration were verified by genomic PCR analysis of transformants using primers that annealed specifically in the overlap regions (Figure 7). Examples of the analysis of more colonies can be found in Figure B1 (Appendix B).

The overlap length had a strong positive effect on the assembly and integration efficiency, reaching up to 85% efficiency when a 100 bp overlap segment was employed (Table 2). Notably, direct assembly by HR requires only one PCR round (full *in vitro* Gibson assembly requires two), and thus can be readily performed in a day.

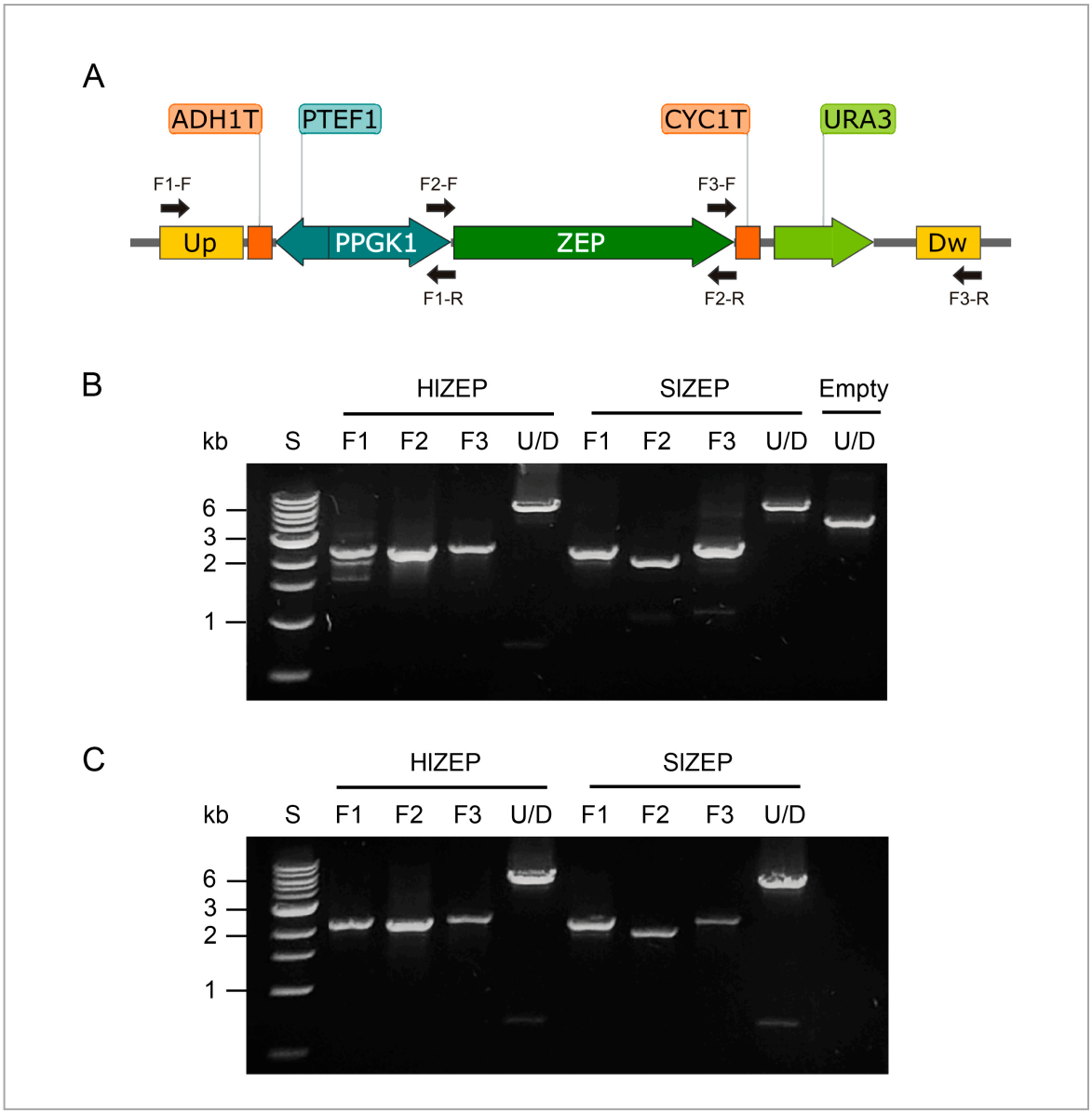

Figure 7. Assembly and integration of ZEP expression cassettes. **(A)** Scheme of assembled ZEP expression cassette with the corresponding verification primers. Genomic PCR analysis of the integrated HlZEP and SlZEP constructs for full *in vitro* Gibson assembly **(B)** and direct assembly by HR **(C)**. S: 1 kb DNA ladder. F1, F2, and F3 refer to Fragments 1, 2, and 3, and U/D represents the UP/DOWN region. Empty refers to the backbone vector linearized by SwaI digestion. Expected PCR products (bp): Hl-F1 (2364), Hl-F2 (2300), Hl-F3 (2428), Hl-U/D (7043), Sl-F1 (2371), Sl-F2 (2041), Sl-F3 (2428), Sl-U/D (6791), Empty-U/D (4781).

Table 2. Integration efficiencies of assembled ZEP expression constructs based on PCR analysis of genomic DNA of 20 colonies (10 for HlZEP and 10 for SlZEP).

Method	Integration efficiency (%)
Digested empty vector	100
Full *in vitro* Gibson assembly	95
Direct assembly by HR - 40 bp overlap	20
Direct assembly by HR - 60 bp overlap	50
Direct assembly by HR -100 bp overlap	85

The proposed methodology for direct assembly and integration of expression cassettes by HR was adapted from the so-called DNA assembler method presented by Shao et al. (2009), with the incorporation of some important features for streamlining the workflow. In the DNA assembler method, promoter-gene-terminator units are assembled by OE-PCR, while the helper fragment – which contains the marker and integration site – is obtained by enzyme digestion. Since all these elements are included in the backbone vector in this method, additional *in vitro* steps aside of the PCR amplification of the designed fragments are unnecessary. Moreover, here we demonstrated effective *in vivo* assembly with single specific integration site, instead of repeated δ sites as in Shao et al. (2009). The proposed method avoids multiple integrations events that usually occur in δ sites (Sakai, Shimizu, & Hishinuma, 1990; X. Wang, Wang, & Da Silva, 1996), allowing finer control of the gene copy number. Finally, instead of single homology arm integration, we proposed a double

crossing-over configuration, which avoids direct repeats sequences and increases the genomic stability of the construct (Gnügge & Rudolf, 2017; Taxis & Knop, 2006).

As mentioned earlier, yeast recombination cloning has been extensively reported for the construction of yeast extrachromosomal vectors or plasmids for other species. However, this approach is futile when the goal is to integrate expression cassettes, as extrachromosomal replicating elements (CEN/ARS, 2μ) prevent chromosomal integration. To overcome this obstacle, Chou, Patel, & Gartenberg (2015) constructed a series of conditional shuttle vectors where the CEN/ARS elements were flanked by loxP sites. This feature enabled elimination of the replicating sequences when the vectors were transformed in Cre recombinase-expressing bacteria. In this way, the plasmid can be assembled extrachromosomally in yeast and converted to an integrative vector in bacteria, which can be then used to transform the yeast again. This time-consuming cloning strategy requires several transformations and plasmid isolation steps. In contrast, the direct assembly by HR proposed here simplifies the assembly and integration of expression cassettes to only few simple steps.

4.2 Evaluation of different β-carotene hydroxylases for zeaxanthin biosynthesis

β-carotene is the starting metabolite for the biosynthesis of β-xanthophylls. It is first hydroxylated in both rings by β-carotene hydroxylases to yield zeaxanthin through the mono-hydroxylated intermediate cryptoxanthin (Figure 2). Since zeaxanthin is the precursor of epoxidated carotenoids, we evaluated the performance of β-carotene hydroxylases from different species in the β-carotenogenic yeast strain SM14. This strain was previously obtained by adaptative laboratory evolution to hyperaccumulate β-carotene in response to oxidative stress by oxygen peroxide (Reyes et al., 2014).

Two major classes of carotenoid hydroxylases are known: heme-containing cytochrome P450 hydroxylases and non-heme di-iron hydroxylases (Martín, Gudiña, & Barredo, 2008). The former family is involved primarily in β- and ε-ring hydroxylation of α-carotene; whereas the latter group catalyzes the hydroxylation of both β-rings in β-carotene (J. Kim, Smith, Tian, & DellaPenna, 2009). Given that our target molecule requires a double hydroxylation of β-carotene, we focused exclusively on non-heme di-iron hydroxylases. We compared the performance of β-carotene hydroxylases from a broad phylogenetic spectrum, from bacteria (*Pantoea ananatis*, PaCrtz) to algae (*Haematoccocus lacustris*, HlCrtZ) and plants (*Solanum lycopersicum*, CrtR-b2). Considering that algal and plant β-carotene hydroxylases are plastid-targeted enzymes (T. Sun et al., 2018), the impact of the N-terminal truncation of the predicted transit peptide in zeaxanthin biosynthesis was evaluated.

After 48 h of shake flask cultivation, carotenoids were extracted and quantified. The parent strain SM14 accumulated 15.3 mg/g_{DCW} of total carotenoids (Table A3, Appendix A) and, as

expected, it was mainly β-carotene (89.2 % of total carotenoids, Figure 8). The expression of β-carotene hydroxylases in the SM14 strain led to an accumulation of hydroxylated carotenoids, albeit with a 40% reduction in total carotenoid yield (from 15.3 to mean 9.1 $mg/g_{DCW,}$ Table A3, Appendix A). No significant differences were observed in total carotenoid yields between strains expressing different β-carotene hydroxylases. However, the strains displayed distinct carotenoid composition (percent of total carotenoids, Figure 8). PaCrtZ strain showed the best hydroxylating performance, accumulating 75% of zeaxanthin (6.9 mg/g_{DCW}) and 7% of the intermediate cryptoxanthin. In contrast, *S. lycopersicum* (CrtR-b2) and *H. lacustris* (HlCrtZ) hydroxylases exhibited lower activities, reflected in higher β-carotene and cryptoxanthin accumulation. Interestingly, N-truncation of predicted transit peptides (tr-CrtR-b2 and tr-HlCrtZ) incremented the *in vivo* activity of both, algal and plant enzymes, increasing zeaxanthin production by 132% and 37%, respectively.

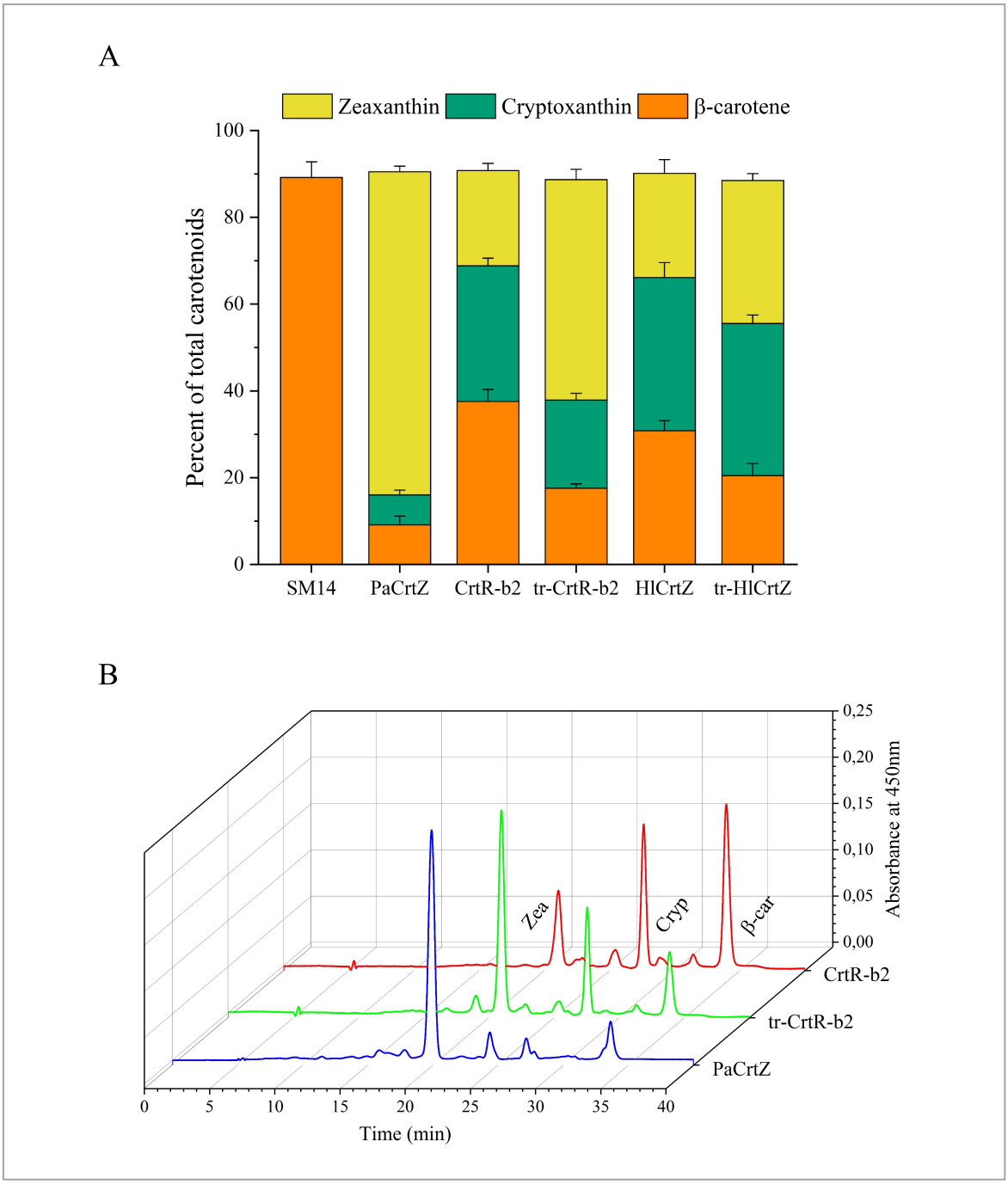

Figure 8. Evaluation of different β-carotene hydroxylases for zeaxanthin biosynthesis. **(A)** Carotenoids content in strains expressing β-carotene hydroxylases from *P. ananatis* (PaCrtZ), S. *lycopersicum* (CrtR-b2) and *H. lacustris* (HlCrtZ). The missing percentage corresponds to unidentified carotenoids. Error bars represent standard deviation from triplicate experiments. **(B)** HPLC elution profiles of the different strains. tr: truncated at predicted transit peptide. SM14: β-carotenogenic parent strain.

Previous studies have shown that bacterial CrtZs effectively transform β-carotene to zeaxanthin with minimal accumulation of intermediates (Choi, Matsuda, Hoshino, Peng, & Misawa, 2006; X. Li, Tian, Shen, & Liu, 2015). On the other hand, high cryptoxanthin accumulation was reported when β-carotene hydroxylases from different algal and plant species are expressed in a β-carotenogenic *E. coli* strain (Galpaz, Ronen, Khalfa, Zamir, & Hirschberg, 2006; Y. K. Kim et al., 2013; Linden, 1999; Qin, Zhang, Dubcovsky, & Tian, 2012). This evidence agrees with our results, suggesting that bacterial β-carotene hydroxylases perform better than eukaryotic counterparts for zeaxanthin biosynthesis in both *E. coli* and *S. cerevisiae*. The low hydroxylation yields observed for heterologously expressed plant β-carotene hydroxylases could be explained by the dependence of these enzymes on ferredoxin to obtain reducing equivalents (Bouvier, Keller, D'Harlingue, & Camara, 1998). In the case of bacterial CrtZ, electrons donors have not yet been described, but our results indicate that reducing power can be obtained from the endogenous yeast metabolism. Consequently, PaCrtZ was chosen for further violaxanthin strain construction.

4.3 Evaluation of different zeaxanthin epoxidases for violaxanthin production

Violaxanthin is produced by sequential introduction of 5,6-epoxy groups in the two rings of zeaxanthin, via the mono-epoxidated intermediate antheraxanthin (Figure 2). The reaction is catalyzed by zeaxanthin epoxidase (ZEP) and requires molecular oxygen and reducing equivalents (Büch, Stransky, & Hager, 1995; Siefermann & Yamamoto, 1975). Like other carotenogenic enzymes, ZEPs also have plastid transit peptides and have been found associated to thylakoid membranes (Schaller, Wilhelm, Strzałka, & Goss, 2012). To obtain the first violaxanthin-producing strains, we constructed the corresponding β-xanthophyll

pathway by co-expression of PaCrtZ and ZEPs in the β-carotenogenic strain SM14. We evaluated the epoxidating performance of ZEPs from *H. lacustris* (HlZEP), *A. thaliana* (AtZEP), and *S. lycopersicum* (SlZEP). Additional to the full-length enzymes, we also evaluated four truncated variants of each enzyme, including truncation at the position of the predicted transit peptides.

Expression of different ZEP genes greatly altered carotenoid composition (percent of total carotenoids, Figure 9), with no significant changes in total carotenoid yields (mean 9.3 mg/gDCW, Table A3, Appendix A). Overall, HlZEP strains displayed higher epoxycarotenoids accumulation (violaxanthin and antheraxanthin) than SlZEP and AtZEP strains (Figure 9). The N-terminal truncation dramatically affected the epoxidating activity of all tested ZEPs. Both AtZEP and SlZEP untruncated variants were inactive and required truncation of at least 25-30 residues to epoxidate zeaxanthin. In the case of HlZEP, the full-length enzyme was active, but truncated counterparts showed up to 4-fold increase in violaxanthin yields. Interestingly, truncation at residue 100 abolished the catalytic activity of the three studied ZEPs, while enzymes truncated at residues 25-80 kept their activity. The higher violaxanthin-producing strains were tr30-HlZEP and tr59-HlZEP (predicted transit peptide), with no significant difference between them. The latter strain produced 17.6% of violaxanthin and 10.9% of antheraxanthin, equivalent to 1.6 and 1.0 mg/g_{DCW}, respectively. Additionally to HPLC-DAD analyses, violaxanthin and antheraxanthin identities in tr59-HlZEP strain were confirmed by UPLC-MS (Figure B2 and Figure B3, Appendix B).

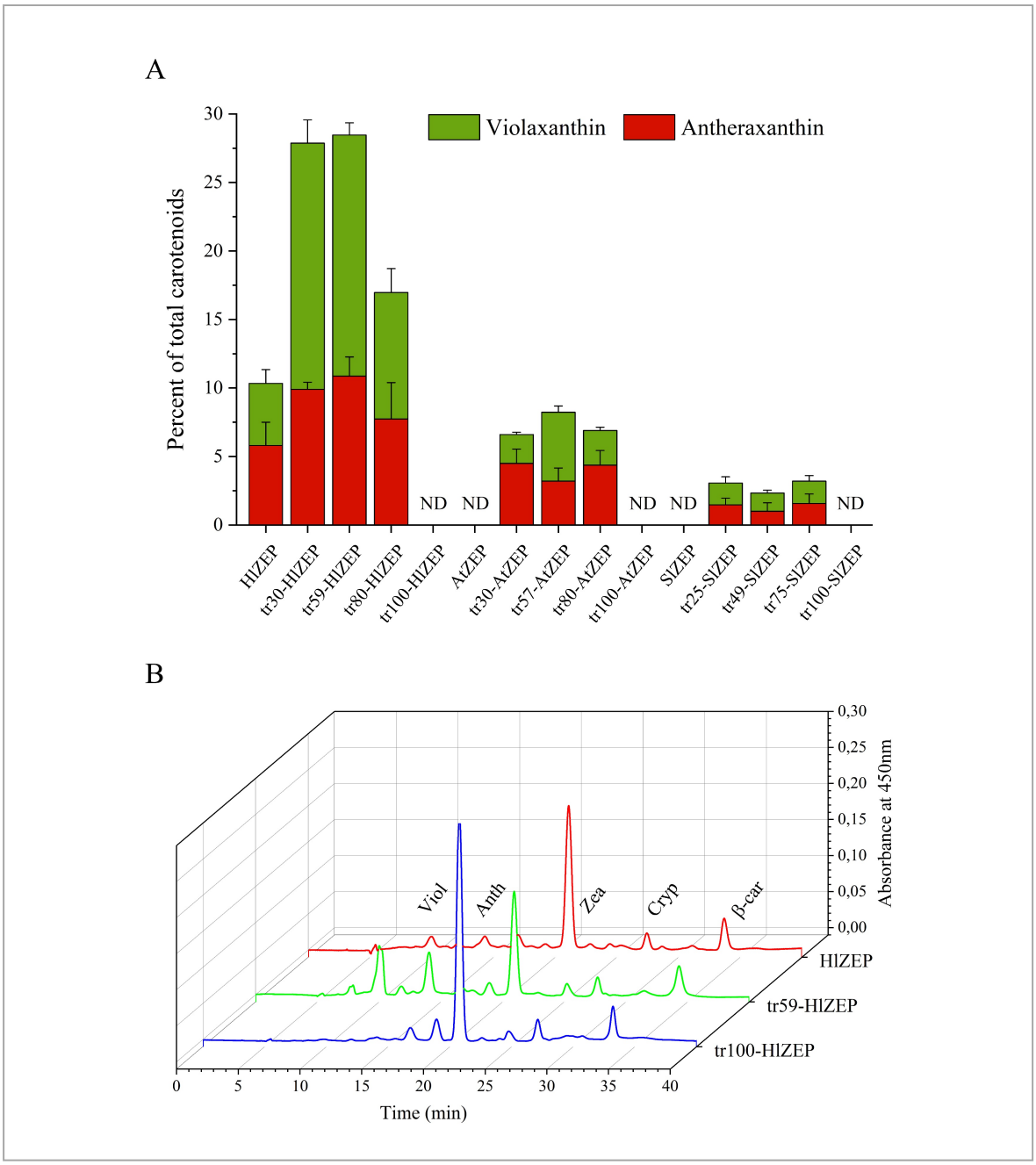

Figure 9. Evaluation of zeaxanthin epoxidases (ZEPs) for violaxanthin production. **(A)** Epoxycarotenoids content (antheraxanthin and violaxanthin) in strains co-expressing PaCrtZ and different N-terminal truncated variants of ZEP from *H. lacustris* (HlZEP), *A. thailiana* (AtZEP), and *S. lycopersicum* (SlZEP). TrN represents truncated variants where N is the residue position of truncation. Variants tr59-HlZEP, tr57-HlZEP and tr49-SlZEP correspond to the enzymes truncated at the predicted transit peptides. Error bars represent standard deviation from triplicate experiments. **(B)** HPLC elution profiles of the different strains.

Our results highlight the importance of truncating heterologous-expressed plant enzymes that are naturally localized into plastids. Typically, plastid transit peptides are cleaved by proteases following import (Bruce, 2001; Kunze & Berger, 2015). This implies that the mature protein is shorter, and the absence of the N-terminal transit peptide could be necessary for the activity. The prediction of transit peptides and, thereby, the truncation position, can be assessed by bioinformatic tools (Emanuelsson et al., 1999). However, as these approaches are prone to inaccuracies, the assessment of other truncation positions becomes important. For instance, our results indicate that, in some cases, additional truncation respect to the predicted transit peptide has no detrimental effect on activity. Importantly, truncation analyses are useful for finding essential enzyme regions. Our data demonstrated the presence of an essential domain in ZEPs between residues 80 and 100. In line with these results, it has been proposed that ZEPs contain a transmembrane helix in the N-terminal region (Schaller et al., 2012). Moreover, bioinformatic predictions showed a putative transmembrane helix between residues 80 and 100 for HlZEP (Figure B4, Appendix B). Altogether, these results suggest that membrane association, mediated by an N-terminal transmembrane region is essential for the activity of ZEPs.

4.4 Violaxanthin production through CrtZ-ZEP fusion proteins

Although the previously selected ZEP was able to transform zeaxanthin to violaxanthin in vivo, the main carotenoid accumulated in tr59-HlZEP strain was still zeaxanthin (38.5% of total carotenoids).

Both enzymes, CrtZ and ZEP, are predicted transmembrane enzymes (Figure B4, Appendix B); and carotenogenesis is typically localized in biological membranes (T. Sun et al., 2018). We then hypothesized that zeaxanthin epoxidation might be limited by the low lateral diffusion rates in membranes of either zeaxanthin or biosynthetic enzymes. Thus, we constructed and expressed PaCrtZ-tr59-HlZEP fusions (for simplicity, named CrtZ-ZEP) to evaluate if the covalent joining of these enzymes could improve the overall transformation rate of zeaxanthin into violaxanthin. Successful construction of fusion proteins often depends on the selected order of the enzymes in the fused polypeptide chain (N-terminal or C-terminal), as well as the linkers employed to join these proteins (Yu, Liu, Kim, & Lee, 2015). To tackle these variables, we assembled four fusions proteins, that include both orders (CrtZ-ZEP and ZEP-CrtZ), and two types of linkers (one rigid and one flexible, denoted R and F, respectively). Respective sequences are shown in Table 1.

Schematic representation of the constructed fusions is illustrated in Figure 10A. The relative localization of soluble domains and the topology of transmembrane helices in fusion enzymes were deduced from sequence analysis (Figure B4, Appendix B). Regardless of the linker employed, fusion variants in which ZEP was attached as N-terminal module (ZEP-R-CrtZ and ZEP-F-CrtZ) displayed no epoxidase activity (Figure 10B). These variants also showed reduced hydroxylase activity with respect to the individually expressed enzymes (control strain, tr59-HlZEP). On the contrary, when CrtZ was the N-terminal component (CrtZ-R-ZEP and CrtZ-F-ZEP), both hydroxylase and epoxidase activities were detected. The CrtZ-R-ZEP expressing strain showed the highest violaxanthin content (18.9% of total carotenoids) among all fusion variants, even though it was not significantly different from

the control. Although this strain accumulated the same epoxidated carotenoids than the control strain, its zeaxanthin content was lower. Moreover, it showed a 1.9-fold increase in epoxycarotenoid/zeaxanthin molar ratio, and a 3.5-fold decrease in hydroxycarotenoid/β-carotene molar ratio with respect to the control strain (Figure 10C). These findings suggest effective substrate channeling of zeaxanthin to violaxanthin, albeit with a concomitant decrease in the net hydroxylase activity.

Our results clearly showed the importance of N- and C-terminal arrangement of CrtZ and ZEP enzymes for the activity of the fusion proteins. Transmembrane orientation of zeaxanthin in lipid membranes (Grudzinski et al., 2017), suggests that the epoxidation of exposed polar headgroups of zeaxanthin is catalyzed by the soluble domain of ZEP, which could be peripherally associated to membranes. The absence of epoxidase activity of the ZEP-CrtZ variants could result from misfolding and/or conformational changes in this soluble domain. In these fusions, the tested linkers (15 residues) might have not been long enough to ensure a proper folding or active conformation of the relatively large non-membrane domain of ZEP (653 residues). On the contrary, in epoxidase active CrtZ-ZEP variants, the soluble domain of ZEP is localized in the native configuration, far from the linker and sterically less impeded (Figure 10A). Hydroxylase activity is also higher in CrtZ-ZEP than ZEP-CrtZ variants. A similar effect was recently reported for the hydroxylating activity of CrtZ-CrtW fusions in astaxanthin biosynthesis, which was attributed to N-terminal helix role in active site formation (Nogueira et al., 2019). Accordingly, linker and ZEP proximity could hinder the correct conformation of the active site of CrtZ in ZEP-CrtZ fusions.

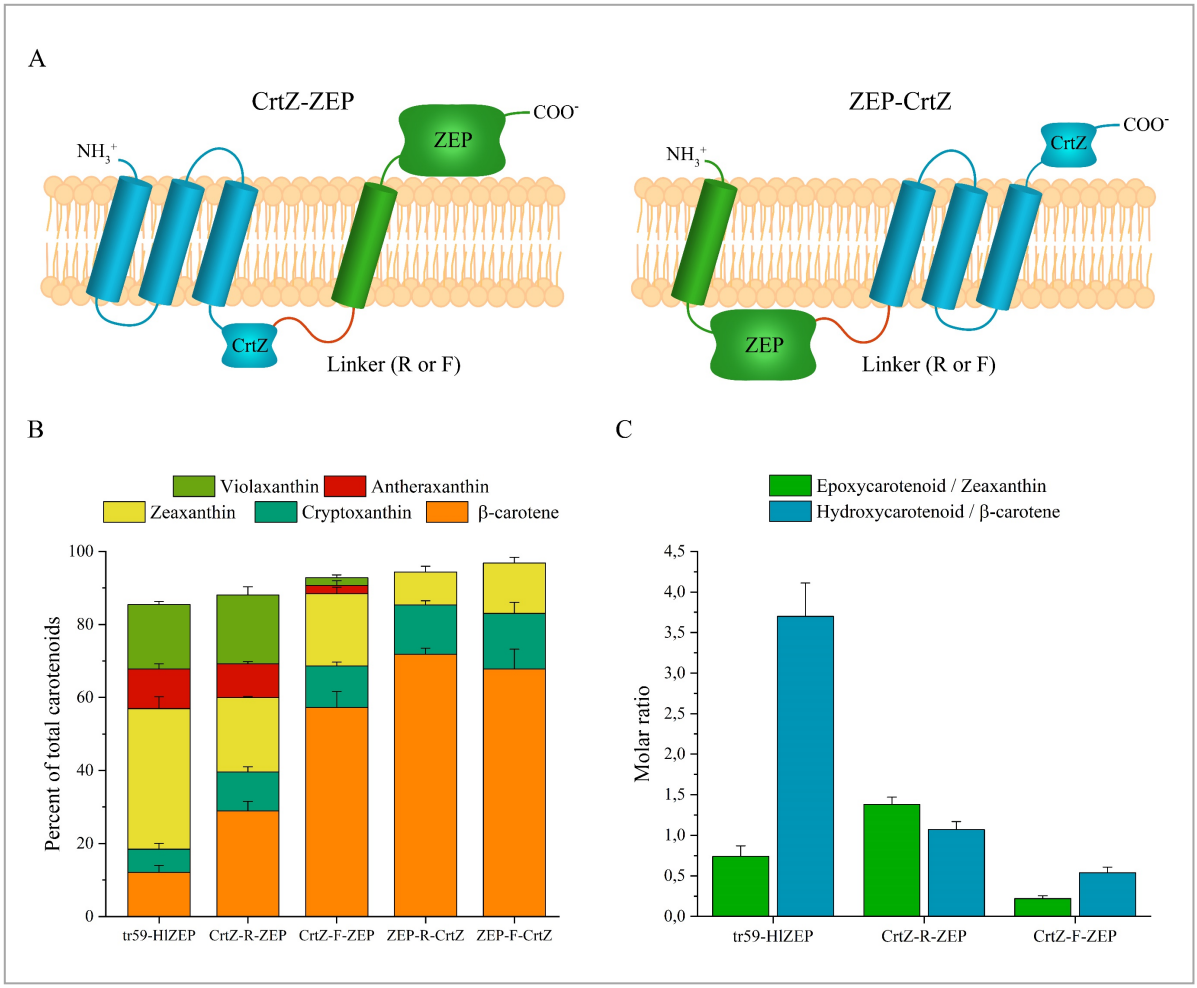

Figure 10. Violaxanthin biosynthesis of strains expressing CrtZ-ZEP fusion proteins. **(A)** Schematic representation of the predicted membrane topology of PaCrtZ-tr59-HlZEP fusion proteins depending on the N- and C-terminal arrangement (CrtZ-ZEP and ZEP-CrtZ). Two linkers (Rigid: R; or Flexible: F) were tested for each enzyme disposition, yielding 4 enzyme variants. **(B)** Carotenoids content in strains expressing fusion variants and separately expressed enzymes (tr59-HlZEP strain). **(C)** Molar ratios of substrate/products, indicative of efficiency of hydroxylase and epoxidase activities in CrtZ-ZEP fusion variants. Error bars represent standard deviation from triplicate experiments.

The linker flexibility in the CrtZ-ZEP fusions impacted directly on both enzymatic activities, but mainly on the epoxidase activity. Flexible linkers enable movement and interaction between fusion partners; while rigid linkers provide distance and reduce the interference between them (X. Chen, Zaro, & Shen, 2013). The higher epoxidase activity found with the α-helical rigid linker (EAAAK)3 indicates that ZEP requires some spatial separation from CrtZ, probably necessary for correct folding and insertion of the N-terminal transmembrane region. Although the constructed fusions did not improve the violaxanthin yield reached by the independent enzymes, the observed substrate channeling reveals the high potential of these CrtZ-ZEP fusion variants. Further testing of additional linker sequences and lengths might yield a more effective fusion variant. Additionally, a modular enzyme assembly such as the recently described strategy based in shorth peptides interactions (RIAD and RIDD; Kang et al., 2019) could reduce the enzymes interference observed in fusion variants and therefore improve violaxanthin yields.

4.5 Co-expression of redox partners and gene copy number adjustment for improvement of violaxanthin production

ZEP incorporates one oxygen from O_2 to the β-rings of zeaxanthin or antheraxanthin and require an electron donor to reduce the second oxygen atom to water. Early studies have showed that the epoxidation reaction in chloroplasts is NADPH dependent and requires FAD as cofactor (Büch et al., 1995; Siefermann & Yamamoto, 1975). Later, Bouvier et al. (1996) demonstrated that ZEP was unable to accept reducing equivalents directly from NADPH and, therefore, the required electrons are transferred from ferredoxin to the ZEP-bound FAD. Likewise, ferredoxin initially obtains its reducing power from ferredoxin-NADPH

oxidoreductase. Thus, in plants, the epoxidation reaction works as an electron transport chain composed of ferredoxin-NADPH oxidoreductase (FNR), ferredoxin (FD) and ZEP (Figure 11A).

Our results demonstrate that the heterologously expressed ZEP can partially obtain the required reducing equivalents directly from the yeast endogenous metabolism. However, the low violaxanthin yields obtained (up to 17.6% of total carotenoids) could be indicative of limited electron transfer to ZEP. Therefore, to improve the violaxanthin production, we co-expressed the ZEP redox partners FNR and FD in the tr59-HlZEP strain. Between all the FNRs and FDs plant isoforms, we selected RFNR1 and FD3 (from *A. thaliana*) due to their high catalytic efficiency in reductive reactions (Hanke et al., 2005; Onda et al., 2000). We also tested combinations of truncated variants lacking the predicted plastid transit peptides. In addition to plant redox partners, we assessed if the yeast mitochondrial ferredoxin-like YAH1 and its reductase ARH1 could also promote electron transfer in ZEP catalyzed reaction. To localize ARH1 and YAH1 to the cytoplasm, we expressed truncated variants without the predicted mitochondrial transit peptides. Prediction of the transit peptide was not possible for ARH1, so in this case we expressed two truncated variants: tr25-ARH1 and tr50-ARH1.

The expression of the different redox partners in tr59-HlZEP strain did not produce significant changes in total carotenoid yields (mean 9.3 mg/gDCW, Table A3, Appendix A). Yeast truncated ARH1 and YAH1 were unable to change carotenoid composition, nor to increase violaxanthin production (Figure 11B). On the other hand, co-expression of untruncated plant redox partners RFNR1 and FD3 increased by 47% the violaxanthin content

with respect to tr59-HlZEP strain (from 17.6 to 25.8% of total carotenoids). Further truncation of ferredoxin (RFNR1/tr-FD3) or both ferredoxin and oxidoreductase (tr-RFNR1/tr-FD3) resulted in 2.2-fold-increase in violaxanthin accumulation (from 17.6 to 38.3% of total carotenoids), achieving 3.5 mg/g_{DCW}. The three strains that expressed the RFNR1/FD3 system showed a reduction of 15% in final biomass. Importantly, both tr-RFNR1 and tr-FD3 expressed separately had no effect on epoxycarotenoids biosynthesis nor final biomass.

The increment of violaxanthin production resulting from the co-expression of RFNR1/FD3 system validated *in vivo* the *in vitro* model of ZEP catalyzed epoxidation, proposed several years ago by Bouvier et al (1996). Moreover, we found that the absence of one redox partner cannot be complemented by the endogenous yeast metabolism, suggesting a high specificity in the electron transfer between RFNR1 and FD3. However, unspecific FD3 reducing activity is still possible and can trigger more NADPH consumption, which could explain the slightly reduced growth of RFNR1/FD3 strains. Takemura et al. (2019) reported that the expression of cyanobacterial redox partners in a violaxanthin-producing *E. coli* strain was unable to increase violaxanthin yields. Our data indicates that *S. cerevisiae* is a suitable host to express fully functional FNR/FD/ZEP system.

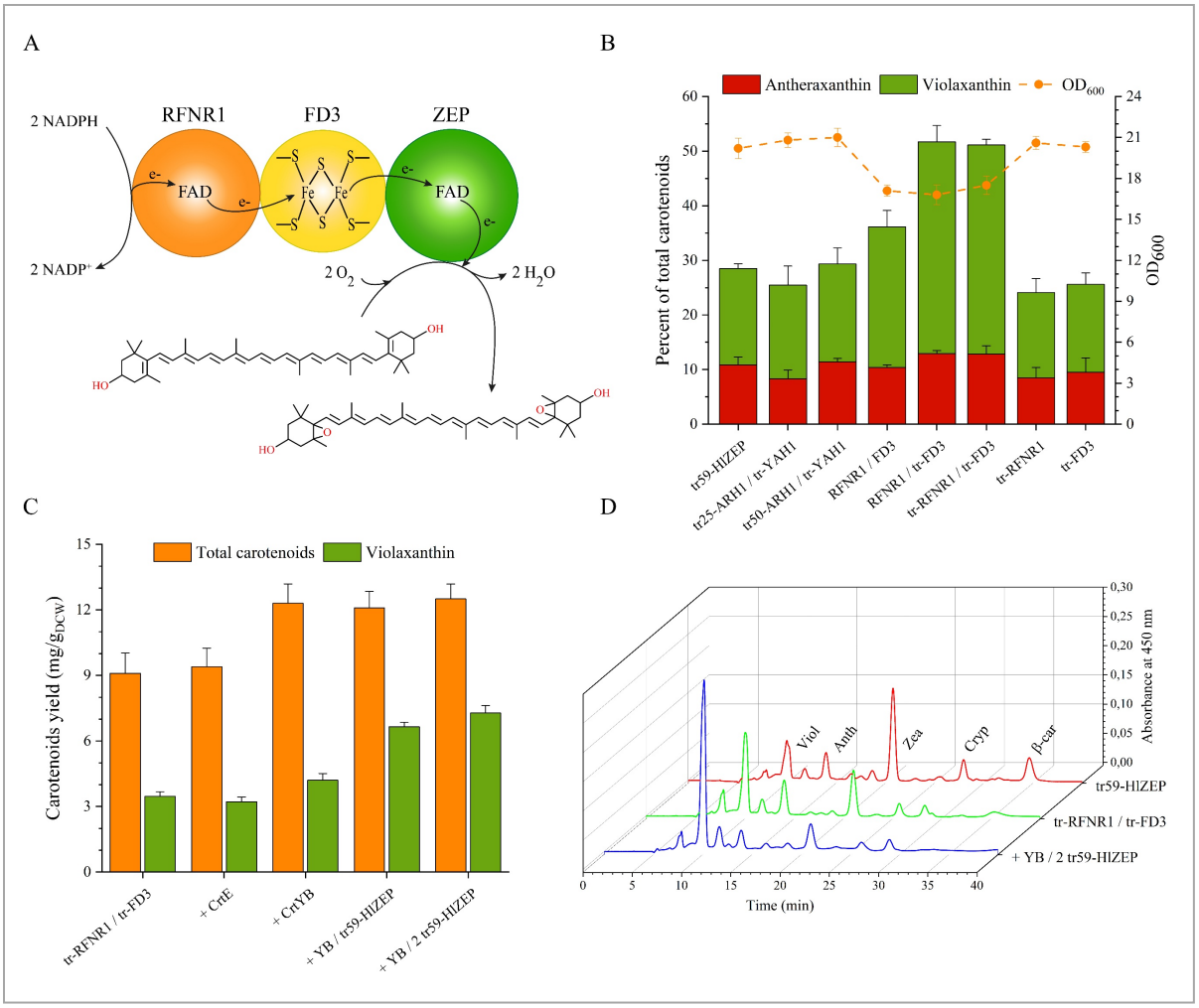

Figure 11. Improvement in violaxanthin production by co-expression of redox partners and gene copy number adjustment. **(A)** Schematic representation of plant redox partners involved in transferring reducing equivalents to ZEP. **(B)** Epoxycarotenoids content (antheraxanthin and violaxanthin) in strains obtained by co-expression of different redox partners in the violaxanthin-producing strain tr59-HlZEP. **(C)** Violaxanthin yields obtained by expression of additional copies of genes from β-carotene and β-xanthophyll pathway in the tr-RFNR1/tr-FD3 strain. Error bars in **(B)** and **(C)** represent standard deviation from triplicate experiments. **(D)** HPLC elution profiles of the different strains.

After the improvement of ZEP activity by redox balancing, we evaluated the impact of adjusting the gene dosage on violaxanthin yield. For this purpose, we initially redirected the carbon flux from upstream precursors, by adding an extra copy of the β-carotenogenic genes CrtE, CrtI and CrtYB to the tr-FD3/tr-RFNR1 expressing strain. An additional copy of the CrtE gene had no effect on carotenogenesis, while an extra copy of the CrtYB gene increased significantly both total carotenoids (from 9.1 to 12.3 mg/g_{DCW}) and violaxanthin yields (from 3.5 to 4.2 mg/g_{DCW}, Figure 11C). No colonies grew in plates after an extra copy of CrtI was included. Further improvement of violaxanthin accumulation was carried out by expressing additional copies of tr59-HlZEP. The strain harboring CrtYB and tr59-HlZEP extra copies (+YB/tr59-HlZEP) accumulated 6.7 mg/g_{DCW} of violaxanthin. Expression of a third copy of tr59-HlZEP (+YB/2 tr59-HlZEP) further increased the violaxanthin yield to 7.3 mg/$_{gDCW}$ which corresponds to 58.4% of total carotenoids. Overall, the gene dosage strategy allowed a 2.1-fold increase in the violaxanthin yields.

β-carotenogenic parent strain SM14 with an additional CrtI copy showed a 2.8-fold increase in total carotenoids (Table A3). Therefore, the lethality observed in violaxanthin + CrtI strain suggests that *S. cerevisiae* is more tolerant to hydrophobic carotenes than to polar xanthophylls. The latter can also explain the reduced total carotenoids observed in xanthophyll-accumulating strains respect the parent β-carotenogenic strain (section 4.2 and Table A3). In yeast, lycopene has been observed enriched in lipid droplets and its production was successfully improved by promoting triacylglycerol biosynthesis and lipid droplet size (Ma et al., 2019). Hydrocarbon carotenes, such as lycopene and β-carotene, might be stabilized in the neutral hydrophobic core of lipid droplets. However, the polar rings of

xanthophylls make them less soluble in neutral lipid phases than carotenes (Borel et al., 1996). Additionally, xanthophylls are well stabilized in lipid bilayers, and are localized in a transmembrane orientation with the oxygen ends interacting with the polar groups of phospholipids (Grudzinski et al., 2017). Hence, high levels of xanthophylls accumulated in cellular membranes might be causing changes in membrane dynamics and function, triggering cellular toxicity. Future studies on subcellular localization of xanthophylls, and efforts on membrane and lipid engineering strategies should boost xanthophyll production in *S. cerevisiae*.

4.6 Kinetics of β-xanthophylls accumulation in batch bioreactors

The constructed xanthophyll-producing strains (zeaxanthin and violaxanthin) accumulated between 10 and 20% of unidentified carotenoids after 48 h cultivation in shake flasks (e.g., Figure 8 and Figure 10). We investigated the origin of these unidentified carotenoids, as well as the kinetics of violaxanthin accumulation, in 1 L batch fermentations with the highest violaxanthin producer (+YB/2 tr59-HlZEP).

The strain showed the classic diauxic growth of *S. cerevisiae* with glucose depletion after 14 h cultivation, followed by ethanol consumption until 30 h (Figure 12A). Total carotenoid yield remained constant at about 9 mg/g_{DCW} until 20 h of cultivation. After that, and in concordance with ethanol consumption, the total carotenoid yield increased up to 15.3 mg/g_{DCW}. The strain exhibited increasing violaxanthin yields since 6 h cultivation with a more pronounced increment from 20 h, reaching up to 7.6 mg/g_{DCW} at 32 h. A more detailed analysis of carotenoid composition over time is given in Figure 12B. A decrease in

zeaxanthin proportion and corresponding increase in violaxanthin were observed between 6 and 28 h of fermentation. On the other hand, β-carotene and cryptoxanthin remained below 6 % during the 48 h of cultivation. Interestingly, the cells were increasingly enriched in unidentified carotenoids during the fermentation. These carotenoids reached a maximum accumulation of 20% after 36 h of cultivation.

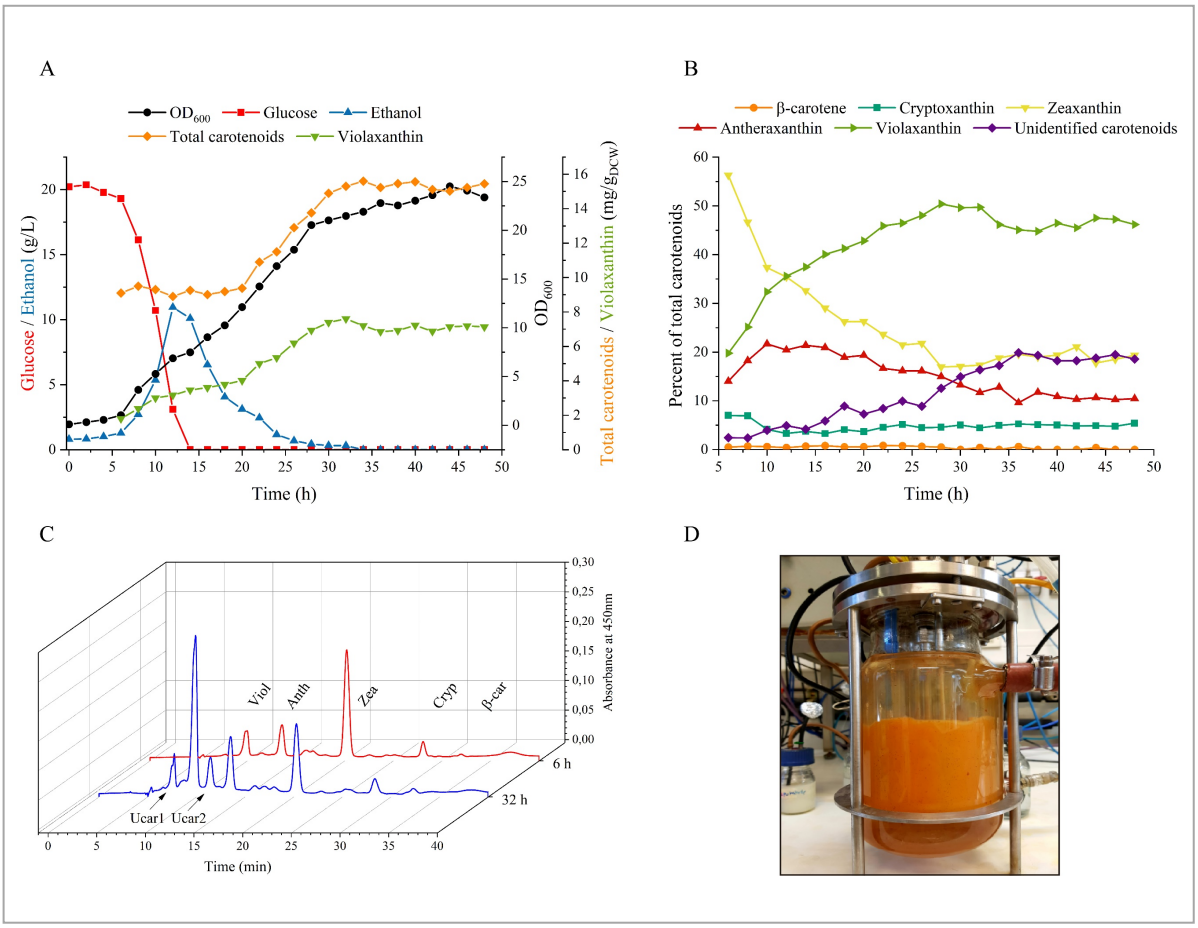

Figure 12. Kinetics of xanthophyll accumulation. **(A)** Batch fermentation kinetics of the highest violaxanthin-accumulating strain (+YB/2 tr59-HlZEP). **(B)** Evolution of different carotenoids during the batch fermentation. **(C)** HPLC elution profiles at 6 and 32 h of culture, showing the accumulation of unidentified carotenoids. **(D)** Orange-pigmented yeast cells growing in the bioreactor.

We reached a higher yield of total carotenoids in bioreactors than in shake flask cultures (15.3 vs. 12.5 mg/g_{DCW}). However, the violaxanthin relative content was lower in bioreactors (49.7 vs 58.4%). Thus, there was no significant difference in the violaxanthin yields between bioreactors and shake flask cultivations (7.6 vs 7.3 mg/g_{DCW}). Despite the resulting yields, batch fermentation data revealed insights about the kinetics of the constructed violaxanthin pathway. The high proportion of zeaxanthin and the low proportion of β-carotene and cryptoxanthin observed at early fermentation times indicate a highly efficient hydroxylating activity of CrtZ. Conversely, epoxidation reaction was slower, yielding the maximum levels of violaxanthin at 28-32 h of fermentation, strongly suggesting that the ZEP reaction is the rate-limiting step in the heterologous expressed β-xanthophyll pathway. Notably, the constant-rate accumulation of unidentified carotenoids observed until 36 h of cultivation suggests that these compounds are metabolic derivatives of the β-xanthophyll pathway. The two predominant unidentified carotenoids, named UCar1 and Ucar2 (Figure 12C) eluted two minutes before violaxanthin and antheraxanthin, respectively. Probably these carotenoids were generated by biotransformation of epoxycarotenoids by the endogenous yeast metabolism. Such carotenoid modifications could include double bond saturations to generate dihydro derivatives or additional introduction of oxygen groups. Future identification of these reactions will enable the disruption of the involved genes and further increase violaxanthin yields.

5. CONCLUSION

In this thesis, we described the successful construction of the first violaxanthin-producing yeast. Violaxanthin production was achieved by integration of the β-xanthophyll pathway in a β-carotenogenic yeast strain. Further stepwise improvements, including enzyme truncation, redox partners co-expression, and gene copy number adjustment enabled 18-fold increase in violaxanthin yields. The higher producing strain expressed 10 heterologous genes and reached 7.3 mg/g_{DCW} of violaxanthin. Besides the epoxycarotenoid production, we obtained fundamental insights into zeaxanthin epoxidase structure and catalysis. Our data supports that ZEP catalyzed zeaxanthin epoxidation is a membrane-associated reaction, which requires accessory redox partners to effectively obtain the reducing equivalents needed for the reaction. The yeast engineering strategy presented in this study may be employed to optimize microbial production of other valuable epoxycarotenoids, such as neoxanthin and fucoxanthin.

Additionally, we developed two simple, rapid, and effective workflows for cloning-free assembly and integration of gene expression cassettes in *S. cerevisiae*. While both approaches are inspired on reported assembly strategies, the introduced adaptations enabled substantial reductions in experimental efforts while maintaining high integration efficiencies. The first method – termed full *in vitro* Gibson assembly – showed the best integration efficiency (95%), while the second – direct assembly by HR – was faster (it can be performed in a day) with a reasonably high efficiency (85%). Importantly, both techniques can be readily employed to join more than three fragments, e.g., construction of bidirectional expression cassettes by a four-fragment assembly. Although the tools presented here are particularly

tailored for genes that are unclonable in *E. coli*, they can also be used as general-purpose, rapid and efficient gene integration alternatives methods.

6. PERSPECTIVES

The β-carotenogenic parent strain (SM14) used in this work was useful to demonstrate and improve the heterologous production of violaxanthin in batch fermentations. However, we have reported that this strain displays an exacerbated Crabtree effect, affecting its performance in fed-batch fermentations and limiting carotenoid productivity. Apparently, the adaptative laboratory evolution used to increase β-carotene production also generated mutations that impaired mitochondrial respiration, which ultimately reduced the oxidative capacity of this strain (López et al., 2019). Hence, the strains constructed in this thesis cannot be used in high-density fermentations. However, the knowledge acquired about the enzymes involved in the β-xanthophyll pathway can be directly applied for the construction of violaxanthin cell factories in different, more robust parent strains, such as *S. cerevisiae* CEN.PK family.

The highest violaxanthin yeast producer reported in this thesis accumulates 7.3 mg/g_{DCW} of product, which is significantly lower than the maximum yields reported for lycopene (75 mg/g_{DCW}, Shi et al., 2019) and β-carotene (25 mg/g_{DCW}, Olson et al., 2016) in *S. cerevisiae*. Our data also support the idea that polar xanthophylls exert higher cytotoxicity than non-polar carotenes. Therefore, a promising strategy for improving the specific yield and productivity for this kind of carotenoids might consider the genomic integration of the whole violaxanthin pathway under the control of inducible promoters (e.g., galactose). This approach will split the fermentation into two phases: a growth phase and a production phase, preparing the cells for the carotenoid accumulation and reducing the overall toxicity. Additionally, since xanthophylls are expected to be localized in lipid bilayers, their toxicity

could be triggered by physicochemical changes in cell membranes. Therefore, more comprehensive research of how these molecules affect the dynamics and function of biological membranes could give insights into membrane engineering strategies to increase xanthophyll accumulation.

Bioreactor fermentations clearly showed that ZEP is the rate-limiting enzyme of the heterologous pathway constructed in *S. cerevisiae*. Improving the catalytic efficiency of this enzyme seems crucial to increase violaxanthin productivities. Enzyme engineering can be a reliable strategy to achieve this goal. However, no carotenogenic enzymes have been crystalized yet, and there are no structural models available. Additionally, the absence of a template with enough sequence homology and the relatively large size of ZEP enzymes (over 600 residues), makes both homology and *de novo* structural modelings particularly challenging. To tackle the characterization of carotenogenic enzymes, we are currently working on the structural modeling of PaCrtZ. This enzyme is smaller (175 residues) than ZEPs and has an available structural template that enables the homology modeling. Further evolutionary trace analysis will detect conserved residues, while experimental directed mutagenesis will confirm the key functional roles of these residues. These results will set structural bases of carotenogenic enzymes that could be useful for design rational enzyme engineering.

Violaxanthin is a metabolic intermediate in the biosynthesis of two high-value compounds: the carotenoid fucoxanthin and the apocarotenoid β-damascenone. In the biosynthetic pathways of both molecules violaxanthin is converted to neoxanthin. Recently, violaxanthin de-epoxidase–like (VDL) proteins were identified as the enzymes that catalyze neoxanthin

biosynthesis in chromalveolate algae (Dautermann et al., 2020). The integration of a VDL gene in our violaxanthin-producing strains should extend the pathway to neoxanthin. Then, the expression of a carotenoid cleavage dioxygenase 1 (CCD1) will enable the oxidative cleavage of neoxanthin to form the apocarotenoid grasshopper ketone. An enzymatic reduction and two acid-catalyzed dehydrations will transform the grasshopper ketone into β-damascenone (Sefton et al., 2011).

The transformation of neoxanthin to fucoxanthin requires an acetylation of the 3′-OH group and ketolation at C8 position (Mikami and Hosokawa, 2013). Unfortunately, the enzymes that catalyze these reactions have not yet been discovered. Bioinformatic analyses could become an efficient strategy to obtain candidates' coding region sequences (CDS) for fucoxanthin biosynthetic enzymes. This approach takes advantage of the different carotenoid pathways in heterokont algae, particularly in the ochrophyta phylum. Fucoxanthin is accumulated by some ocrophytes such as the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. By contrast, other ocrophytes such as the eustigmatophytes *Nannochloropsis oceanica* and *Nannochloropsis gaditana* biosynthesize vaucheriaxanthin esters instead of fucoxanthin (Dautermann et al., 2020). Presumably, these two carotenoids are biosynthesized from neoxanthin 3-acetate (dinoxanthin), in reactions catalyzed by different enzymes. By bioinformatically analyzing and crossing all putative protein sequences (translated from CDS) of these four species, we might obtain gene clusters of orthologs. Genes shared by the two diatoms, but absent in the two eustigmatophytes, are potential enzymes involved in fucoxanthin biosynthesis. To further reduce the number of candidates, several filters can then be applied. For example, the classification of the first

round of candidates in protein families based on documented domains will discard several proteins involved in well-known cellular functions. Then, the second round of candidates can be further filtered using sequence-based prediction of subcellular localization. Our target enzymes, like all other carotenogenic enzymes, should be localized to plastids. Finally, the selected candidates can then be cloned and expressed to test their enzymatic activity.

7. ABBREVIATIONS

β-car	β-carotene
AmpR	Ampicillin resistance gene
Anth	Antheraxanthin
ARH1	Yeast mitochondrial ferredoxin oxidoreductase
At	*Arabidopsis thaliana*
bp	Base pair
CrtE	Geranylgeranyl diphosphate synthase
CrtI	Phytoene desaturase
CrtYB	Bifunctional lycopene cyclase/phytoene synthase
CrtZ/CrtR-b2	β-carotene hydroxylase
Cryp	Cryptoxanthin
DMAPP	Dimethylallyl diphosphate
DR	Direct repeat sequence
FD3	Ferredoxin 3
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
GPP	Geranyl diphosphate

Hl	*Haematococcus lacustris*
HR	Homologous recombination
IPP	Isopentenyl diphosphate
kb	Kilobase
MEP	Methylerythritol 4-phosphate
MVA	Mevalonate
OE-PCR	Overlap extension PCR
Ori	Replication origin
Pa	*Pantoea ananatis*
RFNR1	Ferredoxin-NADPH oxidoreductase
Sl	*Solanum lycopersicum*
tr	Truncated
URA3	Orotidine-5'-phosphate decarboxylase marker
Viol	Violaxanthin
YAH1	Yeast mitochondrial ferredoxin
Zea	Zeaxanthin
ZEP	Zeaxanthin epoxidase

8. PUBLICATIONS AND MEETINGS

During the progress of this thesis the following papers were published:

- **Cataldo, V.F.**, López, J., Cárcamo, M., & Agosin, E. (2016). Chemical vs. biotechnological synthesis of C_{13}-apocarotenoids: current methods, applications and perspectives. *Applied Microbiology and Biotechnology*, *100*(13), 5703–5718. https://doi.org/10.1007/s00253-016-7583-8
- López, J., **Cataldo, V. F.**, Peña, M., Saa, P. A., Saitua, F., Ibaceta, M., & Agosin, E. (2019). Build your bioprocess on a solid strain - β-Carotene production in recombinant *Saccharomyces cerevisiae*. *Frontiers in Bioengineering and Biotechnology*, *7*, 171. https://doi.org/10.3389/fbioe.2019.00171
- **Cataldo, V.F.**, Salgado, V., Saa, P. A., & Agosin, E. (2020). Genomic integration of unclonable gene expression cassettes in *Saccharomyces cerevisiae* using rapid cloning-free workflows. *MicrobiologyOpen*, *9*(3), e978. https://doi.org/10.1002/mbo3.978
- **Cataldo, V.F.**, Arenas, N., Salgado, V., Camilo, C., Ibáñez, F., & Agosin, E. (2020). Heterologous production of the epoxycarotenoid violaxanthin in *Saccharomyces cerevisiae*. *Metabolic Engineering*, *59*, 53–63. https://doi.org/10.1016/j.ymben.2020.01.006

This thesis work were also partially presented in the following acadamic meetings:

- **Cataldo, V.F.**, Arenas, N., López, J., Camilo, C., & Agosin, E. (2018, March 4-8). Sustainable production of β-xanthophylls in *Saccharomyces cerevisiae*. Microbial Engineering, Santa Fe, U.S.A.
- **Cataldo, V.F.**, Arenas, N., Salgado, V., López, J., Camilo, C., & Agosin, E. (2018, June 24-28). Heterologous production of β-Xanthophylls in *Saccharomyces cerevisiae*. Metabolic Engineering 12, Munich, Germany.
- **Cataldo, V.F.**, Arenas, N., Salgado, V., López, J., Camilo, C., & Agosin, E. (2018, September 18-21). Metabolic engineering of *Saccharomyces cerevisiae* for production of plant β-Xanthophylls. Biotechnology of Flavours, Fragrances and Functional Ingredients, Frankfurt, Germany.
- **Cataldo, V.F.**, Arenas, N., Salgado, V., Camilo, C., Íbañez, F., & Agosin, E. (2019, October 1-4). Heterologous production of violaxanthin in *Saccharomyces cerevisiae*. International Flavor and fragrance conference, Viña del Mar, Chile.

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APPENDIX

APPENDIX A: SUPPLEMENTARY TABLES

Table A1. Gene sequences used for the construction of integrative plasmids and expression cassettes. Organisms of origin are indicated in parentheses. All genes were codon-optimized for expression in *S. cerevisiae*.

PaCrtZ (*Pantoea ananatis)*
ATGTTGTGGATCTGGAACGCTTTGATCGTTTTCGTTACTGTTATTGGTATGGAAGTTGTTGCTG
CATTGGCTCATAAATATATTATGCATGGTTGGGGTTGGGGTTGGCATTTGTCTCATCATGAAC
CAAGAAAGGGTGCTTTCGAAGTTAACGATTTGTACGCAGTTGTTTTCGCTGCATTGTCTATCTT
GTTGATATATTTGGGTTCAACTGGCATGTGGCCATTACAATGGATTGGTGCTGGTATGACAGC
TTATGGTTTGTTGTACTTCATGGTTCATGATGGTTTGGTTCATCAAAGATGGCCTTTTAGATAC
ATCCCAAGAAAGGGTTATTTGAAGAGATTGTACATGGCTCATAGAATGCATCATGCAGTTAG
AGGTAAAGAAGGTTGTGTTTCTTTTGGTTTCTTGTACGCTCCACCATTGTCAAAATTGCAAGCA
ACATTAAGAGAAAGACATGGTGCTAGAGCAGGTGCTGCAAGAGATGCTCAAGGTGGTGAAG
ATGAACCAGCATCAGGTAAATAA
CrtR-b2 (*Solanum lycopersicum)*
ATGGCTGCAGGTATTTCTGCATCAGCTTCTTCAAGAACTATCAGATTGAGACATAACCCATTT
TTATCTCCAAAGTCTGCATCAACTGCTCCACCAGTTTTGTTTTTCTCTCCATTGACAAGAAACT
TCGGTGCTATCTTGTTATCAAGAAGAAAGCCAAGATTAGCAGTTTGTTTCGTTTTGGAAAACG
AAAAATTAAATTCTACAATCGAATCTGAATCAGAAGTTATTGAAGATAGAATACAAGTTGAA
ATTAATGAAGAAAAATCTTTGGCTGCATCATGGTTGGCTGAAAAATTAGCAAGAAAGAAATC
TGAAAGATTCACTTATTTGGTTGCTGCAGTTATGTCTTCATTAGGTATCACATCTATGGCAATC
TTGGCTGTTTACTACAGATTTTCATGGCAAATGGAAGGTGGTGAAGTTCCATTTTCTGAAATG
TTGGCTACTTTTACATTATCATTTGGTGCTGCAGTTGGTATGGAATACTGGGCAAGATGGGCT
CATAGAGCATTGTGGCATGCTTCTTTATGGCACATGCATGAATCACATCATAGACCAAGAGAA
GGTCCATTCGAAATGAACGATGTTTTCGCTATCACTAACGCAGTTCCAGCAATTGCTTTGTTGT
CTTATGGTTTCTTTCATAAGGGTATCGTTCCAGGTTTGTGTTTTGGTGCTGGTTTAGGTATTAC
AGTTTTTGGTATGGCATACATGTTCGTTCATGATGGTTTGGTTCATAAGAGATTTCCAGTTGGT
CCAATCGCTAACGTTCCATACTTCAGAAGAGTTGCTGCAGCTCATCAATTGCATCATTCTGAT
AAGTTCGATGGTGTTCCATACGGTTTGTTTTTAGGTCCAAAAGAATTGGAAGAAGTTGGTGGT
TTGGAAGAATTAGAAAAAGAAGTTAATAGAAGAATTAAAATTTCAAAAGGTTTGTTATAA

HlCrtZ (*Haematoccocus lacustris)*

ATGACTTTTCATAAACCAGTTTCTGGTGCATCAGCTTTACCACATATTGGTCCACCACCACATT
TGCATAGATCTTTTGCTGCAACTACAATGTTGTCAAAATTGCAATCTATCTCAGTTAAGGCTA
GAAGAGTTGAATTAGCAAGAGATATCACAAGACCAAAGGTTTGTTTGCATGCTCAAAGATGT
TCTTTGGTTAGATTAAGAGTTGCTGCACCACAAACTGAAGAAGCATTGGGTACAGTTCAAGCT
GCAGGTGCAGGTGACGAACATTCTGCAGATGTTGCTTTGCAACAATTAGATAGAGCAATTGCT
GAAAGAAGAGCTAGAAGAAAGAGAGAACAATTGTCATACCAAGCTGCAGCTATTGCAGCTTC
TATTGGTGTTTCAGGTATCGCAATCTTCGCTACTTACTTGAGATTCGCTATGCACATGACAGTT
GGTGGTGCTGTTCCTTGGGGTGAAGTTGCAGGTACTTTGTTATTGGTTGTTGGTGGTGCATTGG
GTATGGAAATGTATGCAAGATACGCTCATAAAGCAATTTGGCATGAATCTCCATTGGGTTGGT
TATTGCATAAATCACATCATACTCCAAGAACAGGTCCATTCGAAGCTAACGATTTGTTCGCAA
TTATTAACGGTTTGCCAGCTATGTTGTTGTGTACTTTCGGTTTCTGGTTGCCAAATGTTTTAGGT
GCAGCTTGTTTTGGTGCTGGTTTGGGTATCACATTGTACGGTATGGCATACATGTTTGTTCATG
ATGGTTTGGTTCATAGAAGATTTCCAACTGGTCCAATTGCTGGTTTGCCATACATGAAGAGAT
TGACAGTTGCACATCAATTGCATCATTCTGGTAAATACGGTGGTGCTCCTTGGGGCATGTTTTT
GGGTCCACAAGAATTACAACATATTCCAGGTGCAGCTGAAGAAGTTGAAAGATTGGTTTTGG
AATTGGATTGGTCAAAAAGATAA

HlZEP (*Haematoccocus lacustris)*

ATGTTGTTACATACTTCTTCATTGCCAAGATGTCAAGCTGCAGGTCATGTTAAGTCAACAGTTT
CTATCCATGTTCCAGCTTCTCCAAGATTAGTTCCATCATGTCATCATGGTTCTGCTGCACCAGT
TTCACCAAGAAGATGGACTCCACCATCAGTTTCTTGTCCAGCTGTTTTGGAAGCTGCAAGACC
AGGTCAACAAGAAAGATTAGAAGGTGCAGTTCCAGAATTGTGTCCAGGTTTAACTATTGTTAT
TGCAGGTGCTGGTATTTCTGGTTTGACATTAGCTTTGTCATTGTTGAAGAAAGGTGTTAAGTGT
CAAGTTTTGGAAAGAGATTTGACAGCTATTAGAGGTGAAGGTAAAATTAGAGGTCCAATTCA
AGTTCAATCAAATGCTTTAGCTGCATTGGAAGCAATTGATCCAGTTGTTGCTGATGATATTAT
GGCACATGGTTGTATTACTGGTGACAGAATTAATGGTTTGTGTGATGGTGTTTCTGGTGACTG
GTATGTTAAATTTGATACATTTCATCCAGCTGTTGAAAGAGGTTTGCCAGTTACTAGAGTTATT
AATAGAGTTACATTGCAACAATTGTTAGCAGAAGCTGTTATTAGATTGGGTGGTGAAGATATG
ATTTTAGGTGGTTGTCATGTTACTGCTTATGAAGAATTTGTTGATAGAGCATCAGGTAAACAA
CAAGTTGCTGCAATTTTGGAAGATGGTAGAAGATTTGAGGGTGACTTGTTAGTTGGTACAGAT
GGTATTTGGTCTAAGATCAGACAACAAATGATTGGTGACGCACCAGCTCATTACTCTGAATAC
ACTTGTTACACAGGTATCTCAGAATACGTTCCAGCTGATATTGATGTTGTTGGTTACAGAGTTT
TCTTGGGTAACAGACAATACTTCGTTTCTTCAGATGTTGGTGAAGGTAGAATGCAATGGTATG
CTTTTCATCAAGAACCAGCAGGTGGTCAAGATACTTTGGGTCAAAGAAAGGCTAGATTGTTGC
AATTGTTCGGTCATTGGAACTACAACGTTGTTGATTTGATCAGAGCTACACCAGAAGAAGATG

TTTTGAGAAGAGATATCTATGATAGAGCACCAATTTTTAAGTGGGCTCAAGGTAGAGTTGCAT
TGATGGGTGACTCTGCACATGCTATGCAACCAAATTTGGGTCAAGGTGGTTGTATGGCAATGG
AAGATGCTTTTCAATTGGCAAATGATATTGCTGCAATGGCAGAAAAAGCTGGTCAACAAGGT
GCTTTAGGTCCATTGGCAGTTCAACAATGTTTGAGAAGATACCAAGATCAAAGAATCATGAG
AGTTTCTGCTATTCATGGTATGGCTGGTATGGCTGCTTTTATGGCTTCAACTTACAAAGCATAT
TTGGGTGAAGGTTTAGGTCCATTGTCTTGGTTGACAAGATACAAGATCCCACATCCAGGTAGA
GTTGTTGGTCAATGGGTTATGAAATTGACTATGCCAGGTGTTTTGGGTTGGGTTTTAGGTGGTA
ATACAGATAAATTGGAAGCTGCAAGAGCTCCACATTGTAGATTGTCTGATAAGCCAAGATGTT
TCCAAGAATCAGAATTTGAATTGTTGATGAGAGATGATGATTTGTTAGCTGAAAGAGCAAAT
GCTGATTGGTTGTTAGTTGCTGAAAGATTGGCAAGACCACCAACTGCTTTAAATGCTGCACAA
GGTCAAGGTCAACATGTTTACGCATTGGCTATGATGGATACATTAGTTCCAGGTTCAGGTTCT
TCATCTTCATCTGGTGGTTCATCTTTTCCATTGGCTGCAGCTGGCATGTCTAGAGCTGAAGAAG
AAGGTGTTACTTTGCCAAGACCAGGTGGTTTCGGTTTAGCACCATCAGAATACAAAGGTGTTT
ATTTGAATCCAGCACCAGAAGCTACTCCAGCAGCTGAACCAGGTGTTACATTAGTTGGTAGAT
CACCATCTTGTCATTTGGTTTTGGATAATCCATCTTGTGCTGAACAACATGCAAGAATTGAAA
TGCAATCTGCTGGTAGATACTTCGCACATGATTTGGGTTCAAACAATGGTACATGGGTTAACG
GTCATAGATTGGAAAAGGGTGAAAGAGCTATGTTGCATCCAGGTGACGTTTTAAGATTTGGTA
GACAAGGTTCTGAAGTTTTTACTGTTAAATTGCAACATACATCATACAGAAATGCTGAAGTTA
GAGGTGACTGTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAA

AtZEP (*Arabidopsis thaliana)*

ATGGGTTCTACACCATTTTGTTACTCAATTAATCCATCTCCATCAAAATTGGATTTCACAAGAA
CTCATGTTTTCTCTCCAGTTTCAAAGCAATTCTATTTGGATTTGTCTTCTTTTTCTGGTAAACCA
GGTGGTGTTTCTGGTTTTAGATCAAGAAGAGCTTTGTTAGGTGTTAAAGCTGCAACAGCATTA
GTTGAAAAAGAAGAAAAGAGAGAAGCTGTTACTGAAAAGAAAAAGAAATCAAGAGTTTTGG
TTGCTGGTGGTGGTATTGGTGGTTTGGTTTTTGCATTAGCTGCTAAAAAGAAAGGTTTCGATGT
TTTGGTTTTCGAAAAAGATTTGTCTGCTATCAGAGGTGAAGGTAAATACAGAGGTCCAATTCA
AATTCAATCAAATGCTTTAGCTGCATTGGAAGCAATCGATATTGAAGTTGCTGAACAAGTTAT
GGAAGCAGGTTGTATTACAGGTGACAGAATTAATGGTTTGGTTGATGGTATTTCTGGTACTTG
GTATGTTAAGTTCGATACTTTTACTCCAGCTGCTTCAAGAGGTTTGCCAGTTACAAGAGTTATT
TCAAGAATGACTTTGCAACAAATTTTAGCTAGAGCAGTTGGTGAAGATGTTATCAGAAACGA
ATCTAACGTTGTTGATTTTGAAGATTCAGGTGACAAGGTTACAGTTGTTTTAGAAAACGGTCA
AAGATACGAGGGTGACTTGTTAGTTGGTGCTGATGGTATTTGGTCTAAAGTTAGAAACAATTT
GTTTGGTAGATCAGAAGCAACTTATTCAGGTTACACATGTTACACTGGTATCGCTGATTTCAT
CCCAGCAGATATTGAATCAGTTGGTTACAGAGTTTTCTTGGGTCATAAGCAATACTTCGTTTCT
TCAGATGTTGGTGGTGGTAAAATGCAATGGTACGCTTTTCATGAAGAACCAGCAGGTGGTGCT

GATGCACCAAATGGTATGAAGAAAAGATTGTTCGAAATTTTTGATGGTTGGTGTGATAACGTT
TTAGATTTGTTGCATGCTACTGAAGAAGAAGCAATCTTGAGAAGAGATATATATGATAGATCA
CCAGGTTTTACATGGGGTAAAGGTAGAGTTACTTTGTTGGGTGACTCAATTCATGCTATGCAA
CCAAATATGGGTCAAGGTGGTTGTATGGCAATCGAAGATTCTTTCCAATTGGCTTTAGAATTG
GATGAAGCATGGAAACAATCAGTTGAAACTACAACTCCAGTTGATGTTGTTTCTTCATTGAAG
AGATACGAAGAATCAAGAAGATTGAGAGTTGCTATTATTCATGCTATGGCAAGAATGGCTGC
AATCATGGCTTCTACATACAAAGCATATTTGGGTGTTGGTTTGGGTCCATTGTCATTTTTAACT
AAGTTTAGAGTTCCACATCCAGGTAGAGTTGGTGGTAGATTTTTCGTTGATATTGCTATGCCAT
CTATGTTGGATTGGGTTTTAGGTGGTAATTCTGAAAAATTGCAAGGTAGACCACCATCATGTA
GATTGACAGATAAAGCTGATGATAGATTAAGAGAATGGTTTGAAGATGATGATGCATTGGAA
AGAACTATTAAAGGCGAATGGTACTTAATTCCACATGGTGACGATTGTTGTGTTTCTGAAACA
TTGTGTTTGACTAAGGATGAAGATCAACCATGTATTGTTGGTTCAGAACCAGATCAAGATTTT
CCTGGTATGAGAATCGTTATCCCATCTTCACAAGTTTCTAAGATGCATGCTAGAGTTATATAT
AAGGATGGTGCTTTCTTTTTGATGGATTTGAGATCAGAACATGGTACATACGTTACTGATAAT
GAAGGTAGAAGATATAGAGCTACACCAAATTTTCCAGCAAGATTCAGATCATCTGATATTATC
GAATTTGGTTCTGATAAGAAAGCTGCTTTTAGAGTTAAAGTTATTAGAAAGACACCAAAATCT
ACTAGAAAGAATGAATCAAATAATGATAAATTGTTACAAACTGCTTAA

SlZEP (*Solanum lycopersicum)*

ATGTACTCTACTGTTTTCTATACATCAGTTCATCCATCTACTTCAGTTTTGTCAAGAAAGCAAT
TGCCATTGTTAATTTCTAAGGATTTCTCAGCTGAATTGTACCATTCTTTGCCATGTAGATCATT
AGAAAACGGTCATATCAATAAGGTTAAGGGTGTTAAGGTTAAGGCTACTATCGCTGAAGCAC
CAGTTACTCCAACAGAAAAGACTGATTCTGGTGCAAATGGTGACTTGAAAGTTCCACAAAAG
AAATTGAAGGTTTTGGTTGCTGGTGGTGGTATTGGTGGTTTAGTTTTTGCATTGGCTGCTAAGA
AAAGAGGTTTCGATGTTTTGGTTTTCGAAAGAGATTTGTCTGCTATTAGAGGTGAAGGTCAAT
ACAGAGGTCCAATTCAAATTCAATCAAATGCTTTGGCTGCATTAGAAGCAATCGATTTGGATG
TTGCTGAAGATATTATGAATGCAGGTTGTATCACAGGTCAAAGAATTAATGGTTTGGTTGATG
GTATTTCTGGTAACTGGTACTGTAAGTTCGATACTTTTACACCAGCTGTTGAAAGAGGTTTGCC
AGTTACTAGAGTTATTTCAAGAATGACATTGCAACAAATCTTGGCTAGAGCAGTTGGTGAAGA
AATCATCATGAACGAATCAAACGTTGTTGATTTCGAAGATGATGGTGAAAAGGTTACTGTTGT
TTTAGAAAACGGTCAAAGATTCACTGGTGACTTGTTAGTTGGTGCTGATGGTATTAGATCTAA
AGTTAGAACTAATTTGTTTGGTCCATCTGAAGCTACATATTCAGGTTACACTTGTTATACAGGT
ATTGCTGATTTTGTTCCAGCAGATATTGATACTGTTGGTTACAGAGTTTTCTTGGGTCATAAGC
AATACTTCGTTTCTTCAGATGTTGGTGGTGGTAAAATGCAATGGTACGCTTTCTACAACGAAC
CAGCAGGTGGTGCTGATGCACCAAACGGTAAAAAGGAAAGATTGTTGAAGATCTTCGGTGGT
TGGTGTGATAACGTTATCGATTTGTTGGTTGCTACAGATGAAGATGCAATCTTGAGAAGAGAT

ATATATGATAGACCACCAACTTTTTCTTGGGGTAGAGGTAGAGTTACATTGTTGGGTGACTCA
GTTCATGCTATGCAACCAAATTTGGGTCAAGGTGGTTGTATGGCTATTGAAGATTCTTACCAA
TTAGCATTGGAATTAGAAAAAGCATGTTCAAGATCAGCAGAATTTGGTTCACCAGTTGATATT
ATTTCTTCATTAAGATCTTATGAATCAGCTAGAAAATTGAGAGTTGGTGTTATTCATGGTTTGG
CAAGAATGGCTGCAATCATGGCTTCTACTTACAAAGCATATTTGGGTGTTGGTTTGGGTCCAT
TATCATTTTTGACACAATACAGAATACCACATCCAGGTAGAGTTGGTGGTAGAGTTTTTATTG
ATTTGGGTATGCCATTGATGTTATCTTGGGTTTTAGGTGGTAATGGTGACAAATTGGAAGGTA
GAATTAAACATTGTAGATTATCAGAAAAGGCTAACGATCAATTGAGAAAGTGGTTCGAAGAT
GATGATGCATTGGAAAGAGCTACTGATGCAGAATGGTTGTTATTGCCAGCTGGTAATGGTTCT
TCAGGTTTAGAAGCAATTGTTTTGTCAAGAGATGAAGATGTTCCATGTACTGTTGGTTCTATTT
CACATACAAACATCCCTGGTAAATCAATCGTTTTGCCATTACCACAAGTTTCTGAAATGCATG
CTAGAATTTCATGTAAAGATGGTGCTTTCTTTGTTACTGATTTGAGATCTGAACATGGTACTTG
GGTTACAGATAACGAAGGTAGAAGATATAGAACTTCACCAAATTTTCCAACAAGATTCCATC
CATCTGATGTTATCGAATTTGGTTCAGATAAAGCTGCTTTTAGAGTTAAGGCTATGAAGTTCC
CATTGAAGACATCTGAAAGAAAGGAAGAAAGAGAAGCAGTTGAAGCTGCATAA

ARH1 (*Saccharomyces cerevisiae*)

ATGAGCTTTGTTCAAATAAGGCACATTTCTTCACAAATAAACCGTAAGACTGTATCCATTGTT
GGATCGGGGCCTTCCGGCTTTTATACAGCGTACCATTTACTCAAGAAGTCACCGATTCCATTA
AATGTTACTATATGGGAAAAGTTACCTGTTCCTTTTGGTTTAAGTAGATATGGTGTGGCACCT
GATCATCCAGAAGTCAAAAATTGTGAAGAAACGTTTACCACATGTGCAGAAGAGTTTTCTTCC
CCTACAAACCAAAAGCATAAATTTTCCTTTGTTGGTGGTATAACCATTGGAAAAGAAATATTG
TTGAAGGAATTGCTGGATAATCAAGATGCTGTTATTTTAAGTTATGGTTGTACAGGGGACAGA
AAGCTGAATATCCCTGGCGAACTCGGAACAAAAGGGGTGTTTAGCAGTAGAGAATTTGTCAA
TTGGTACAATGGTCATCCTGATTTCGCAAAGGATAAGCGATTTACCGATTTTGACTGGAGCAA
AGTTTCGAAAGTTGGTATTATAGGAAACGGTAACGTTGCTCTTGATATTACGCGTGTACTTAT
TTCTAATCAAATTGATGAAATATGGGAAAATACGGACATCTCATCTCTTGCACTAAATTTGCT
AAGAAGGGCACCTGTAAAGGACGTTAAGCTAATTGCACGAAGGGATTTCGTTCATTCCAAAT
TCACCAACAAAGAATTAAGAGAACTATGGGAGTTAGAAAAGTATGGCATACGTGGCCGTATT
GATCCTAAATTTTTCCAGAAAGAAATGTTTGACCCATCTAAGTACGATCGTGCATTCAATAGA
CGAGTAGAGATGTGTAGTGAGTATCTCAAGCCATTTAATGAACGTTCGAAGAAAAACTATAA
AAAGGCTCCTCCTCCAAGCAGCGGATATGACAAATTCTGGGAGTTAGATTATTTGAAGACTCC
CTTGAAAATTAATAGAGACGATTTTGGTGCAATCAACTCTTTGAGTTTATGTAACAATCGATT
AAATGAAGATAACAGTTTGCAACCCTTGAAGGACGTCAATAATATTATGACATATAAAGTGG
ATTTGCTGATTACTTCATTGGGATATGCAGGCGTTCCCATGCCTGAATTCTCTAAGTTGTCTAT
TGGATTTGACAAAGATCATATAGCTAATAAACAGGGTCGTGTTTTAACTTCCAGCGGAGAAAT

ATTCCCACATTTATATGCATCTGGTTGGATCCGTAAGGGCAGCCAGGGCGTTATTGCCTCGAC
AATGCAAGATGCTTTTGAAGTTGGAGACAGAGTAATACAAGACTTGGTGGTCAGCGGAGCGC
TATCCTTAGAGAATTCTATCGACCTCTCTAATATCAAGCACACCACATGGAAGGATTGGGAAA
GAATCAACAAGAAGGAATTGCTTCGGGGCAAAAAGGAACACAAAACTCGGTCAAAGTTTTTA
ACTTTTGAAGAGTTGTGGAACGGTGTAGAAGGCATATAA

YAH1 (*Saccharomyces cerevisiae*)

ATGCTGAAAATTGTTACTCGGGCTGGACACACAGCTAGAATATCGAACATCGCAGCACATCTT
TTACGCACCTCTCCATCTCTGCTCACACGCACCACCACAACCACAAGATTTCTGCCCTTCTCTA
CGTCTTCGTTCTTAAACCATGGCCATTTGAAAAAACCGAAACCAGGCGAAGAACTGAAGATA
ACTTTTATTCTGAAGGATGGCTCCCAGAAGACGTACGAAGTCTGTGAGGGCGAAACCATCCTG
GACATCGCTCAAGGTCACAACCTGGACATGGAGGGCGCATGCGGCGGTTCTTGTGCCTGCTCC
ACCTGTCACGTCATCGTTGATCCAGACTACTACGATGCCCTGCCGGAACCTGAAGATGATGAA
AACGATATGCTCGATCTTGCTTACGGGCTAACAGAGACAAGCAGGCTTGGGTGCCAGATTAA
GATGTCAAAAGATATCGATGGGATTAGAGTCGCTCTGCCCCAGATGACAAGAAACGTTAATA
ACAACGATTTTAGTTAA

RFNR1 (*Arabidopsis thaliana)*

ATGGCATTGTCAACTACACCATCACAAATGTCTGTTGCTTTACCAACTAGAATCGATGGTTCTT
CAAGATCAATGATCAAGGTTCAATCTATTTCTTTTACTGATAAATCTTGGGGTCCACCATTGTT
GAGATTGGATTCTAAGTCAAGATCATTGGGTGTTAAGAAAAGATCAACTATTTGTATGTCTTT
GCAACAATCTTCAAAGTCTAAGGTTTTGGTTACTCCATTGGAATTAGAAGATCCAAAGGAAAC
ACCATTGAATTTGTTTAGACCAAAGGAACCATACACTGCAACAATTGTTTCAGTTGAAAGAAT
TGTTGGTCCACAAGCTCCAGGTGAAACATGTCATATCGTTATCGATCATGATGGTAACGTTCC
ATACTGGGAAGGTCAATCTTACGGTGTTATTCCACCAGGTGAAAATCCAAAGAAACCAGGTG
CACCACATAACGTTAGATTGTACTCAATCGCTTCTACTAGATACGGTGACTCATTTGATGGTA
AAACAGCATCTTTATGTGTTAGAAGAGCTATCTATTACGATCCAGAAACTGGTAAAGAAGATC
CATCAAAAGCAGGTGTTTGTTCTAATTTCTTGTGTAACGCTAAACCAGGTGACAAAGTTAAAA
TTACTGGTCCATCTGGTAAAGTTATGTTGTTACCAGAAGATGATCCAAAAGCTACACATATTA
TGATTGCAACTGGTACAGGTGTTGCTCCATACAGAGGTTACTTGAGAAGAATGTTCATGGAAA
ACGTTCCAAACTTCAAGTTCGATGGTTTAGCATGGTTGTTTTTAGGTGTTGCTAATTCAGATTC
TTTGTTGTACGATGAAGAATTTGCAGGTTATAGAAAGGATTACCCAGAAAACTTCAGATACGA
TAAAGCATTGTCAAGAGAAGAAAAGAATAAGAAAGGTGGTAAAATGTACGTTCAAGATAAA
ATTGAAGAATACTCTGATGAAATTTTTAAATTGTTAGATAACGGTGCACATATCTATTTCTGTG
GTTTGAAGGGTATGATGCCAGGTATTCAAGATACTTTGAAGAGAGTTGCTGAAGAAAGAGGT
GAATCTTGGGAACAAAAATTGACACAATTGAGAAAGAATAAGCAATGGCATGTTGAAGTTTA
CTAA

FD3 (*Arabidopsis thaliana)*

ATGGCTACTGTTAGAATTTCTTCAACTTCTATGACAAAAGCAGTTTTGAGATCACAAACTACA
AATAAGTTGATCACTAATAAGTCTTACAATTTGTCTGTTGGTTCAACAAAGAGAGTTTCAAGA
TCATTTGGTTTGAAGTGTTCTGCTAATTCAGGTGGTGCTACAATGTCAGCAGTTTACAAGGTTA
AATTGTTAGGTCCAGATGGTCAAGAAGATGAATTTGAAGTTCAAGATGATCAATACATTTTAG
ATGCTGCAGAAGAAGCTGGTGTTGATTTGCCATACTCTTGTAGAGCTGGTGCATGTTCAACTT
GTGCAGGTCAAATTGTTTCTGGTAACGTTGATCAATCTGATGGTTCATTTTTGGAAGATTCACA
TTTGGAAAAGGGTTACGTTTTGACATGTGTTGCATACCCACAATCTGATTGTGTTATTCATACT
CATAAAGAAACAGAATTGTTTTAA

CrtE (*Xanthophyllomyces dendrorhous)*

ATGGACTATGCCAACATCTTAACTGCTATCCCTTTAGAATTTACCCCTCAAGATGACATTGTAT
TATTAGAACCTTATCACTATTTGGGTAAAAATCCTGGTAAAGAAATCAGATCACAATTGATCG
AAGCCTTTAACTACTGGTTGGATGTTAAGAAAGAAGACTTAGAAGTTATTCAAAACGTTGTCG
GCATGTTGCATACTGCATCTTTGTTAATGGATGACGTTGAAGATTCTTCAGTCTTGAGGAGAG
GTTCTCCAGTAGCCCACTTAATCTATGGTATTCCTCAAACTATTAATACCGCCAACTACGTTTA
CTTTTTGGCTTACCAAGAAATTTTCAAATTAAGACCAACACCTATCCCAATGCCTGTCATTCCA
CCTTCCAGTGCATCTTTGCAATCTTCAGTATCCAGTGCTTCTTCTTCTTCTGCTTCATCCGA
AAATGGTGGTACTTCAACACCAAACTCCCAAATCCCTTTTTCTAAGGATACATATTTGGACAA
GGTCATCACCGATGAAATGTTGTCATTACATAGAGGTCAAGGTTTGGAATTATTTTGGAGAGA
TAGTTTGACCTGTCCATCTGAAGAAGAATACGTAAAGATGGTTTTGGGTAAAACAGGTGGTTT
ATTCAGAATCGCAGTTAGATTGATGATGGCCAAGTCAGAATGCGATATAGACTTCGTCCAATT
GGTAAATTTGATATCTATCTATTTCCAAATCAGAGATGACTACATGAACTTGCAAAGTTCTGA
ATACGCTCATAATAAGAACTTCGCAGAAGATTTGACTGAGGGTAAATTTTCTTTCCCAACAAT
CCATTCCATCCACGCTAATCCTTCATCCAGATTGGTTATTAACACTTTGCAAAAGAAATCTACT
TCTCCTGAAATATTACATCACTGTGTTAACTACATGAGAACCGAAACTCACTCATTTGAATAC
ACCCAAGAAGTCTTGAACACTTTATCCGGTGCATTGGAAAGAGAATTGGGTAGATTACAGGG
TGAATTTGCTGAAGCAAATAGTAAGATTGATTTGGGTGACGTTGAATCTGAAGGTAGAACAG
GTAAAAACGTAAAGTTAGAAGCAATCTTGAAAAAGTTGGCAGACATCCCATTATGA

CrtI (*Xanthophyllomyces dendrorhous)*

ATGGGTAAAGAACAAGATCAAGACAAGCCTACTGCTATTATCGTCGGTTGCGGTATTGGTGG
TATCGCTACTGCTGCCAGATTGGCCAAGGAAGGTTTCCAAGTCACTGTATTCGAAAAGAATGA
TTACTCCGGTGGTAGATGTAGTTTGATAGAAAGAGATGGTTACAGATTTGACCAAGGTCCTTC
TTTGTTGTTGTTGCCAGATTTGTTTAAACAAACTTTCGAAGACTTAGGTGAAAAGATGGAAGA
TTGGGTTGACTTGATCAAATGTGAACCAAACTATGTCTGCCATTTCCACGATGAAGAAACTTT
TACATTGTCTACAGACATGGCTTTGTTGAAGAGAGAAGTTGAAAGATTCGAGGGTAAAGATG

GTTTCGACAGATTCTTATCTTTTATTCAAGAAGCTCATAGACACTACGAATTGGCAGTTGTCCA
CGTTTTACAAAAGAATTTCCCTGGTTTTGCTGCATTCTTGAGATTACAATTCATCGGTCAAATA
TTGGCATTGCATCCATTCGAATCAATTTGGACTAGAGTTTGCAGATACTTCAAGACAGATAGA
TTGAGAAGAGTATTTTCCTTCGCCGTTATGTATATGGGTCAATCTCCTTACTCAGCTCCAGGTA
CCTACAGTTTGTTGCAATACACCGAATTAACTGAAGGTATTTGGTACCCTAGAGGTGGTTTTT
GGCAAGTACCAAACACTTTGTTGCAAATCGTTAAGAGAAATAACCCTTCTGCCAAGTTTAATT
TCAACGCTCCTGTCTCTCAAGTATTGTTATCACCAGCAAAAGATAGAGCCACAGGTGTTAGAT
TGGAATCTGGTGAAGAACATCACGCTGATGTAGTTATTGTCAATGCTGACTTGGTATATGCAT
CAGAACATTTGATTCCAGATGACGCCAGAAACAAAATAGGTCAATTGGGTGAAGTCAAGAGA
TCTTGGTGGGCTGATTTGGTTGGTGGTAAAAAGTTGAAGGGTTCATGTTCTTCATTATCCTTCT
ACTGGAGTATGGATAGAATCGTTGACGGTTTGGGTGGTCATAACATTTTCTTGGCAGAAGATT
TCAAAGGTTCCTTCGACACCATCTTTGAAGAATTGGGTTTACCTGCCGATCCAAGTTTTTATGT
TAACGTCCCATCCAGAATTGATCCTAGTGCCGCTCCAGAGGGTAAAGACGCAATTGTTATATT
AGTCCCTTGCGGTCATATTGATGCCTCCAATCCACAAGACTACAACAAATTGGTTGCAAGAGC
CAGAAAGTTCGTCATACACACATTATCTGCTAAGTTGGGTTTGCCTGATTTCGAAAAGATGAT
CGTCGCAGAAAAGGTACATGATGCCCCATCCTGGGAAAAGGAGTTTAATTTGAAGGATGGTA
GTATTTTGGGTTTAGCTCATAACTTCATGCAAGTCTTGGGTTTTAGACCTTCTACAAGACACCC
AAAGTACGATAAGTTATTTTTCGTTGGTGCATCAACACATCCTGGTACCGGTGTACCAATAGT
TTTGGCTGGTGCAAAGTTGACCGCTAACCAAGTTTTAGAATCTTTCGATAGATCACCAGCACC
TGACCCAAATATGTCCTTGAGTGTACCTTATGGTAAACCATTAAAGTCTAACGGTACTGGTAT
CGATTCACAAGTTCAATTGAAATTCATGGACTTAGAAAGATGGGTTTACTTGTTAGTCTTGTT
AATAGGTGCCGTTATCGCTAGATCAGTAGGTGTTTTGGCTTTTTAA

CrtYB (*Xanthophyllomyces dendrorhous)*

ATGACTGCCTTAGCATACTATCAAATCCACTTAATCTACACCTTGCCTATCTTGGGTTTATTGG
GTTTATTGACATCACCTATCTTGACAAAGTTCGATATCTATAAGATCTCTATCTTGGTTTTTAT
CGCTTTCTCAGCAACTACACCATGGGACTCCTGGATTATAAGAAATGGTGCTTGGACTTACCC
TTCTGCAGAATCAGGTCAAGGTGTTTTTGGTACATTCTTGGATGTTCCATATGAAGAATACGC
ATTTTTCGTCATCCAAACTGTAATTACAGGTTTGGTCTACGTATTGGCCACCAGACATTTGTTA
CCATCATTGGCTTTACCTAAAACTAGATCTTCAGCATTGTCCTTAGCCTTGAAGGCTTTGATCC
CATTGCCTATCATATATTTGTTTACTGCTCATCCATCTCCTTCACCAGATCCTTTAGTTACCGAT
CACTATTTCTACATGAGAGCATTGAGTTTGTTAATTACCCCACCTACTATGTTGTTAGCTGCAT
TATCTGGTGAATATGCCTTTGATTGGAAATCAGGTCGTGCTAAGTCCACTATTGCCGCTATAA
TGATCCCAACAGTATATTTGATCTGGGTTGATTACGTTGCAGTCGGTCAAGATTCCTGGAGTA
TCAATGACGAAAAGATTGTCGGTTGGAGATTAGGTGGTGTATTGCCAATCGAAGAAGCTATG
TTTTTCTTGTTGACAAACTTAATGATTGTTTTAGGTTTGTCAGCCTGTGATCATACTCAAGCTTT

GTATTTGTTGCACGGTAGAACAATATACGGTAATAAGAAAATGCCATCCAGTTTTCCTTTGAT
CACTCCACCTGTTTTGTCTTTGTTTTTCTCTTCAAGACCATATTCCAGTCAACCTAAGAGAGAT
TTGGAATTGGCTGTAAAGTTGTTAGAAGAAAAATCAAGATCATTTTTCGTTGCTAGTGCAGGT
TTTCCATCTGAAGTCAGAGAAAGATTAGTAGGTTTGTACGCCTTCTGCAGAGTTACTGATGAC
TTAATTGATTCTCCAGAAGTCTCTTCAAACCCTCATGCTACAATCGATATGGTTTCAGACTTTT
TAACCTTGTTGTTCGGTCCACCTTTGCATCCATCCCAACCTGATAAAATTTTGTCCAGTCCATT
GTTACCACCTTCCCACCCAAGTAGACCTACAGGCATGTATCCATTACCACCTCCACCTTCCTTG
AGTCCTGCTGAATTAGTTCAATTCTTGACTGAAAGAGTACCAGTTCAATACCACTTTGCATTC
AGATTGTTAGCCAAATTACAAGGTTTGATCCCAAGATACCCTTTAGATGAATTGTTGAGAGGT
TACACCACTGACTTAATCTTTCCATTGTCTACCGAAGCAGTTCAAGCCAGAAAGACTCCTATT
GAAACAACCGCAGATTTGTTAGACTATGGTTTATGTGTTGCTGGTTCTGTCGCAGAATTGTTA
GTCTACGTATCTTGGGCCTCAGCTCCATCCCAAGTTCCTGCTACCATTGAAGAAAGAGAAGCC
GTTTTGGTCGCTTCAAGAGAAATGGGTACTGCATTGCAATTGGTTAACATCGCCAGAGATATC
AAAGGTGACGCTACAGAAGGTAGATTCTATTTGCCATTGTCTTTCTTTGGTTTGAGAGATGAA
TCTAAGTTGGCAATTCCTACAGACTGGACCGAACCAAGACCTCAAGATTTCGACAAGTTGTTA
TCTTTATCACCATCTTCAACCTTGCCTTCCAGTAACGCATCCGAAAGTTTTAGATTCGAATGGA
AGACTTACTCTTTACCATTGGTCGCATACGCCGAAGATTTGGCTAAGCATTCATACAAGGGTA
TAGACAGATTGCCAACAGAAGTTCAAGCTGGTATGAGAGCAGCCTGCGCCTCATACTTGTTGA
TAGGTAGAGAAATTAAAGTTGTCTGGAAGGGTGACGTTGGTGAAAGAAGAACTGTCGCTGGT
TGGAGAAGAGTAAGAAAGGTTTTGAGTGTAGTTATGTCTGGTTGGGAAGGTCAATAA

Table A2. Primers used in this thesis.

Name	Sequence (5'-3')
Primers used for validation of cloning-free methods (sections 3.3 and 4.1)	
UP-F	GCGGAGAAGTCGTTGATAGCA
DOWN-R	GATCATAGATCCGGCACTTAGAG
Hl-UP(40)-R	GAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(40)-F	CAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(60)-R	TCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(60)-F	AATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(100)-R	ACATGACCTGCAGCTTGACATCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(100)-F	GTGACTGTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Sl-UP(40)-R	TAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTT

Sl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(40)-F	AAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
Sl-UP(60)-R	AACTGATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(60)-F	GAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
Sl-UP(100)-R	AAAACTGAAGTAGATGGATGAACTGATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(100)-F	TGAAGACATCTGAAAGAAAGGAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
F1-F	GCGGAGAAGTCGTTGATAGCA
F3-R	GATCATAGATCCGGCACTTAGAG
Hl-F1-R	AACATTTTTTTACGTATCGCTTTGTTTTAT
Hl-F2-F	ATAAAACAAAGCGATACGTAAAAAAATGTT
Hl-F2-R	GAATGCACGCGATTTAAGC
Hl-F3-F	GCTTAAATCGCGTGCATTCAT
Sl-F1-R	AGTAGAGTACATTTTTTTACGTATCGC
Sl-F2-F	GCGATACGTAAAAAAATGTACTCTACT
Sl-F2-R	GATGAATGCACGCGATTTATG

Sl-F3-F	GCATAAATCGCGTGCATTCAT
UP-F	GCGGAGAAGTCGTTGATAGCA
DOWN-R	GATCATAGATCCGGCACTTAGAG
Hl-UP(40)-R	GAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(40)-F	CAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(60)-R	TCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(60)-F	AATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(100)-R	ACATGACCTGCAGCTTGACATCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(100)-F	GTGACTGTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Sl-UP(40)-R	TAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTT
Sl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(40)-F	AAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC

Sl-UP(60)-R	AACTGATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(60)-F	GAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
Sl-UP(100)-R	AAAACTGAAGTAGATGGATGAACTGATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(100)-F	TGAAGACATCTGAAAGAAAGGAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
F1-F	GCGGAGAAGTCGTTGATAGCA
F3-R	GATCATAGATCCGGCACTTAGAG
Hl-F1-R	AACATTTTTTTACGTATCGCTTTGTTTTAT
Hl-F2-F	ATAAAACAAAGCGATACGTAAAAAAATGTT
Hl-F2-R	GAATGCACGCGATTTAAGC
Hl-F3-F	GCTTAAATCGCGTGCATTCAT
Sl-F1-R	AGTAGAGTACATTTTTTTACGTATCGC
Sl-F2-F	GCGATACGTAAAAAAATGTACTCTACT
Sl-F2-R	GATGAATGCACGCGATTTATG
Sl-F3-F	GCATAAATCGCGTGCATTCAT
UP-F	GCGGAGAAGTCGTTGATAGCA
DOWN-R	GATCATAGATCCGGCACTTAGAG

Hl-UP(40)-R	GAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(40)-F	CAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(60)-R	TCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(60)-F	AATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(100)-R	ACATGACCTGCAGCTTGACATCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(100)-F	GTGACTGTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Sl-UP(40)-R	TAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTT
Sl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(40)-F	AAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
Sl-UP(60)-R	AACTGATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT

Sl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(60)-F	GAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
Sl-UP(100)-R	AAAACTGAAGTAGATGGATGAACTGATGTATAGAAAACAGTAGAGTA CATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAA AAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGA TTTATGCAGCTTCAACTGCT
Sl-DOWN(100)-F	TGAAGACATCTGAAAGAAAGGAAGAAAGAGAAGCAGTTGAAGCTGCA TAAATCGCGTGCATTCATCC
F1-F	GCGGAGAAGTCGTTGATAGCA
F3-R	GATCATAGATCCGGCACTTAGAG
Hl-F1-R	AACATTTTTTTACGTATCGCTTTGTTTTAT
Hl-F2-F	ATAAAACAAAGCGATACGTAAAAAAATGTT
Hl-F2-R	GAATGCACGCGATTTAAGC
Hl-F3-F	GCTTAAATCGCGTGCATTCAT
Sl-F1-R	AGTAGAGTACATTTTTTTACGTATCGC
Sl-F2-F	GCGATACGTAAAAAAATGTACTCTACT
Sl-F2-R	GATGAATGCACGCGATTTATG
Sl-F3-F	GCATAAATCGCGTGCATTCAT
UP-F	GCGGAGAAGTCGTTGATAGCA
DOWN-R	GATCATAGATCCGGCACTTAGAG
Hl-UP(40)-R	GAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG

Hl-DOWN(40)-F	CAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(60)-R	TCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(60)-F	AATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Hl-UP(100)-R	ACATGACCTGCAGCTTGACATCTTGGCAATGAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTT
Hl-ZEP(100)-F	TAATTATCTACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTG
Hl-ZEP(100)-R	AGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTG
Hl-DOWN(100)-F	GTGACTGTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
Sl-UP(40)-R	TAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTT
Sl-ZEP(40)-F	ACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(40)-R	AGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(40)-F	AAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC
Sl-UP(60)-R	AACTGATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT
Sl-ZEP(60)-F	CAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTCTATACAT
Sl-ZEP(60)-R	TTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT
Sl-DOWN(60)-F	GAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCC

Primers used for violaxanthin strains construction (section 3.2, 4.2-4.6)	
SlZEP-HF	CAAATATAAAACAAAGCGATACGTAAAAAAATGTACTCTACTGTTTTC TATACATCAG
SlZEP-HR	TTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCTTCAACTGCT TCT
V-SlZEP-HF	GAAGAAAGAGAAGCAGTTGAAGCTGCATAAATCGCGTGCATTCATCCG
V-SlZEP-HR	GATGTATAGAAAACAGTAGAGTACATTTTTTTACGTATCGCTTTGTTTT ATATT
V-tr49-SlZEP-HF	CATCTGAAAGAAAGGAAGAAAGAGAAGCAGTTGAAGCTGCATAAATC GCGTGCATTCATC
V-tr49-SlZEP-HR	TTAACACCCTTAACCTTCATTTTTTTACGTATCGCTTTGTTTTATATTTG TTG
tr49-SlZEP-HF	ACAAAGCGATACGTAAAAAAATGAAGGTTAAGGGTGTTAAGGTTAAG
tr49-SlZEP-HR	CTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTATGCAGCT TCAACTGCTTC
V-tr25-SlZEP-HR	AGAATGGTACAATTCAGCTGAGAAATCCTTAGAAATCATTTTTTTACGT ATCGCTTTGTT
tr25-SlZEP-HF	TTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGATTTCTAAG GATTTCTCAGCT
V-tr75-SlZEP-HR	CTTCAATTTCTTTTGTGGAACTTTCAAGTCACCATTCATTTTTTTACGTA TCGCTTTGTT
tr75-SlZEP-HF	TTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGAATGGTG ACTTGAAAGTTCC
V-tr100-SlZEP-HR	ACCAAAACATCGAAACCTCTTTTCTTAGCAGCCAATGCCATTTTTTTAC GTATCGCTTTG
tr100-SlZEP-HF	TACTTTTTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGGCA TTGGCTGCTAAG

V-AtZEP-HF	TAATGATAAATTGTTACAAACTGCTTAAATCGCGTGCATTCATCC
V-AtZEP-HR	CAAAATGGTGTAGAACCCATTTTTTTACGTATCGCTTTGTTT
AtZEP-HF	TATAAAACAAAGCGATACGTAAAAAAATGGGTTCTACACCATTTTGTTACTCAATT
AtZEP-HR	TCGGTTAGAGCGGATGAATGCACGCGATTTAAGCAGTTTGTAACAATTTATCATTATTTG
V-tr57-AtZEP-HR	AAAGACCATCTATGATCCAACATTTTTTTACCCGTTGATTTGTAATTAA
tr57-AtZEP-HF	AAGCGATACGTAAAAAAATGGCTGCAACAGCATTAGTTGAAAA
tr57-AtZEP-HR	AGCGGATGAATGCACGCGATTTAAGCAGTTTGTAACAATTTATCATTATTTG
V-tr30-AtZEP-HR	AAATCCAAATAGAATTGCATTTTTTTACGTATCGCTTTGTTTTATATTTG
tr30-AtZEP-HF	ACAAAGCGATACGTAAAAAAATGCAATTCTATTTGGATTTGTCTTCTTTTTC
V-tr80-AtZEP-HR	ACCACCACCAGCAACCAAAACTCTTGACATTTTTTTACGTATCGCTTTGTT
tr80-AtZEP-HF	CAAATATAAAACAAAGCGATACGTAAAAAAATGTCAAGAGTTTTGGTTGCT
V-tr100-AtZEP-HR	GAAAACCAAAACATCGAAACCTTTCTTCATTTTTTTACGTATCGCTTTGTT
tr100-AtZEP-HF	CAAATATAAAACAAAGCGATACGTAAAAAAATGAAGAAAGGTTTCGATGTT
V-HlZEP-HF	CAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATCC
V-HlZEP-HR	GAAGAAGTATGTAACAACATTTTTTTACGTATCGCTTTGTTTTATATTT
HlZEP-HF	ACAAAGCGATACGTAAAAAAATGTTGTTACATACTTCTTCATTGCCA
HlZEP-HR	AGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTGC
V-tr59-HlZEP-HF	GTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCGTGCATTCATC

V-tr59-HlZEP-HR	TGACCTGGTCTTGCAGCTTCCATTTTTTTACGTATCGCTTTGT
tr59-HlZEP-HF	AAGCGATACGTAAAAAAATGGAAGCTGCAAGACCAGGT
tr59-HlZEP-HR	CTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGATTTAAGCTGCTTGAACCATTGC
V-tr30-HlZEP-HR	TGGTGCAGCAGAACCATGATGACATGATGGAACTAACATTTTTTTACGTATCGCTTTGTT
tr30-HlZEP-HF	TTACAACAAATATAAAACAAAGCGATACGTAAAAAAATGTTAGTTCCATCATGTCATCAT
V-tr80-HlZEP-HR	GCAATAACAATAGTTAACATTTTTTTACGTATCGCTTTGTT
tr80-HlZEP-HF	ACAAAGCGATACGTAAAAAAATGTTAACTATTGTTATTGCAGGTG
V-tr100-HlZEP-HR	ATCTCTTTCCAAAACTTGACACTTAACACCTTTCTTCATTTTTTTACGTATCGCTTTGTT
tr100-HlZEP-HF	TACAACAAATATAAAACAAAGCGATACGTAAAAAAATGAAGAAAGGTGTTAAGTGTCAAG
UP-PGK1-HF	GCGGAGAAGTCGTTGATAGCA
DW-CYC1-HR	GATCATAGATCCGGCACTTAGAG
CrtZ-R-ZEP-HR	GAAGCCGCAGCAAAAGAAGCCGCAGCTAAAGAAGCAGCTGCAAAGGAAGCTGCAAGACCAGGTC
CrtZ-R-ZEP-HF	AAGTGTCAACAACGTATCTACCAACGGAATGCGTGCGATTTAAGCTGCTTGAACCATTGC
DW-CrtZ-R-ZEP-HF	AGCTGCTTCTTTAGCTGCGGCTTCTTTTGCTGCGGCTTCTTTACCTGATGCTGGTTCATC
CrtZ-F-ZEP-HR	GGCGGTGGTGGTAGTGGTGGCGGAGGTTCTGGTGGAGGAGGTAGCGAAGCTGCAAGACCAGGTC
CrtZ-F-ZEP-HF	AAGTGTCAACAACGTATCTACCAACGGAATGCGTGCGATTTAAGCTGCTTGAACCATTGC

DW-CrtZ-F-ZEP-HF	TCCTCCACCAGAACCTCCGCCACCACTACCACCACCGCCTTTACCTGATGCTGGTTCATC
UP-CrtZ-L-ZEP-HR	TGTTACCAAAGAATTAATAGAGGTGCAATGGTTCAAGCAGCTTAAATCGCACGCATTCCG
ZEP-R-CrtZ-HF	CTTTGCAGCTGCTTCTTTAGCTGCGGCTTCTTTTGCTGCGGCTTCAGCTGCTTGAACCATTGC
UP-ZEP-R-CrtZ-HR	CCGCAGCAAAAGAAGCCGCAGCTAAAGAAGCAGCTGCAAAGATGTTGTGGATCTGGAACG
DW-ZEP-R-CrtZ-HF	ATCTTTCTTGTTGACCTGGTCTTGCAGCTTCCATTTTTTTACCCGTTGATTTGTAATTAA
UP-ZEP-F-CrtZ -HR	GTGGTGGTAGTGGTGGCGGAGGTTCTGGTGGAGGAGGTAGCATGTTGTGGATCTGGAACG
DW-ZEP-F-CrtZ-HF	ATCTTTCTTGTTGACCTGGTCTTGCAGCTTCCATTTTTTTACCCGTTGATTTGTAATTAA
ZEP-F-CrtZ-HF	GCTACCTCCTCCACCAGAACCTCCGCCACCACTACCACCACCGCCAGCTGCTTGAACCATTGC
ZEP-L-CrtZ-HR	ACAAATCAACGGGTAAAAAAATGGAAGCTGCAAGACCAGGTC
XI2-UP-HF	GAGGATTTTCGATGGAGCAGGATGA
XI2-DW-HR	GTGGGAAGATTCCGCTCTACC
V-tr-CrtR-b2-GF	TTTTCCAAAACGAAACACATTTTTTTACCCGTTGATTTGTAATTAAAACTTAG
V-tr-CrtR-b2-GR	TTTCAAAAGGTTTGTTATAAATCGCACGCATTCCGTTG
tr-CrtR-b2-GF	ACAAATCAACGGGTAAAAAAATGTGTTTCGTTTTGGAAAACG
tr-CrtR-b2-GR	ACCAACGGAATGCGTGCGATTTATAACAAACCTTTTGAAATTTTAATTCTTCTATTA
V-HlCrtZ-GF	ACTGGTTTATGAAAAGTCATTTTTTTACCCGTTGATTTGTAATTAA
V-HlCrtZ-GR	TGGATTGGTCAAAAAGATAAATCGCACGCATTCCG

HlCrtZ-GF	ACAAATCAACGGGTAAAAAAATGACTTTTCATAAACCAGTTTCTGG
HlCrtZ-GR	ACCAACGGAATGCGTGCGATTTATCTTTTTGACCAATCCAATTCC
V-tr-HlCrtZ-GF	GTTTGTGGTGCAGCAACTCTCATTTTTTTACCCGTTGATTTGTAATTAA
V-tr-HlCrtZ-GR	TGGATTGGTCAAAAAGATAAATCGCACGCATTCCGT
tr-HlCrtZ-GF	AATCAACGGGTAAAAAAATGAGAGTTGCTGCACCACAAACT
tr-HlCrtZ-GR	ACCAACGGAATGCGTGCGATTTATCTTTTTGACCAATCCAATTCC
CrtR-b2-GF	ACAAATCAACGGGTAAAAAAATGGCTGCAGGTATTTC
CrtR-b2-GR	ACCAACGGAATGCGTGCGATTTATAACAAACCTTTTGAAATT
V-CrtR-b2-GF	GCAGAAATACCTGCAGCCATTTTTTTACCC
V-CrtR-b2-GR	TTTCAAAAGGTTTGTTATAAATCGCACGCATTCCG
PaCrtZ-GF	ACAAATCAACGGGTAAAAAAATGTTGTGAATCTGGAACG
PaCrtZ-GR	ACCAACGGAATGCGTGCGATTTATTTACCTGATGCTGGTTCATC
V-PaCrtZ-GF	GCGTTCCAGATCCACAACATTTTTTTACCCGTTGATTTGTAATT
V-PaCrtZ-GR	AACCAGCATCAGGTAAATAAATCGCACGCATTCCG
tr-YAH1-GF	AAGCGATACGTAAAAAAATGACCACAACCACAAGATTTCTGC
tr-YAH1-GR	AGCGGATGAATGCACGCGATTTAACTAAAATCGTTGTTATTAACGTTTC TT
V-tr-YAH1-GF	ATAACAACGATTTTAGTTAAATCGCGTGCATTCATCC
V-tr-YAH1-GR	AGAAATCTTGTGGTTGTGGTCATTTTTTTACGTATCGCTTTGTTTTATAT TT
tr25-ARH1-GF	AATCAACGGGTAAAAAAATGTCCGGCTTTTATACAGCGTAC
tr25-ARH1-GR	ACCAACGGAATGCGTGCGATTTATATGCCTTCTACACCGTTCCA
V-tr25-ARH1-GF	TACGCTGTATAAAAGCCGGACATTTTTTTACCCGTTGATTTGTAATTAA
V-tr25-ARH1-GR	ACGGTGTAGAAGGCATATAAATCGCACGCATTCCGTT

tr50-ARH1-GF	AATCAACGGGTAAAAAAATGCCTGTTCCTTTTGGTTTAAGTAGATATG
tr50-ARH1-GR	ACCAACGGAATGCGTGCGATTTATATGCCTTCTACACCGTTCCA
V-tr50-ARH1-GF	CTTAAACCAAAAGGAACAGGCATTTTTTTACCCGTTGATTTGTAATTAA
V-tr50-ARH1-GR	ACGGTGTAGAAGGCATATAAATCGCACGCATTCCGTT
FD3-GF	ACAAAGCGATACGTAAAAAAATGGCTACTGTTAGAATTTCTTCAACT
FD3-GR	AGCGGATGAATGCACGCGATTTAAAACAATTCTGTTTCTTTATGAGTATGA
V-FD3-GF	AAGAAACAGAATTGTTTTAAATCGCGTGCATTCATCC
V-FD3-GR	GAAATTCTAACAGTAGCCATTTTTTTACGTATCGCTTTGTTTTATATTT
tr-FD3-GF	AAGCGATACGTAAAAAAATGGCTAATTCAGGTGGTGCTACAATG
tr-FD3-GR	AGCGGATGAATGCACGCGATTTAAAACAATTCTGTTTCTTTATGAGTATGA
V-tr-FD3-GF	AAGAAACAGAATTGTTTTAAATCGCGTGCATTCATCC
V-tr-FD3-GR	GTAGCACCACCTGAATTAGCCATTTTTTTACGTATCGCTTTGTTTTATATTT
RFNR1-GF	ACAAATCAACGGGTAAAAAAATGGCATTGTCAACTACACCAT
RFNR1-GR	ACCAACGGAATGCGTGCGATTTAGTAAACTTCAACATGCCATTGCT
V-RFNR1-GF	GGTGTAGTTGACAATGCCATTTTTTTACCCGTTGATTTGTA
V-RFNR1-GR	GGCATGTTGAAGTTTACTAAATCGCACGCATTCCGTT
tr-RFNR1-GF	AATCAACGGGTAAAAAAATGCAATCTTCAAAGTCTAAGGTTTTGGTT
tr-RFNR1-GR	ACCAACGGAATGCGTGCGATTTAGTAAACTTCAACATGCCATTGCT
V-tr-RFNR1-GF	ACCTTAGACTTTGAAGATTGCATTTTTTTACCCGTTGATTTGTAATTAA
V-tr-RFNR1-GR	GGCATGTTGAAGTTTACTAAATCGCACGCATTCCGTT
CrtE-GF	TAAAACAAAGCGATACGTAAAAAAATGGACTATGCCAACATCTTAAC
CrtE-GR	TAGAGCGGATGAATGCACGCGTCATAATGGGATGTCTGCC

V-CrtE-GF	AAAGTTGGCAGACATCCCATTATGAATCGCGTGCATTCATCCGCTCTAACCG
V-CrtE-GR	AAGATGTTGGCATAGTCCATTTTTTTACGTATCGCTTTGTTTTA
CrtYB-GF	ATAAAACAAAGCGATACGTAAAAAAATGACGGCTCTCGCATATTACCAGATC
CrtYB-GR	CTAATTACATGACTCGAGGTCGACTTACTGCCCTTCCCATCCGCTCATGAC
V-CrtYB-GF	GCGGATGGGAAGGGCAGTAAGTCGACCTCGAGTCATGTAATTAGTTATG
V-CrtYB-GR	TAATATGCGAGAGCCGTCATTTTTTTACGTATCGCTTTGTTTTATATTTGTTGTAAAAAGTAG
CYC1	TTACATGCGTACACGCGTCT
ADH1	GAGCGACCTCATGCTATACCT
TEF1	CTCTTTCGATGACCTCCCATTGAT
PGK1	CAACAACAGCCTGTTCTCACACAC
XI5-DW	CTTAAGGTTGTTGTCACAACCCACG
XI5-UP	GGTAGCAACGAAAGCTAGTCGCA
X2-DW	AGTCGCCCGGTTGTTGAAA
X2-UP	CTCGGAGATGGCGCATCTATTTG
XI2-UP	CGCAAAACTCAGTAATAAGCTTTCTG
XI2-DW	CGCACCAAAAGTAAGAAACG
XI3-DW	GCAAGTTCTGTGGAGTTCCATGT
XI3-UP	GCGTTTATTGTCGCATCGCTAGC

Table A3. Total carotenoid yields in the different strains.

Strain	**Total carotenoids mg/g_{DCW} ± SD**
SM14	15.27 ± 0.56
SM14-ΔURA3	14.79 ± 0.49
PaCrtZ	9.17 ± 0.68
HlCrtZ	8.91 ± 0.83
tr-HlCrtZ	9.43 ± 0.61
CrtR-b2	9.11 ± 0.56
tr-CrtR-b2	9.02 ± 0.66
SlZEP	9.63 ± 0.74
tr25-SlZEP	8.84 ± 0.81
tr49-SlZEP	9.59 ± 0.55
tr75-SlZEP	9.24 ± 0.67
tr100-SlZEP	9.36 ± 0.53
HlZEP	9.19 ± 0.81
tr30-HlZEP	8.96 ± 0.77
tr59-HlZEP	9.15 ± 0.58
tr80-HlZEP	9.45 ± 0.70
tr100-HlZEP	9.20 ± 0.51
AtZEP	9.27 ± 0.57
tr30-AtZEP	9.46 ± 0.61
tr57-AtZEP	9.08 ± 0.75
tr80-AtZEP	9.68 ± 0.49

tr100-AtZEP	9.58 ± 0.60
CrtZ-R-ZEP	9.25 ± 0.71
CrtZ-F-ZEP	9.43 ± 0.57
ZEP-R-CrtZ	9.18 ± 0.69
ZEP-F-CrtZ	9.12 ± 0.77
tr25-ARH1/tr-YAH1	9.55 ± 0.84
tr50-ARH1/tr-YAH1	9.22 ± 0.53
RFNR1/FD3	9.49 ± 0.63
RFNR1/tr-FD3	9.33 ± 0.46
tr-RFNR1/tr-FD3	9.11 ± 0.74
tr-FD3	9.29 ± 0.65
tr-RFNR1	9.37 ± 0.57
+CrtE	9.4 ± 0.85
+CrtYB	12.3 ± 0.89
+YB/tr59-HlZEP	12.1 ± 0.75
+YB/2 tr59-HlZEP	12.5 ± 0.68
+CrtI	Lethal
SM14 + CrtI	42.3 ± 1.26

APPENDIX B: SUPPLEMENTARY FIGURES

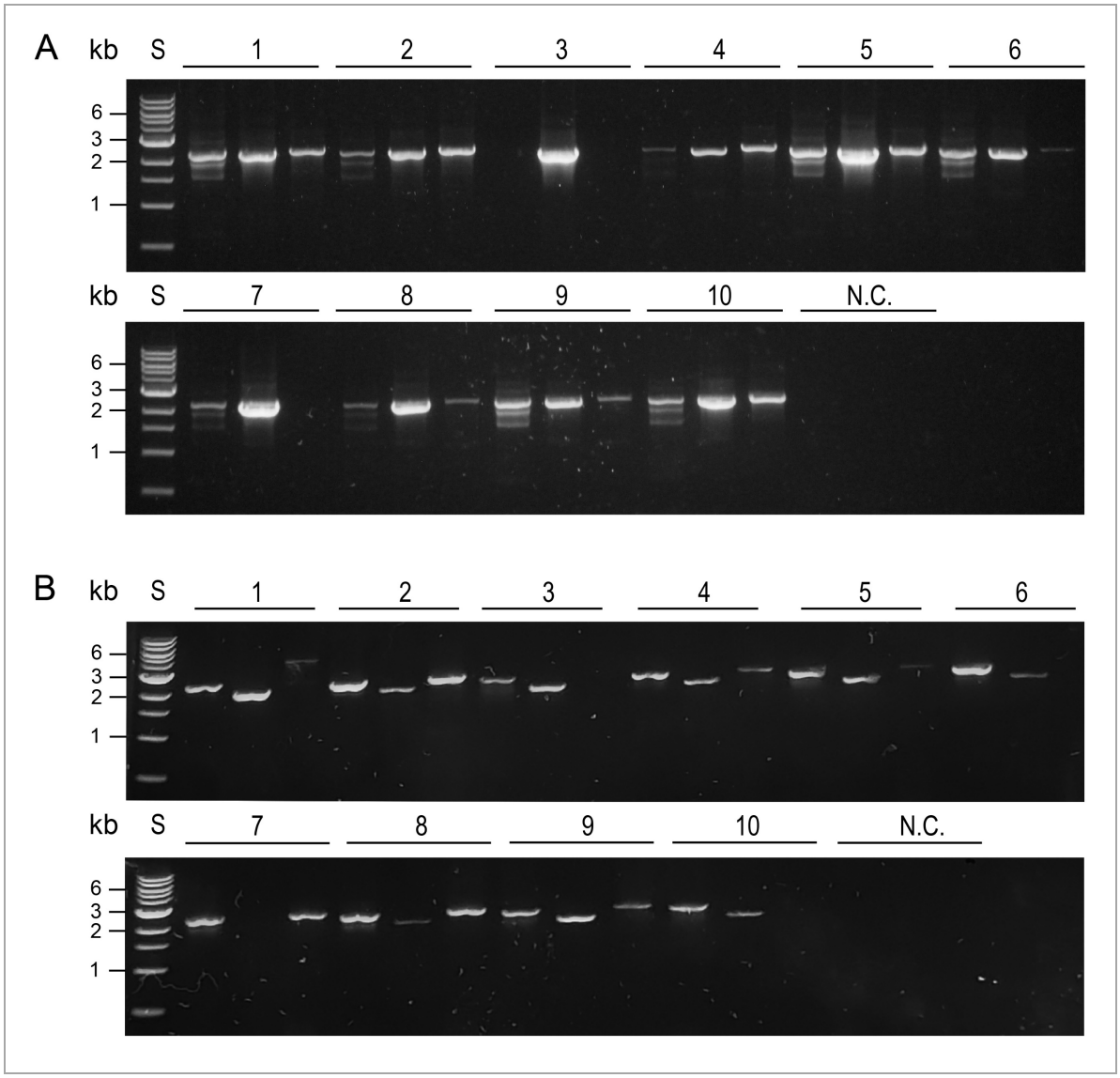

Figure B1. Examples of PCR analysis of genomic DNA of 10 yeast transformants obtained for direct assembly by HR of **(A)** HlZEP with 100 bp of overlap, and **(B)** SlZEP with 60 bp of overlap. PCR of fragments 1, 2, and 3 (refer to Figure 3) are shown for each transformant (1-10). S: 1 kb DNA ladder. N.C.: Negative control.

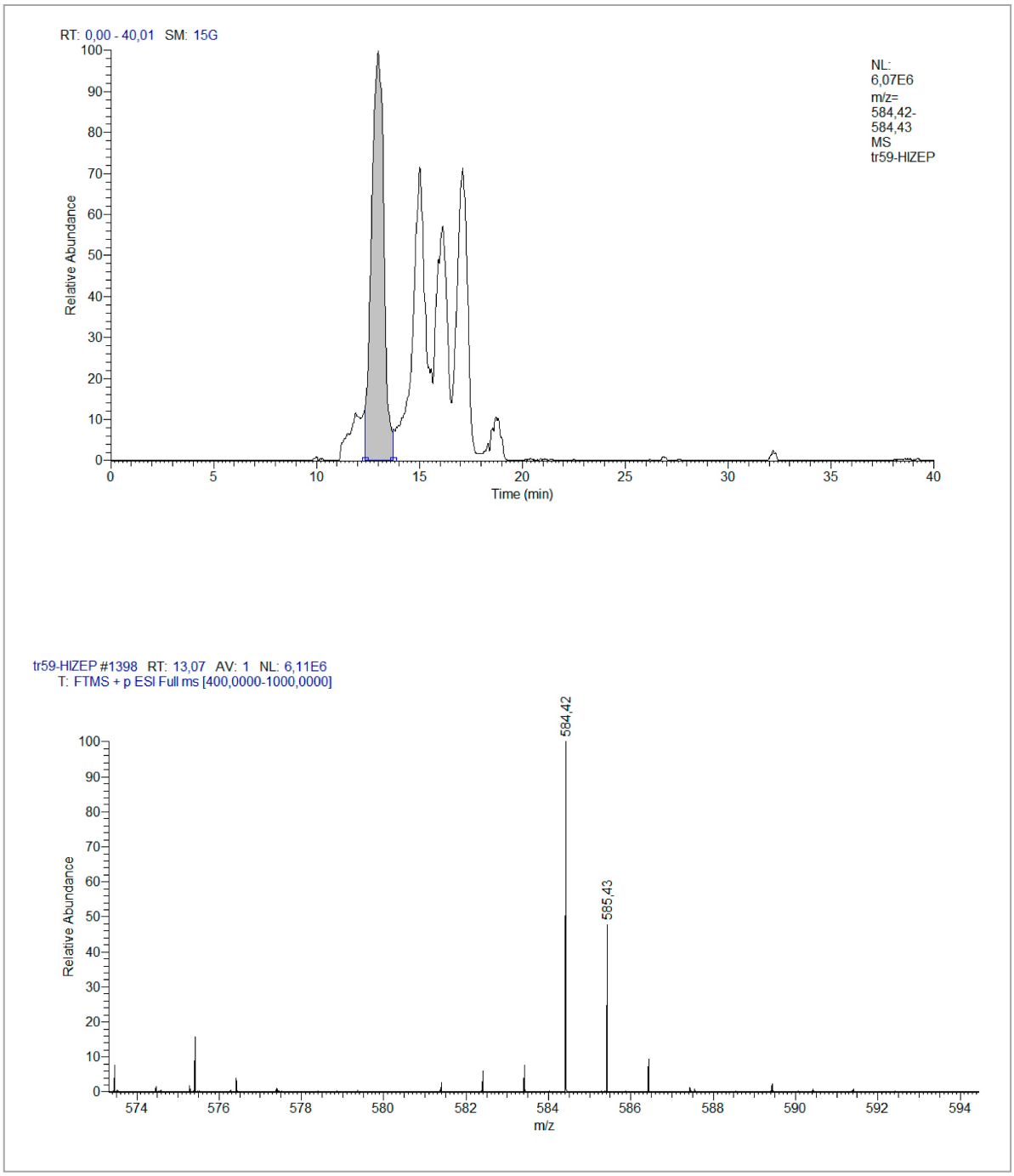

Figure B2. Identification of antheraxanthin by UPLC-MS. Carotenoid extracts of the tr59-HlZEP strain were analyzed by UPLC-MS using the same column and elution program of the HPLC-DAD analysis (see section 2.6). The most abundant masses at the retention time antheraxanthin (13.1 min) were 584.42 and 585.43, corresponding to the predicted exact masses of antheraxanthin molecular cation and the protonated molecule, respectively.

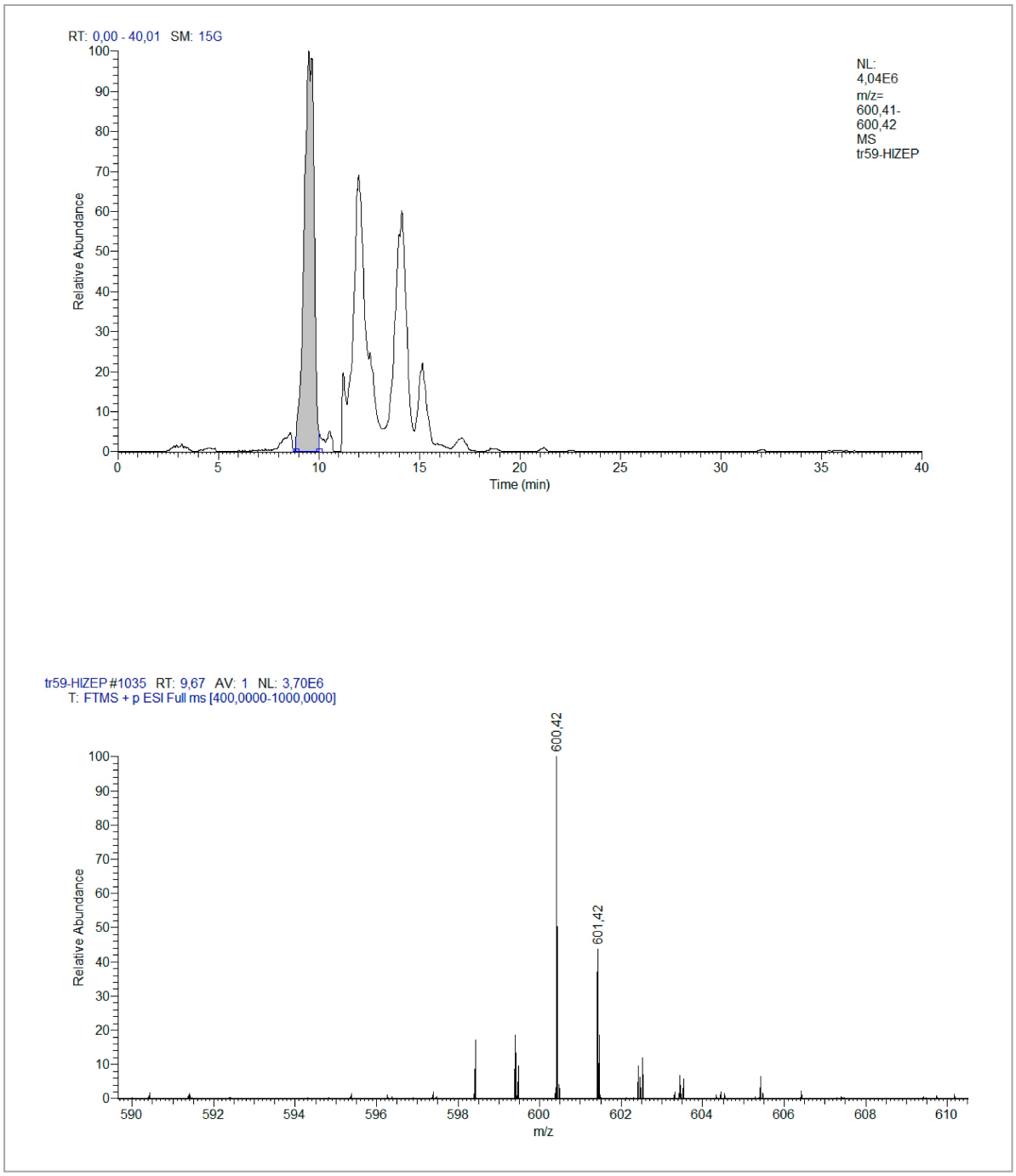

Figure B3. Identification of violaxanthin by UPLC-MS. Carotenoid extracts of the tr59-HlZEP strain were analyzed by UPLC-MS using the same column and elution program of the HPLC-DAD analysis (see section 2.6). The most abundant masses at the retention time of violaxanthin (9.7 min) were 600.42 and 601.42, corresponding to the predicted exact masses of violaxanthin molecular cation and the protonated molecule, respectively.

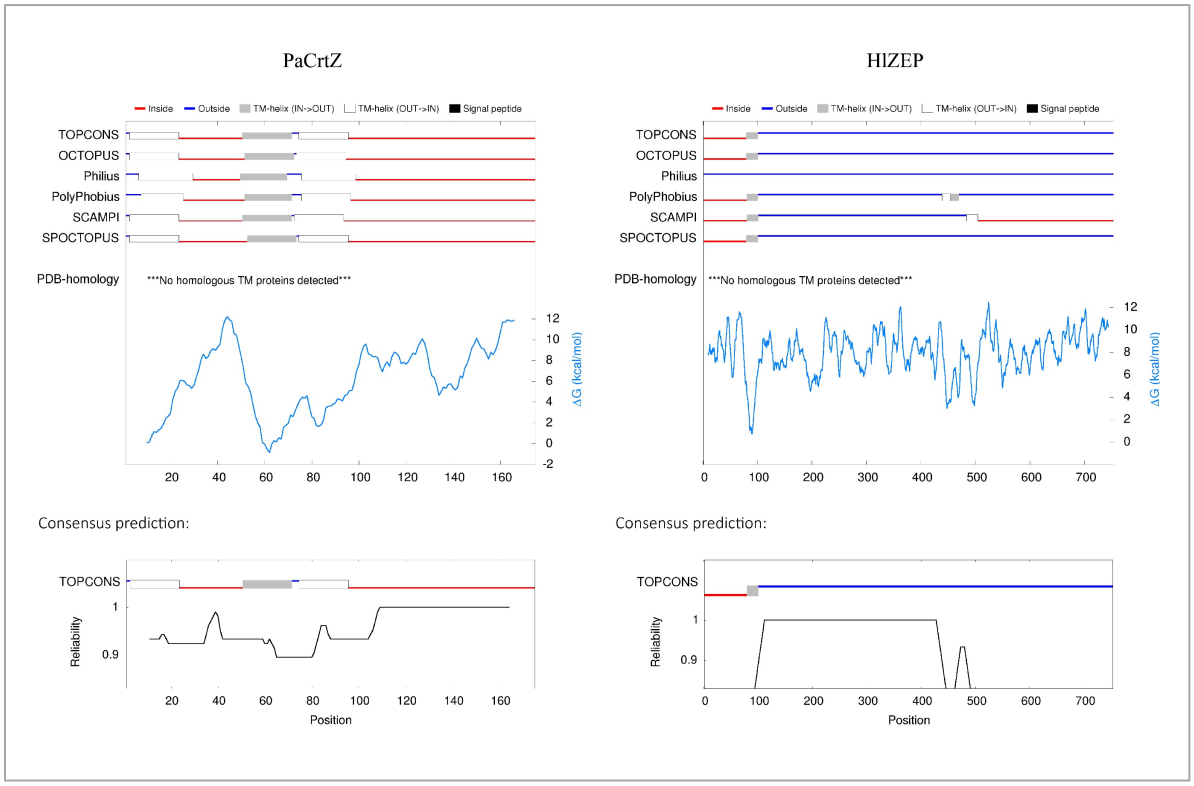

Figure B4. Transmembrane regions prediction by TOPCONS server. Aminoacidic sequences of PaCrtZ and HlZEP were analyzed with TOPCONS server for prediction of membrane protein topology. PaCrtZ showed a consensus prediction of three transmembrane helices, while HlZEP was predicted as single-pass transmembrane protein.