CYAnobacterial platform Optimised for bioproduction

Metabolic engineering of carotenoids biosynthesis in *Synechocystis* sp. PCC 6803: astaxanthin accumulation

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CYAO Closure Meeting

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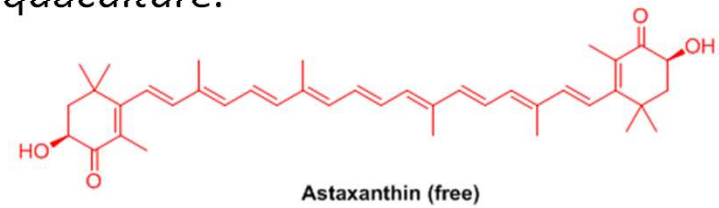
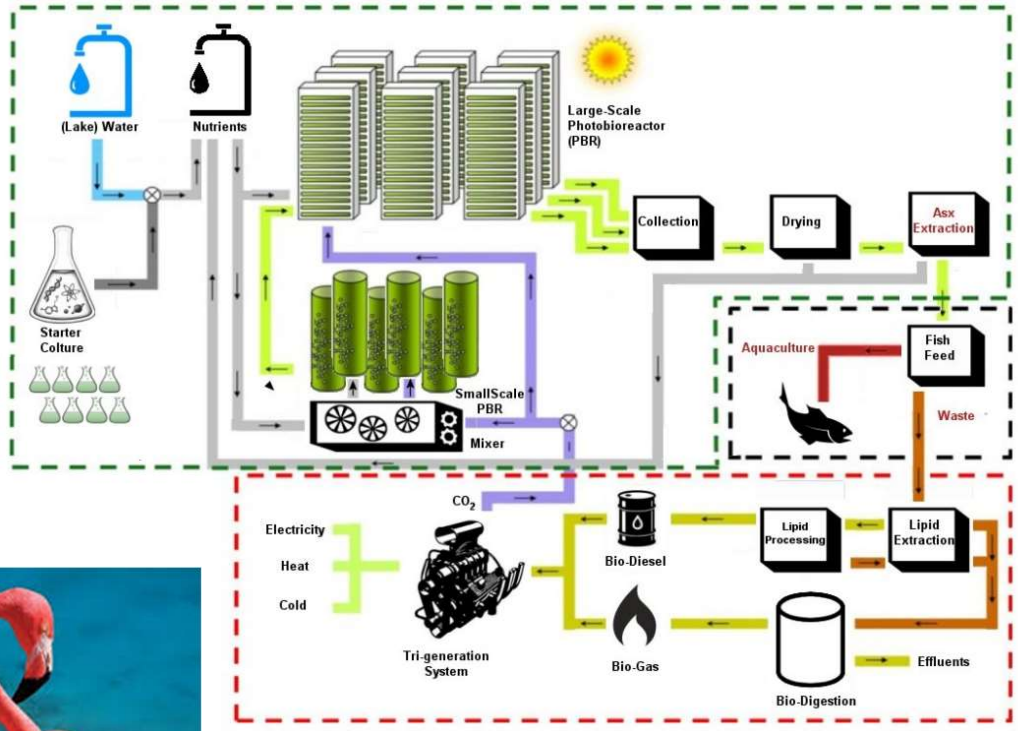
CYAO: Cyanobacterial Platform Optimised for Bioproduction

http://www.cyaoproject.org/

The **CYAO project** aims at improving the biomass productivity of cyanobacateria and at producing an economically attractive added-value product ASTAXANTHIN, to be employed as bio-colourant in aquaculture.



Astaxanthin (free)



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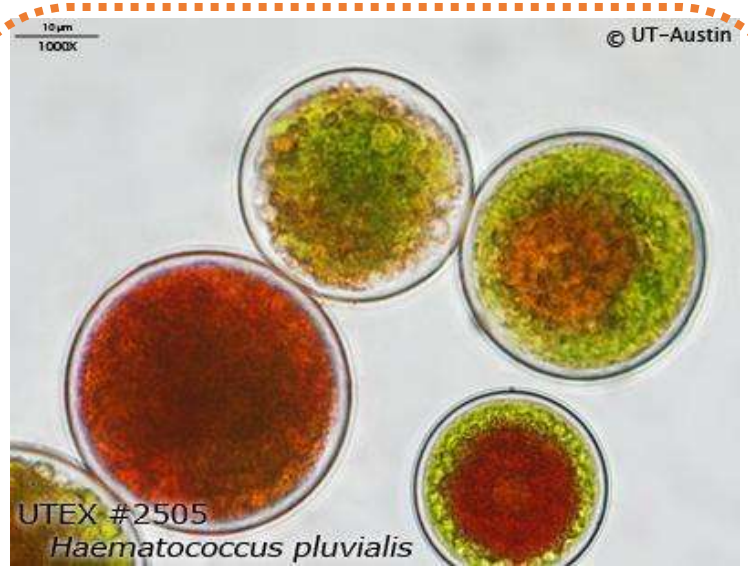
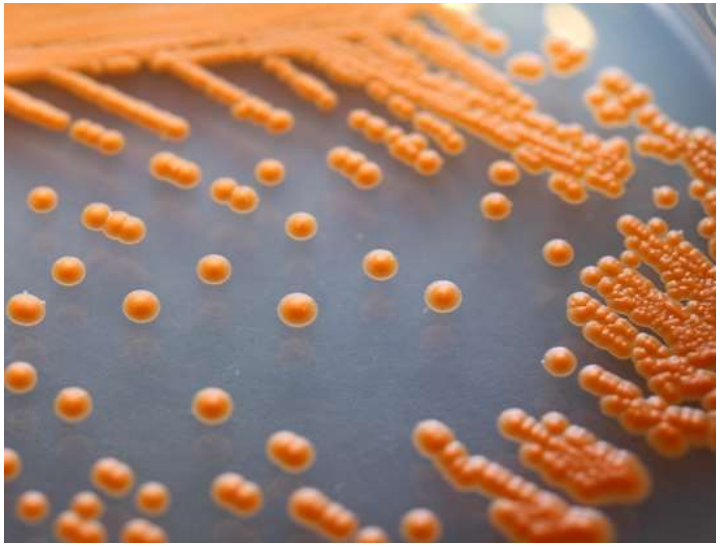
Natural sources for astaxanthin production



green alga

Haematococcus pluvialis



heterobasidiomycetous yeast

Xanthophyllomyces dendrorhous

40 mg/g dry cell weight (DCW)

DRAWBACKS

X Asx produced in esterified form

X long two-stage culture periods

X complicated extraction procedure

X the fermentation process required is expensive and difficult to scale up on an industrial scale

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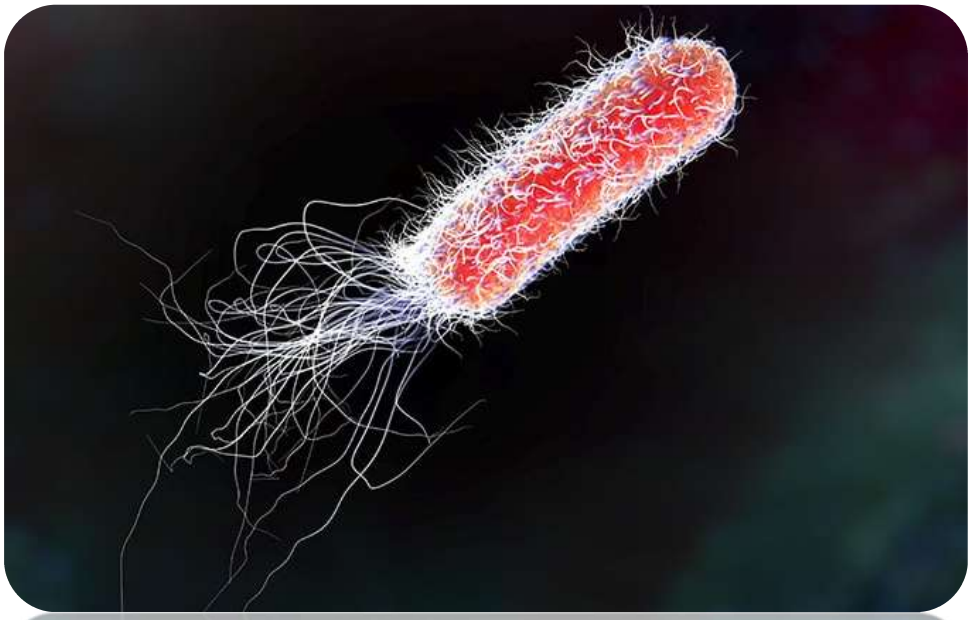
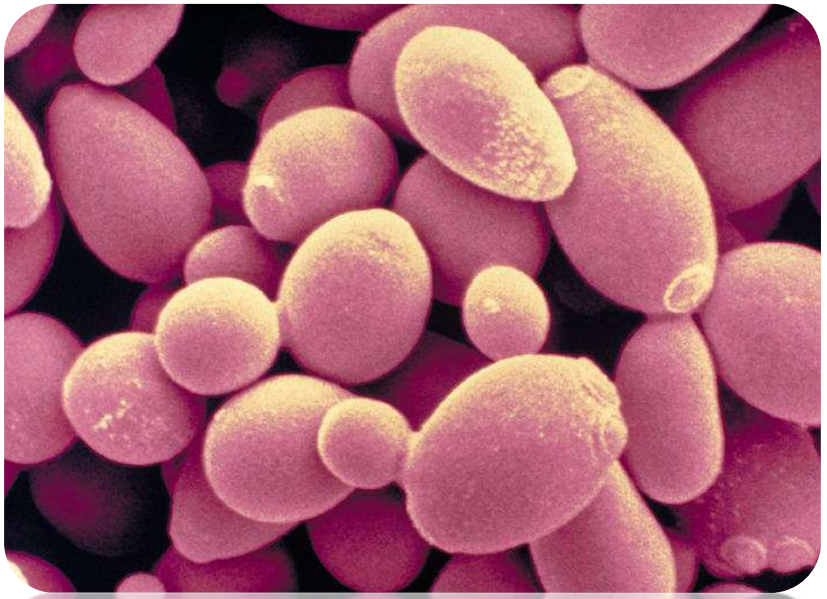
Microbial cell factories for astaxanthin production

introduction of key enzymes of the Asx
biosynthetic pathway from different species

Escherichia coli



Saccharomyces cerevisiae



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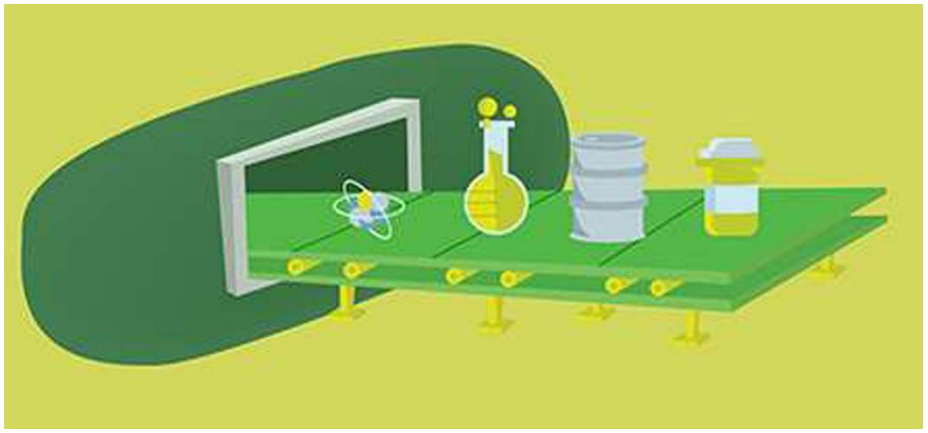
Why cyanobacteria?

Promising

"green *E. coli*"

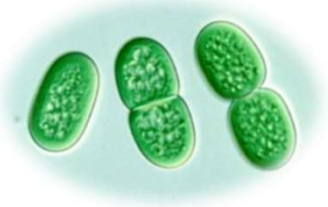


Which microorganisms?

cyanobacterial species:

***Synechocystis* sp. PCC 6803**

***Synechococcus elongatus* PCC 7942**

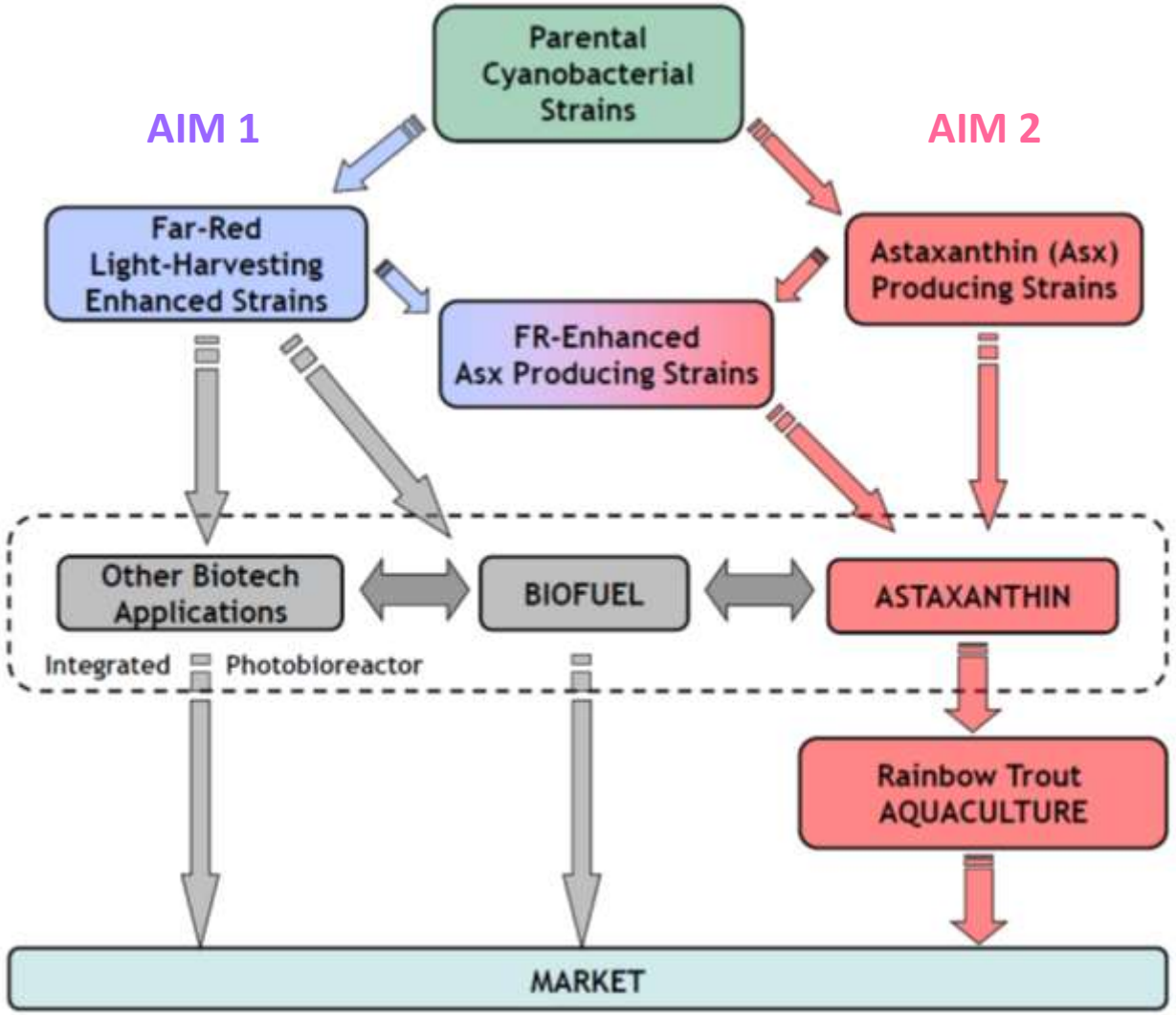


- ✓ *Synechocystis sp*. PCC6803 and *Synechococcus elongatus* PCC 7942 are **model cyanobacteria species** commonly employed for biotechnological applications.
- ✓ They are **easy to be genetically manipulated** and several constructs and tools for genetic engineering are available.
- ✓ They are easy to be cultured and harvested**.**

The goals of the CYAO project are:

1

2



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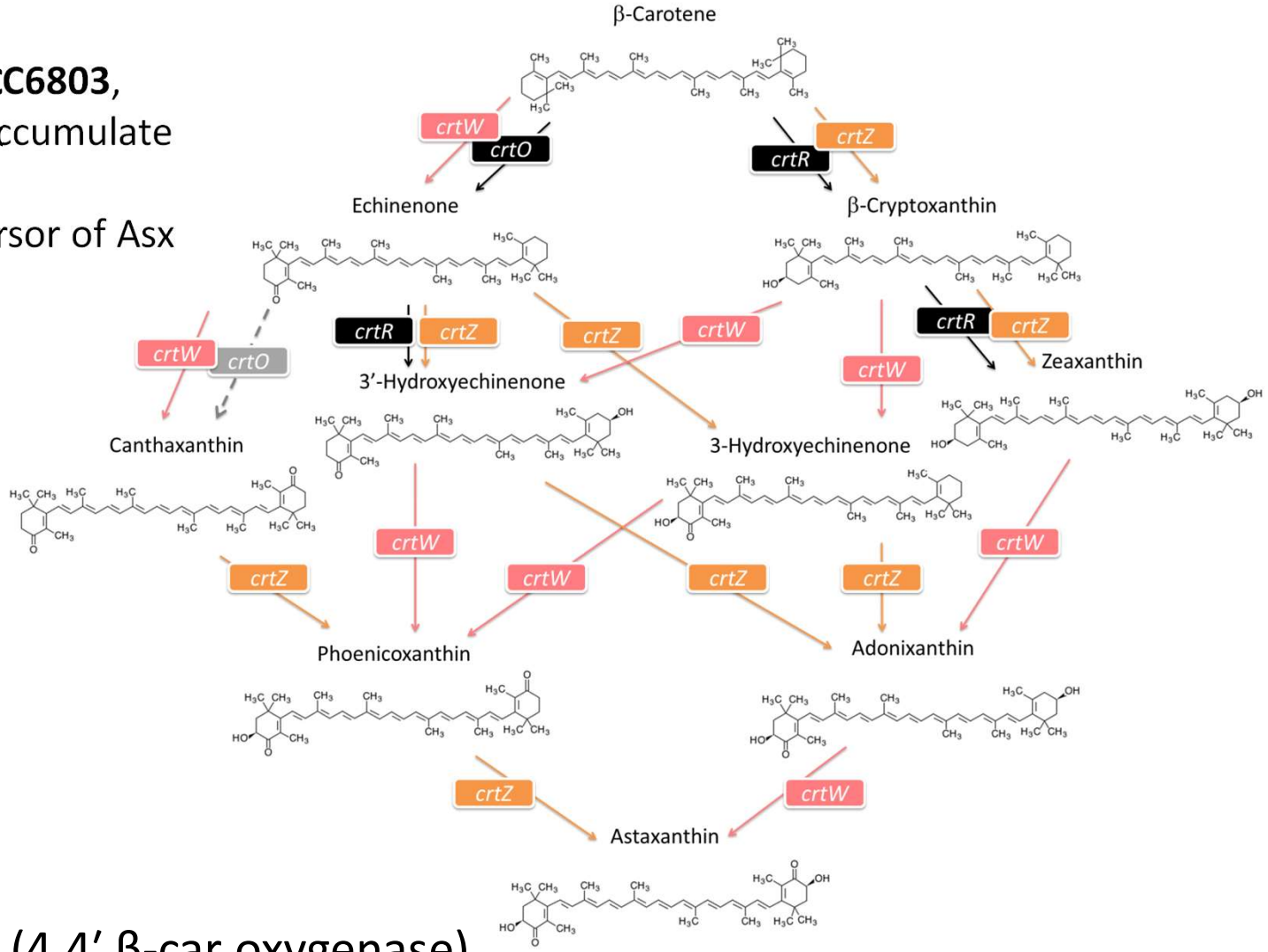
Experimental Strategy: astaxanthin producing strains

***Synechocystis sp*. PCC6803**,
is naturally able to accumulate
zeaxanthin (Zea),
the metabolic precursor of Asx



Introduction of:

- ✓ **CrtW ketolase** (4,4′ β-car oxygenase)
- ✓ **CrtZ hydroxylase** (3,3′ β-car hydroxylase)

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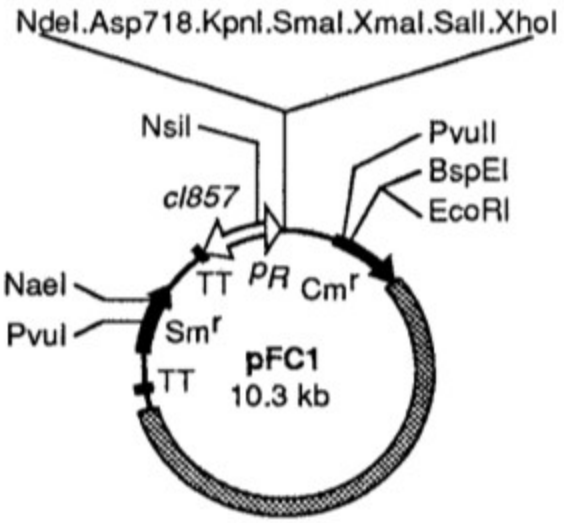
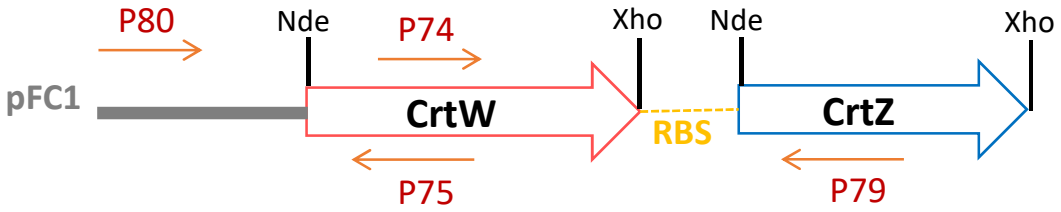
Construction of the **conditional expression vector** pFC1 for the espression of CrtW and CrtZ

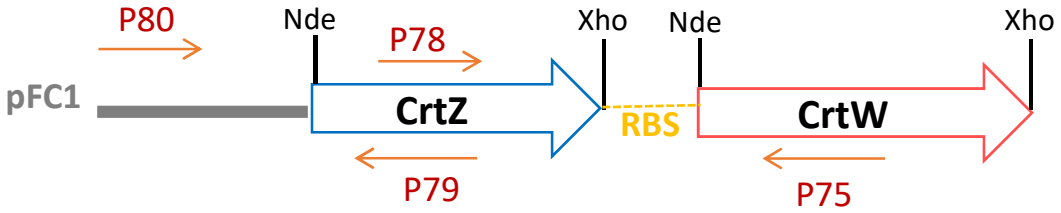
- ✓ pFC1 replicates autonomously in *E. coli* and several cyanobacteria of the genera Synechocystis and Synechococcus, and harbours the **λcI857 temperature-sensitive repressor-encoding gene** that tightly controls the activity of the otherwise strong λpR promoter located downstream
- ✓ Cloning of CrtW ketolase and CrtZ hydroxylase inside the **conditional expression vector pFC1**
- ✓ Design of **specific primer** combinations and **validation of the expression vectors**



(Mermet-Bouvier and Chauvat, 1994 CURRENT MICROBIOLOGY VOL.28)



CrtW/Z



CrtZ/W

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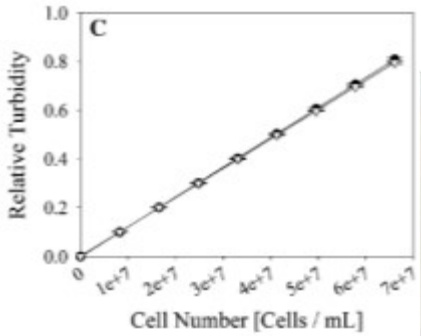
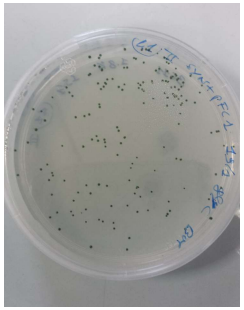
Validation and optimization of the **biparental matings protocol** for *Synechocystis* PCC6803

1. • **Culture preparation:** *E. coli* CM404 pFC 1 (Sp^R and Sm^R) + CrtW/Z (col 41) or + CrtZ/W (col 31) and *Synechocystis* cells were exponentially (3-5 x 10^8)grown
2. • **Coniugation:** The mating mixtures were poured into 6 cm petri dish and incubated under light (3000 lux) at 27°C for 24 h without shaking
3. • **Plating:** 0.1-ml aliquots were spread on heavily poured BG11 plates (40 ml per plate) and further incubated for 20-24 h prior to selection
4. • **Selection:** The antibiotic solution mix (final concentration of 5 μg/ml of Sp/Sm) was spread underneath the agar plates





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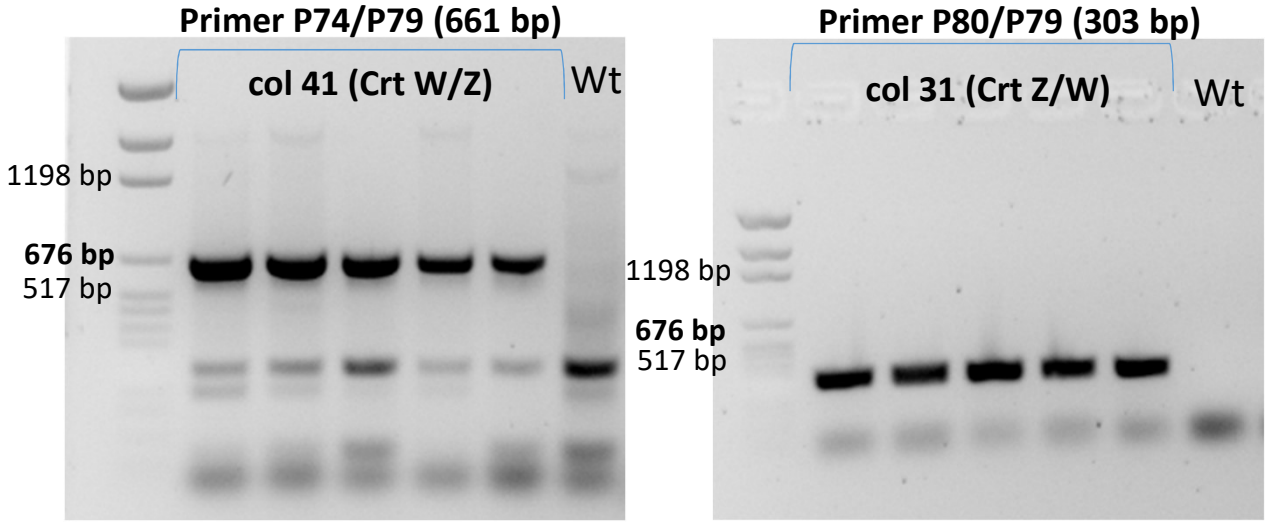
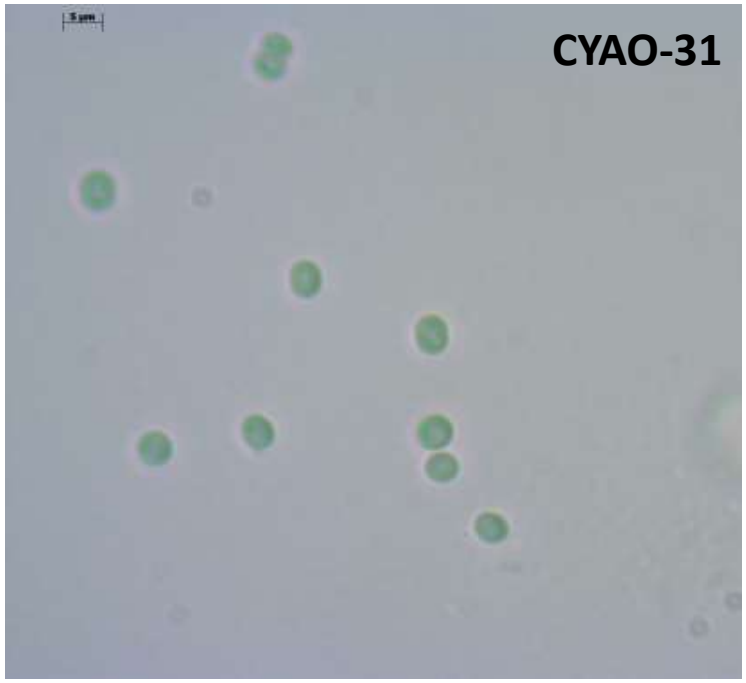
Screening of *Synechocystis* mutants

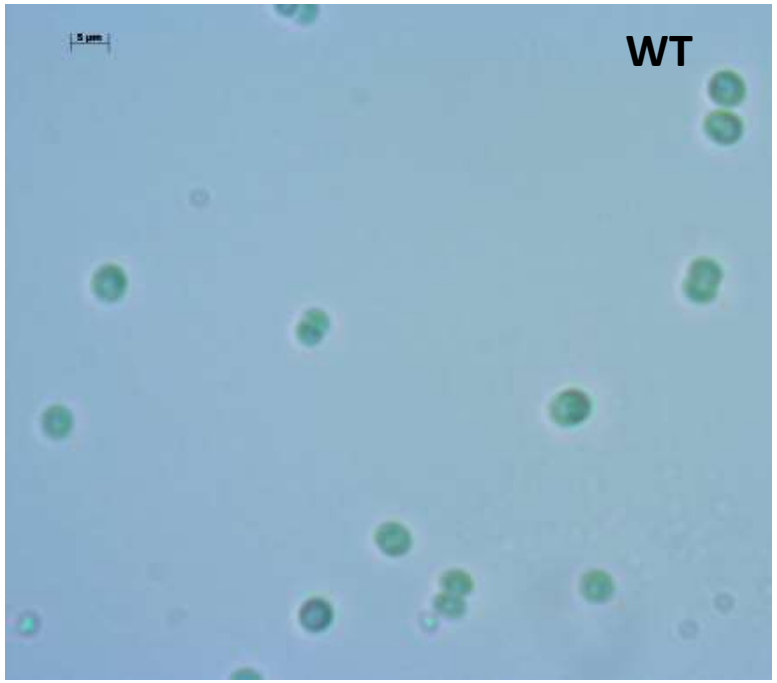
. Synechocystis + pFC1 W/Z (col. 41) → CYAO-41
. Synechocystis + pFC1 Z/W (col. 31) → CYAO-31

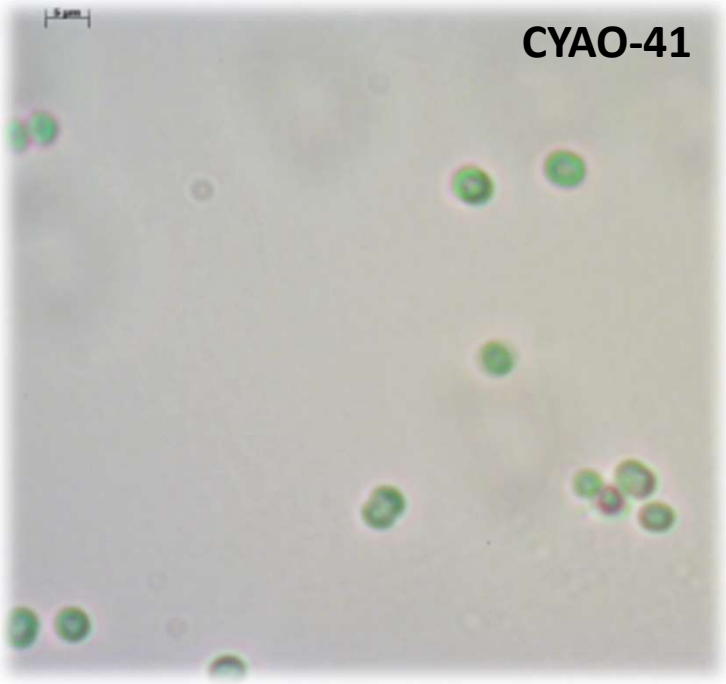


. First, colonies have been subcultured into new BG11+Sp/Sm plates

. Positive colonies have been screened by PCR using specific primer combinations







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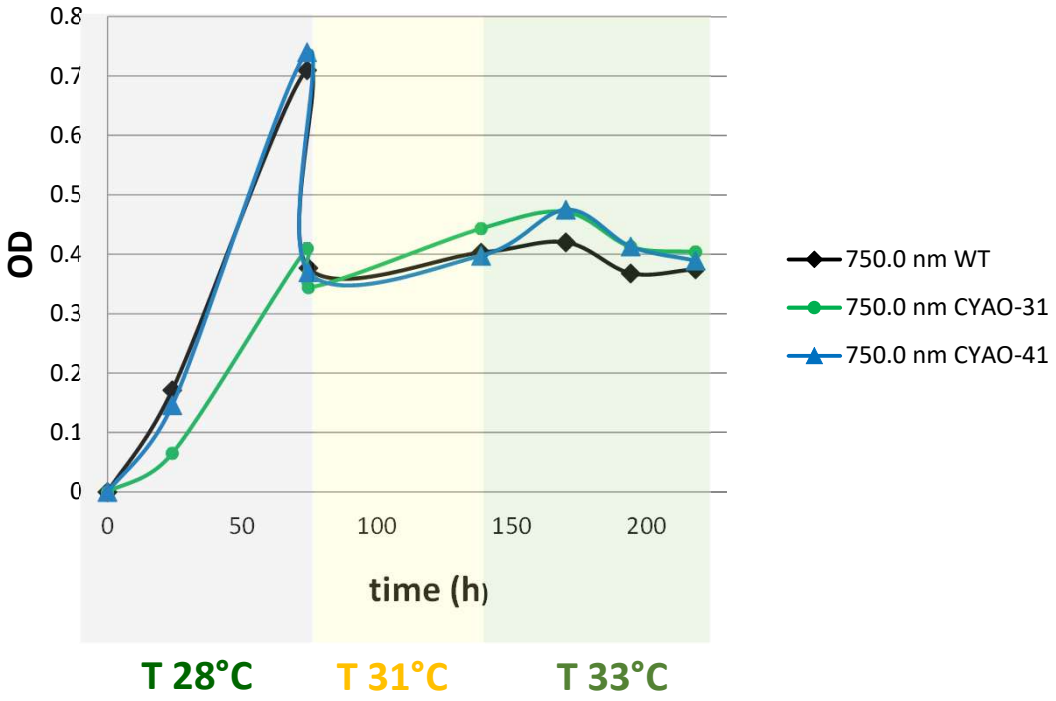
Temperature induction of astaxanthin metabolism in *Synechocystis* mutants

CYAO-31 (pFC1 + CrtZ/W) and CYAO-41 (pFC1 + CrtW/Z)

- ✓ We performed growing trials at different temperature: 28°C, 31°C, 33°C, 35°C and 39°C
- ✓ *Synechocystis* cells growing is strongly dependent from the temperature
- ✓ WT and mutant strains have shown the same growing rate at different temperatures

Growing curve of *Synechocystis* sp. PCC 6803 WT / CYAO-31 and CYAO-41 (750 nm)



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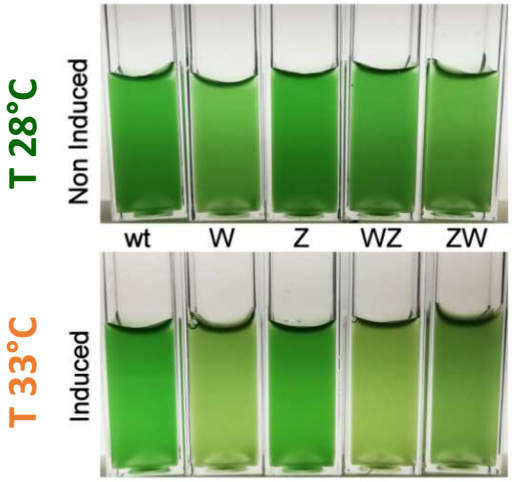
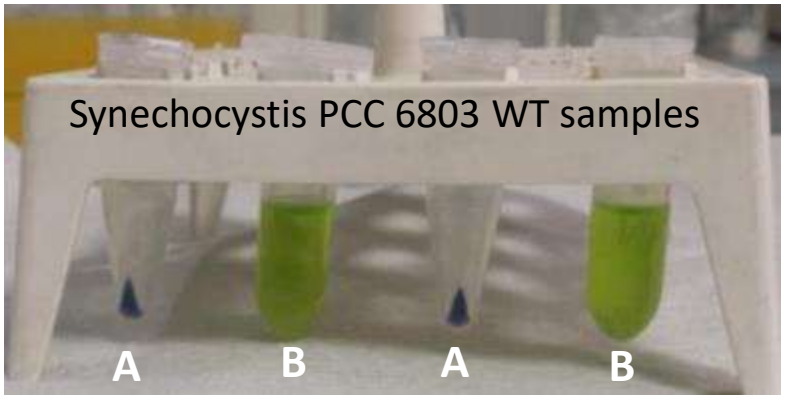
Analysis of total carotenoids and chlorophyll a in *Synechocystis* PCC6803 WT and induced mutant cells



TLC ANALYSIS

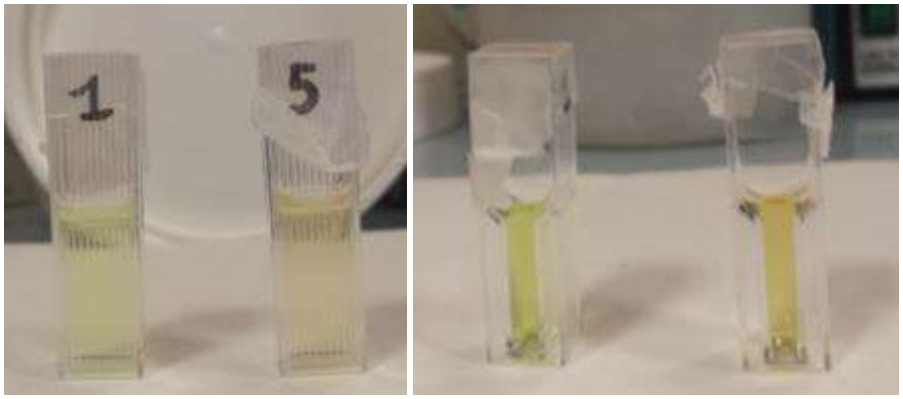
PIGMENTS EXTRACTION

Pigments extraction was performed in MetOH 100%



A) Blue pellets after the extraction
B) Pigments extracted in methanol

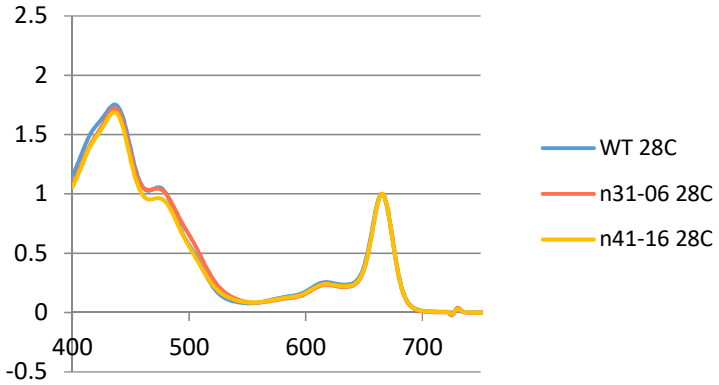
T 28°C T 33°C T 28°C T 33°C



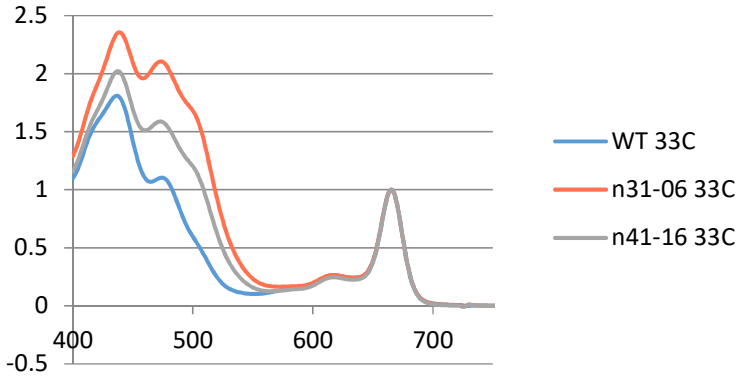
1) Pigments extracted from WT
5) Pigments extracted from CYAO-31

Absorption **spectra of total carotenoids and chlorophyll a** in *Synechocystis* PCC6803 WT and mutant cells

Absorption spectra (400-760 nm) of **total carotenoids** and **Chlorophyll a** in **Syn WT (green)** and mutant strains **Syn CrtZ/W (red)** and **Syn CrtW/Z (yellow)**



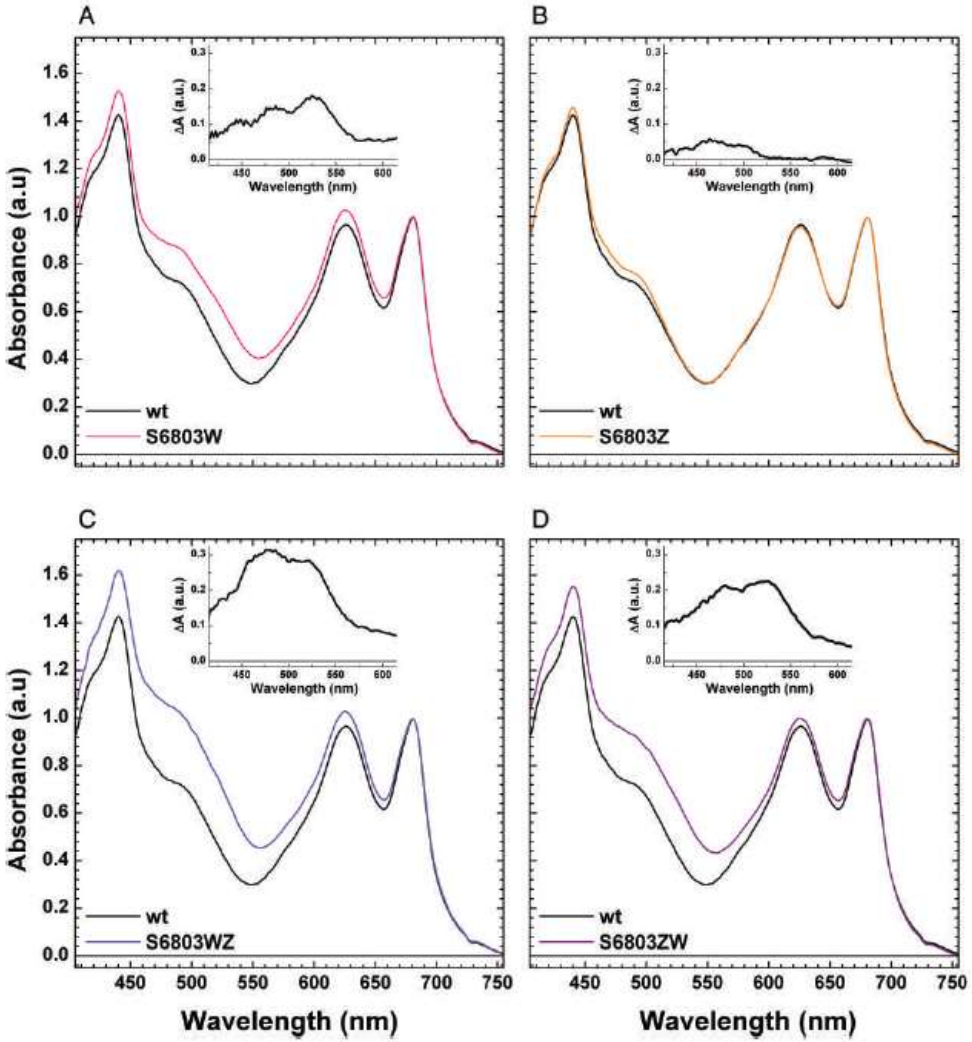
before induction (28°C)



after induction (33°C)

As the temperature increases, the peak of carotenoids increases as well, and changes shape, becoming broader to the right

Comparison of whole cells absorption spectra of *Synechocystis* PCC6803 WT and engineered strains



- All spectra are normalised at their maximal Qy absorbance (680 nm).
- In each panel, the 'mutant minus wt' difference spectra in the 415–615nm window are also shown in the insets.
- To allow direct comparison of the difference-absorbance intensities, the same y-axis scale was used in all insets.

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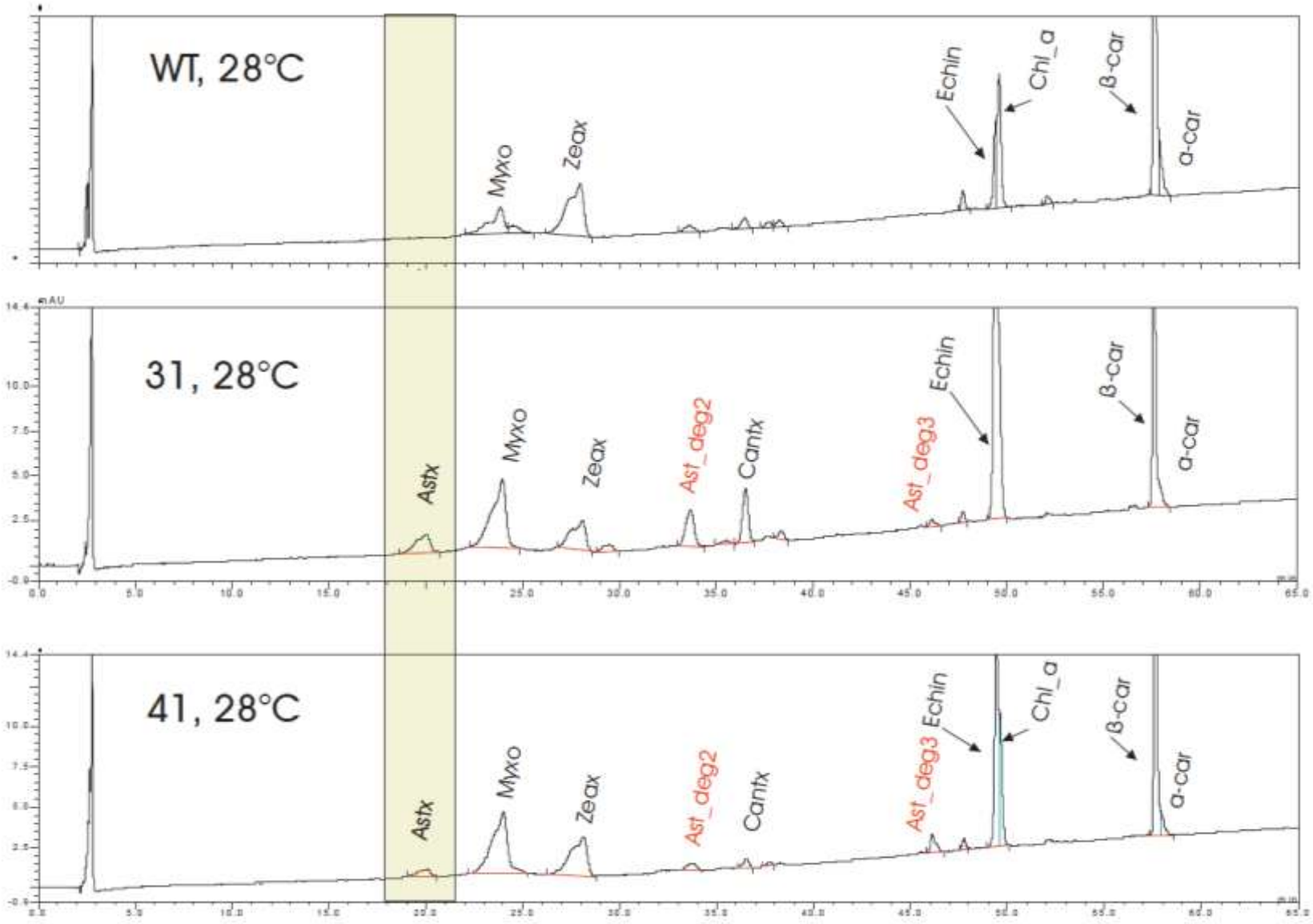
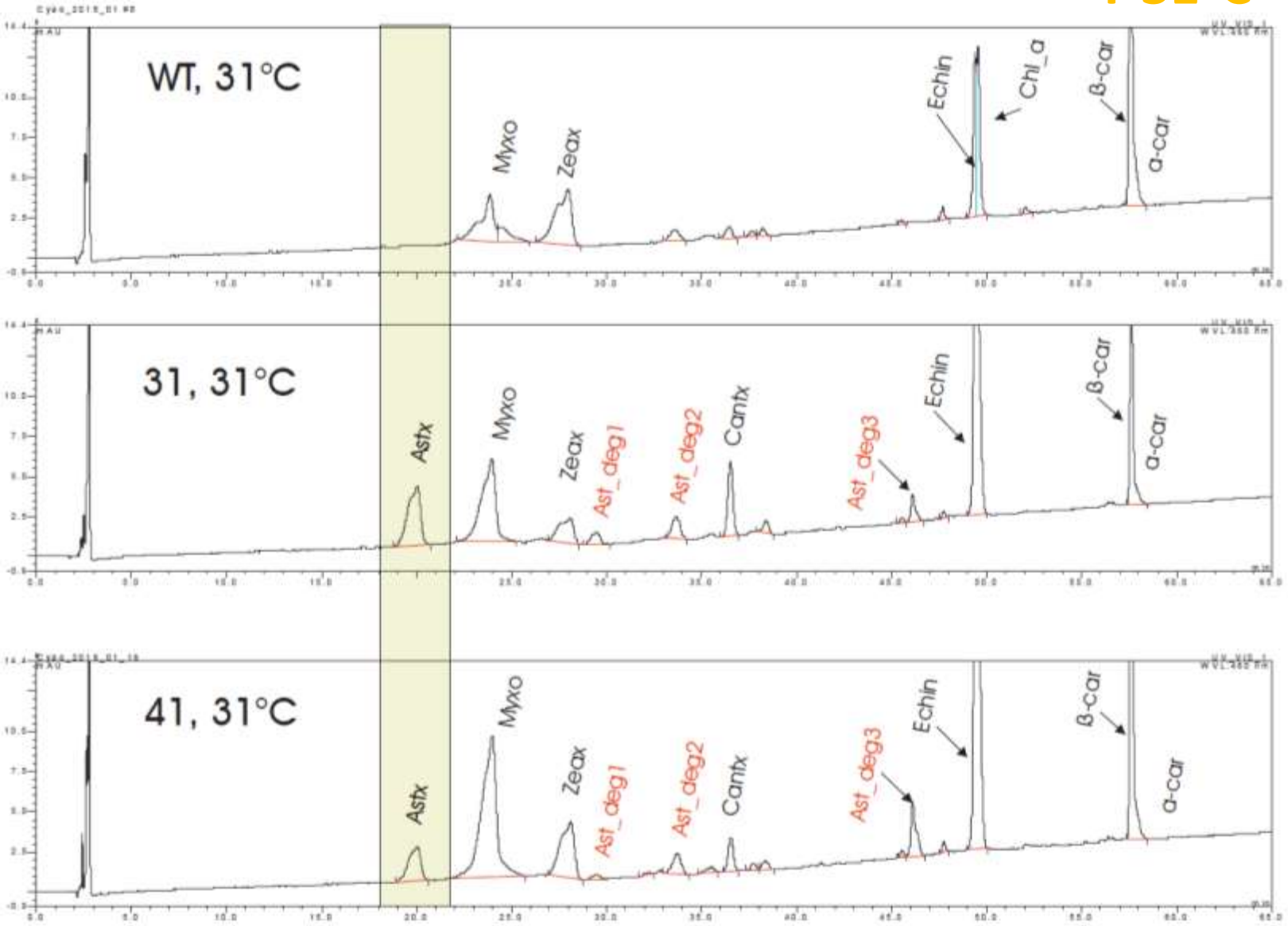
HPLC analysis of carotenoids in *Synechocystis* PCC6803 WT and CYAO-31 and CYAO-41 mutants

T 28°C



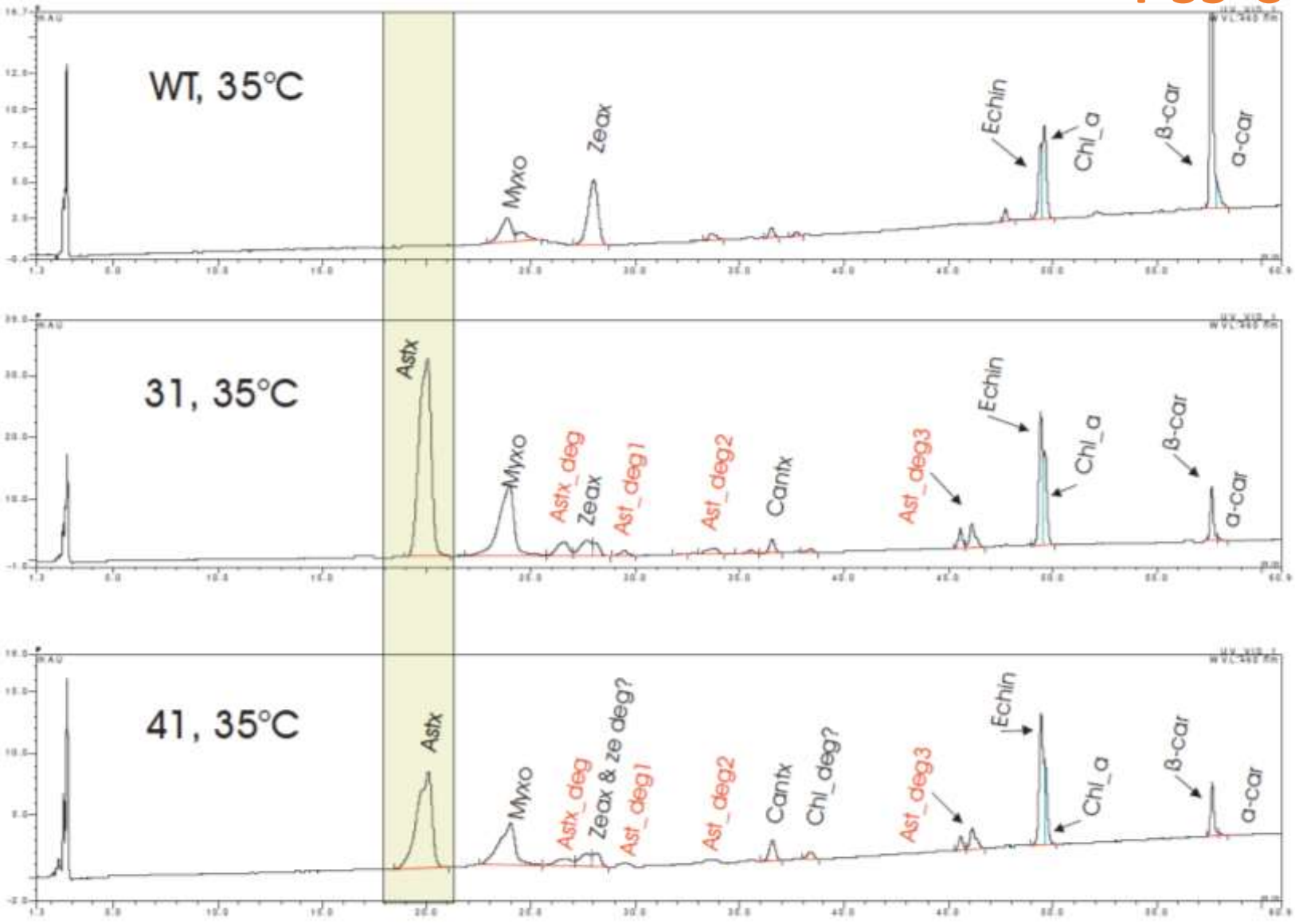
HPLC analysis of carotenoids in *Synechocystis* PCC6803 WT and CYAO-31 and CYAO-41 mutants

T 31°C



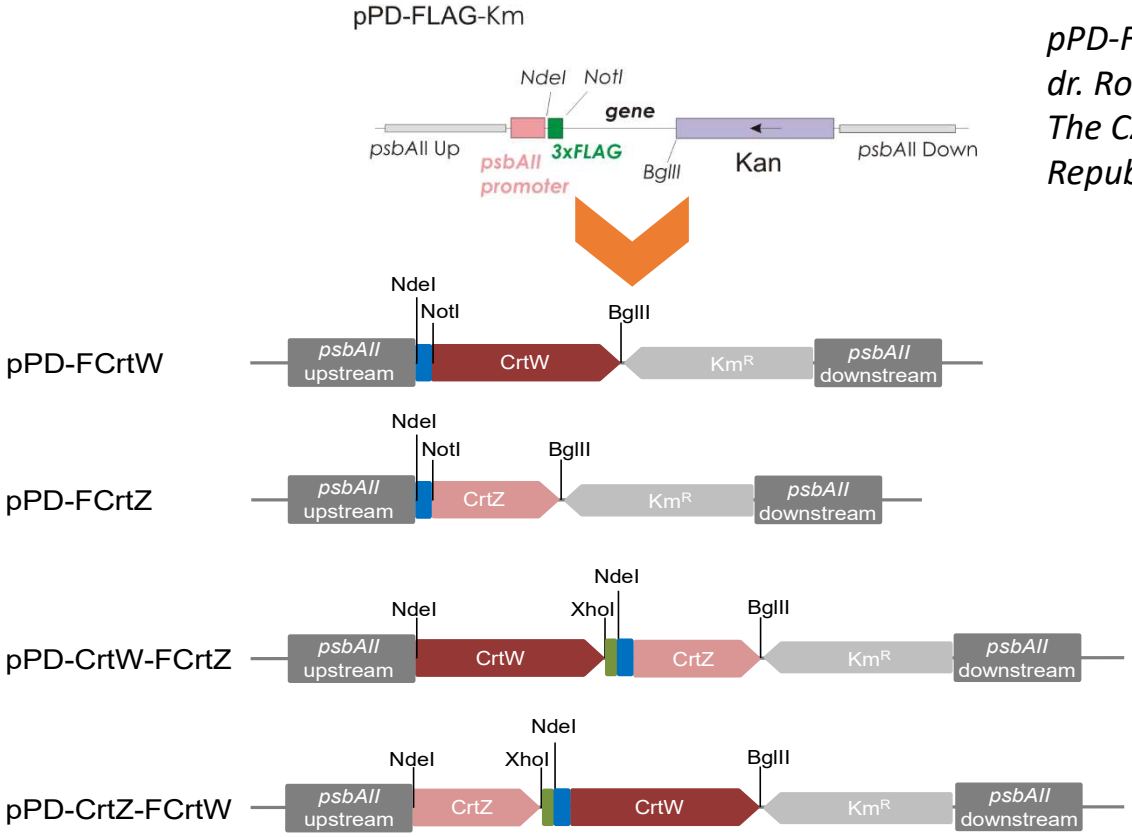
HPLC analysis of carotenoids in *Synechocystis* PCC6803 WT and CYAO-31 and CYAO-41 mutants

T 33°C



Construction of the constitutive expression vector

✓ The CrtW/CrtZ genes were cloned into the **pPD-FLAG plasmid** containing the **3xFLAG tag** and the Synechocystis **psbAII promoter** and up- and down-stream sequences, which promote homologous recombination between the plasmid and the Synechocystis psbAII gene



pPD-FLAG-Km plasmid was kindly provided by dr. Roman Sobotka, Institute of Microbiology, The Czech Academy of Sciences, Třeboň, Czech Republic

✓ Following transformation into Synechocystis WT the 3xFLAG-CrtW/CrtZ genes were **incorporated into the chromosome replacing the original psbAII gene**

✓ Transformants were selected by resistance to **kanamycin**

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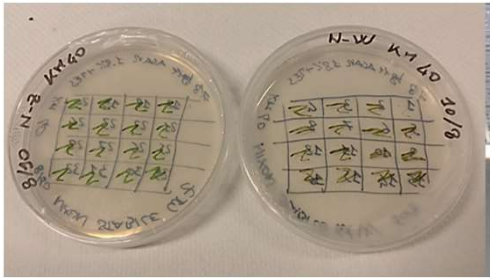
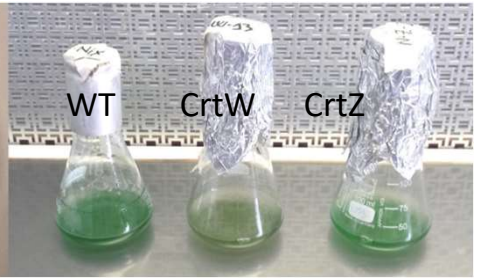
Obtainment of **constitutive** single and double mutants *Synechocystis* sp. 6803

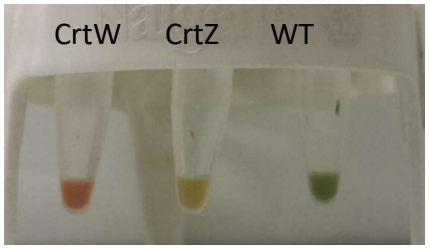
Constitutive mutants

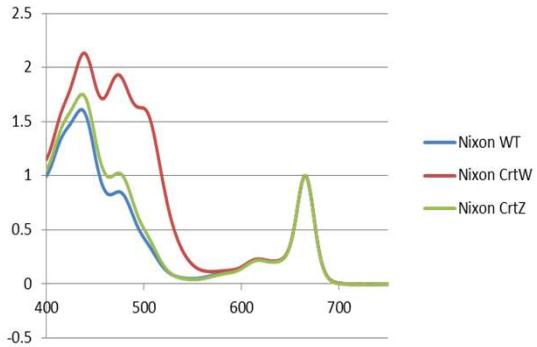
Transformation of cyanobacteria:

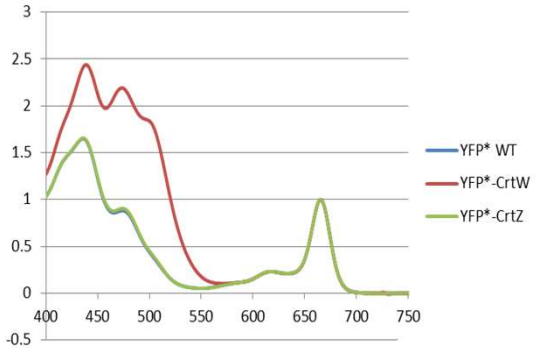
- The integrative plasmids were transformed by natural transformation
- 2 ml of WT Synechocystis (OD_{730} ~2) were spun down at 8000 x 8 min.
- The pellet was resuspended in 100 ml of BG11 and 500 ng of plasmid were added and mixed by pipetting
- The cells were incubated for 3 h under 100 mE at 30°C
- Cells were plated in BG11 agar plates without antibiotics for 24 h at 30°C and approximately 30 mE continuous illumination
- Subsequently, cells were moved to BG11 agar plates with 10 µg/ml kanamycin. Every week colonies were re-streaked onto BG11 plates with double the amount of kanamycin
- Strains were maintained at a final kanamycin concentration of 40 µg/ml











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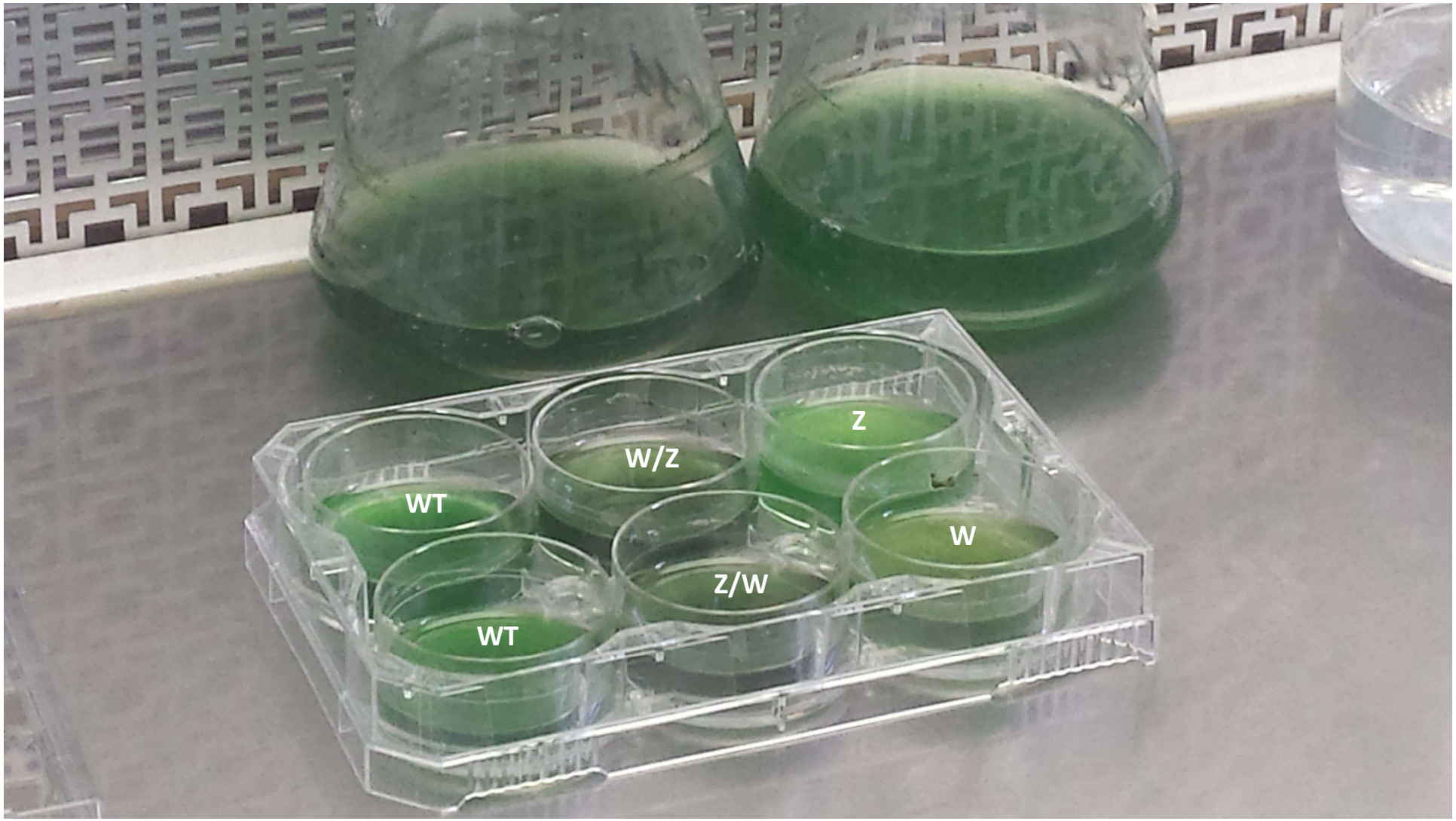

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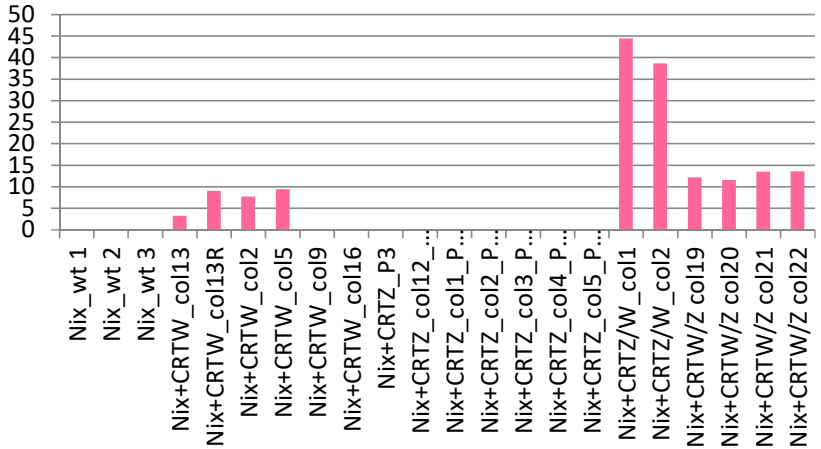
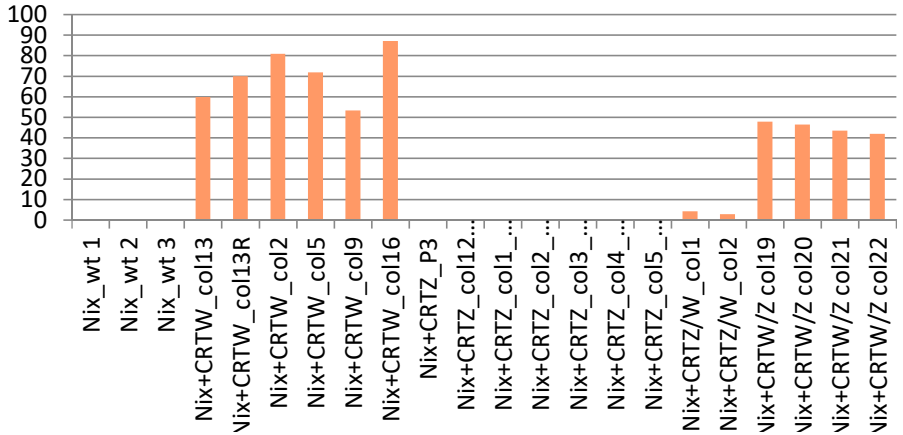
Final results of HPLC analysis: **different carotenoid profile** in *Synechocystis* PCC6803 WT and constitutive mutants

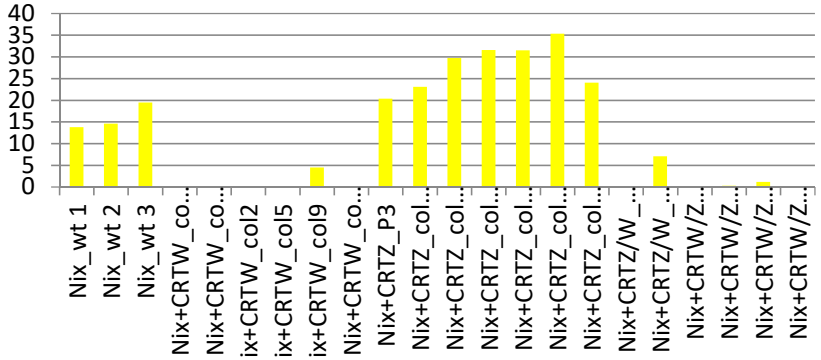
Asx



Canta



Zea



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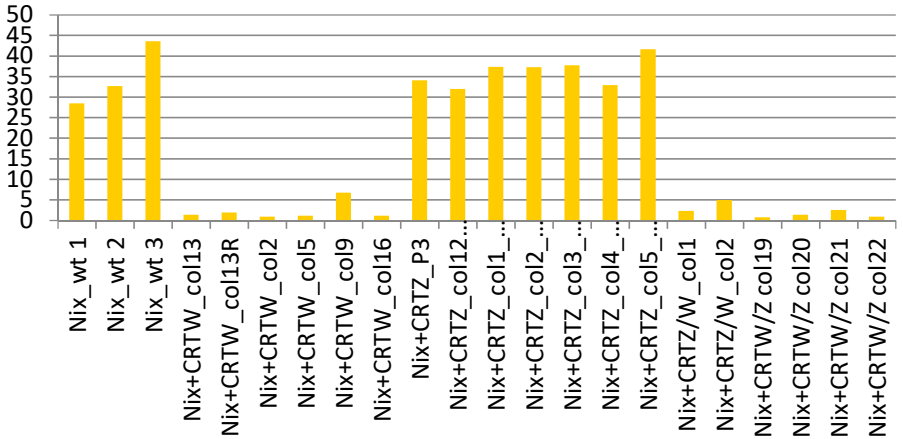

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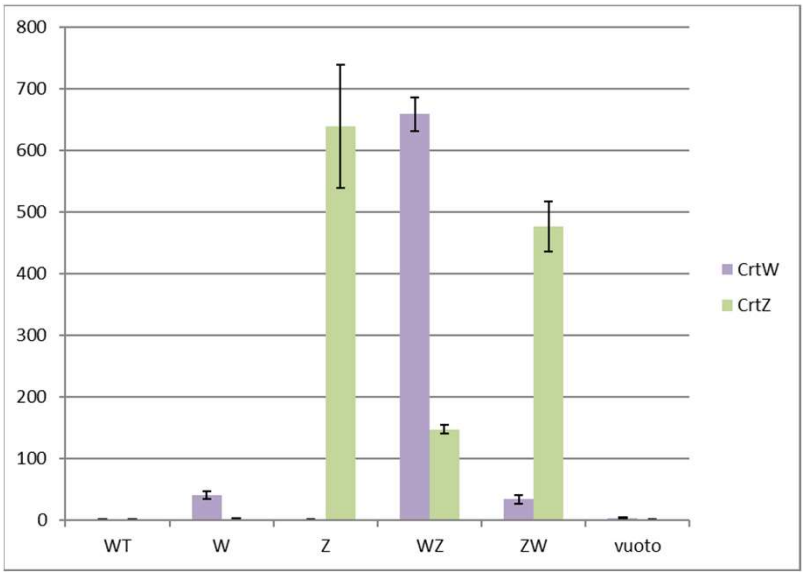
qRT-PCR gene expression analysis in constitutive mutants

Experimental procedure:

- Total RNA was extracted from each strain in the presence of NucleoZOL
- Specific primer were designed to amplify the transgenes (*CrtW* and *CrtZ*) and *psbA2* as internal control
- *rnpB* was used as housekeeping to normalized gene expression levels
- For each gene, cDNA dilution curves were generated (cDNA dilutions: 1/3, 1/9, 1/27 and 1/81) and used to calculate the individual real-time PCR efficiencies
- Reactions were run in the 7300 RealTime PCR System (Applied Biosystems) and data analysed using the 7300 System Software (Applied Biosystems)
- Dissociation analysis was performed at the end of each run to confirm the specificity of the reaction.
- Quantitative variation was evaluated by the $2^{-\Delta\Delta CT}$ method.



Results:

- qRT-PCR analysis of the C-strains indicates a general lack of correlation between the transcript levels of *CrtW* and *CrtZ* and the carotenoid accumulation pattern
- *CrtW* is expressed at rather lower levels in C-W, despite being under the control of the same strong promoter as *CrtZ* in C-Z, but the accumulation of Can was the highest (>60% of total carotenoids)
- *CrtZ* is highly expressed in C-Z, but the observed increase in Zea content is relatively low (a + ~30%)
- In C-WZ the relative abundance of the *CrtZ* transcript is several folds lower than the one of *CrtW*
- In C-ZW, despite *CrtW* transcription being several fold lower than *CrtZ*, the resulting carotenoid pattern phenotype was completely different from that of C-Z

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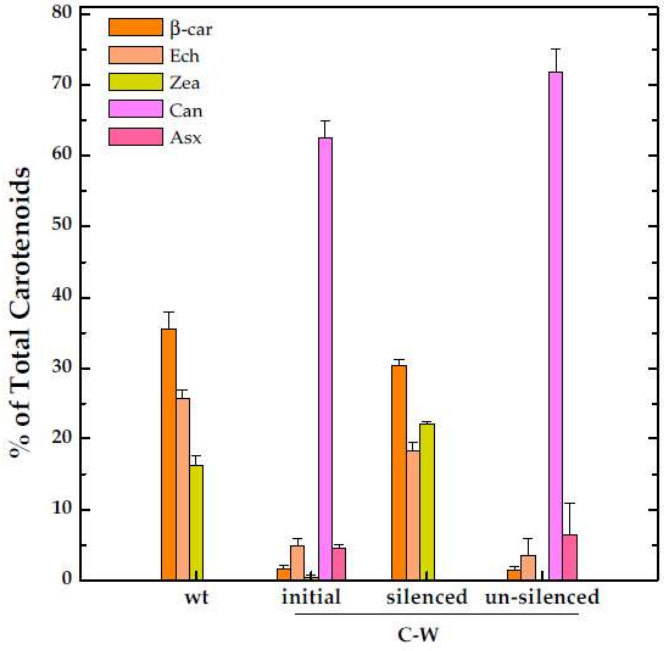
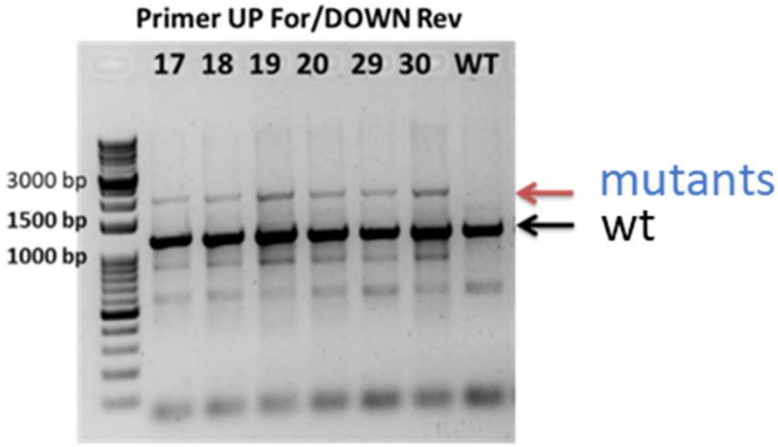
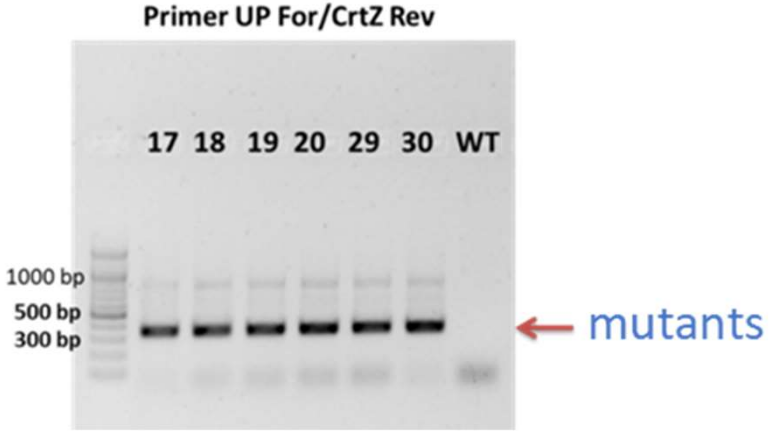
Segregation / Instability issues of the constitutive mutants

- Full segregation could not be attained in these strains
- → high polyploid level?
- → detrimental effects caused by CrtW expression?
- → and/or the perturbation of the endogenous carotenoid biosynthetic pathway?

After **six months** of continuous sub-culturing of the C-W strain its ability to produce the non-endogenous keto-carotenoid Can was almost suppressed and the strain phenotypically reverted to a carotenoid profile resembling the wild-type one







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Comparison of Inducible VS Constitutive expression systems

PRO

- ✓ The **most efficient strain for Astaxanthin production** was the temperature-inducible strain TI-ZW (**1.1 ± 0.2 mg/g DCW** at 39°C), which yielded satisfactory amounts of this compound already at 33 °C (1.0 ± 0.2 mg/g DCW)
- ✓ No instability issues
- ✓ It allows for microbial growth to high cell density before switching on the exogenous biosynthetic pathway

CONS

- x Two-steps cultivation required
- x Antibiotic selection

PRO

- ✓ **Best solution in a logic of scale-up**: one step cultivation / no antibiotics
- ✓ The highest level of **Cantaxanthin** accumulation (**1.3 ± 0.1 mg/g DCW**) was attained in the strain constitutively expressing CrtW

CONS

- x Production **instability** depending on the age of the culture
- x High accumulation levels of non-endogenous metabolites since early-stage culture might exhibit cytotoxic activity or lead to lower the cell fitness

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Non-endogenous ketocarotenoid accumulation in engineered *Synechocystis* sp. PCC 6803

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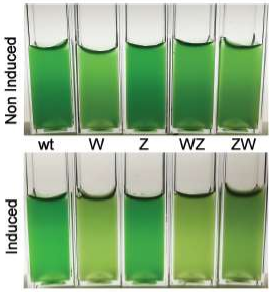

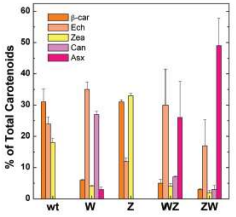

Fig. 4. Relative distribution of the main carotenoids in the wt and the different engineered *Synechocystis* strains after 2 days of induction at 33°C. Strains abbreviated as: wild type (wt), S6803W (W), S6803Z (Z), S6803WZ (WZ) and S6803ZW (ZW). Asx, astaxanthin (pink); β-car, β-carotene (orange); Can, canthaxanthin (light magenta); Ech, echinenone (peach); Zea, zeaxanthin (yellow). Error bars indicate st.

on cell growth and fitness, as reported, for example, for Asx-accumulating transplastomic tobacco plants which showed a reduced growth rate (Hasunuma et al. 2008, Lu et al. 2017). For this reason, in the present study, a temperature-inducible expression system has been used for the metabolic engineering of the carotenoid biosynthetic pathway in *Synechocystis* (Fig. 1) and the expression of the exogenous genes was induced at 33°C, which is a value slightly above the optimal growth temperature for this organism. Under the experimental conditions considered here, no sizable negative effects could be noticed in the engineered strains in response to the accumulation of non-endogenous ketocarotenoids. It can however not be excluded that higher levels of accumulation or constitutive production of Asx and Can can be toxic for *Synechocystis*.

To achieve the most efficient conversion of endogenous β-car into the highly valuable ketocarotenoids Asx and Can and their intermediates, the two carotenogenic enzymes CrtW and CrtZ from *Brevundimonas* sp. SD212 have been chosen (Choi et al. 2005, 2006). This approach has been adopted on the basis of previous reports, which demonstrated the efficacy of *Brevundimonas* CrtW and CrtZ in enabling transgenic higher plants to produce large quantities of Asx (Hasunuma et al. 2008, Harada et al. 2009, 2014, Mortimer et al. 2017, Nogueira et al. 2017). By contrast, previous attempts at engineering ketocarotenoid biosynthesis in cyanobacteria by introducing carotenogenic enzymes from different organisms (Harker and Hirschberg 1997) and/or additional copies of the endogenous ones (Albers 2016) have not been as successful.

In the present study, depending on the introduced gene, *CrtW* or *CrtZ*, or both in tandem as *CrtW-CrtZ* or *CrtZ-CrtW*, significant alterations in the relative abundance of the main endogenous ketolated and hydroxylated carotenoids were observed in the different engineered *Synechocystis* strains, and significant accumulation of non-endogenous ketocarotenoids was present in all strains expressing *CrtW*. When only *CrtZ* was expressed (Figs 3B and 4), a strong enhancement of the Zea content and a concomitant reduction in the Ech level were obtained, indicating that a significant amount of the endogenous β-car was diverted into the metabolic branch that lead to Zea via β-Cryptoxanthin (Fig. 1). Similar results were previously achieved by introducing an additional copy of the endogenous CrtR in *Synechocystis* sp. PCC 6803 (Lagarde et al. 2000), suggesting that in this organism the conversion of β-car into Zea can be modulated by the available amount of 3,3' β-car hydroxylase.

On the other hand, the expression of *CrtW* alone (Figs 3A and 4) redirected the carotenoid biosynthesis almost entirely in favour of Ech and Can, with a concomitant marked reduction of the β-car pool, which is their biosynthetic precursor (Fig. 1). Moreover, in this strain, a small amount of Asx could be detected, which is synthesised from Zea by the exogenous CrtW (Fig. 1). As the relative abundance decrease of β-car and Zea was similar (approximately 80% with respect to wt, Fig. 4), this suggested that the CrtW from *Brevundimonas* had a similar affinity for both substrates. Therefore, Asx accumulation in this strain was limited by the amount of Zea synthesised by the endogenous CrtR.

The coexpression of *CrtW* and *CrtZ* (Figs 3C, D and 4), irrespective of the order in which the two genes have been cloned, enabled the accumulation of consistent amounts of Asx, which could represent up to approximately 50% of the total carotenoids. The position of the *CrtW* and *CrtZ* genes in the expression vector used to transform *Synechocystis* appeared to influence the relative proportion of Asx and Can accumulation, Asx being the predominant ketocarotenoid only in the S6803ZW strain. An analogous position effect has also been reported in Asx-producing transplastomic tobacco plants, where both the transcription level of the two exogenous genes as well as the accumulation level of Asx were higher in the transplastomic plants, where *CrtZ* was integrated upstream of *CrtW* in the plastid genome (Hasunuma et al. 2008). Further investigations are in S6803ZW. Moreover, the difference spectra were noticeably broader and had larger amplitudes in both double mutants. These specific alterations in the absorption spectra of the engineered strains, when compared with the wt, strongly suggested the occurrence of significant changes in carotenoid composition at the cellular level, in response to the expression of the specific exogenous carotenogenic enzymes.

Pigment analysis

To seek further detail on the nature of the carotenoids synthesised in the mutant strains upon temperature induction, these were analysed by HPLC. According to the chromatograms and spectrophotometric analysis of pigment extracts (data not shown), the ratio of total carotenoids to Chla showed only a slight increase which, within the experimental margin of uncertainty, was approximately the same (approximately 5–10%) for all strains. Successively, analysis was therefore focused on the relative distribution of the main carotenoids in rains as well as in the wt ant carotenoid was β-car h and Zea. In the S6803Z hydroxylase of *Brevundi-* e relative abundance of observed, whereas β-car same as in the wt. In the the *CrtW* ketolase of *Bre-* ntent increased markedly appearance of significant the expenses of β-car and ketocarotenoid was Can, w amounts only. In both Z together, the dominant d, although some differ- NZ and S6803ZW, with accumulation (approxi- ds). In these strains, Can ounts. Ech accumulation ghtly larger in S6803WZ still comparable to the as mainly associated to β-car and Zea in both

vere detectable in the or <10% of the total Because of the relative their precise assignment me might be associated iosynthetic pathway (see tion time and absorption ther phoenicoxanthin or

microorganisms
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Article

A Comparison of Constitutive and Inducible Non-Endogenous Keto-Carotenoids Biosynthesis in *Synechocystis* sp. PCC 6803

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Received: 9 August 20

Abstract: The mode an alternative and su category includes k

constitutive expression of the same exogenous *CrtW* and *CrtZ* genes also led to significant changes in the carotenoid composition of the engineered strains.

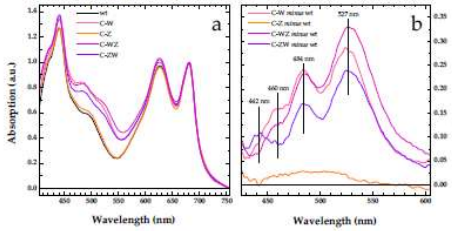


Figure 1. Whole cells absorption spectra. (**a**) Comparison of absorption spectra of wild-type (wt, black line) and engineered *Synechocystis* strains constitutively expressing *Brevundimonas* CrtW (C-W, pink line), CrtZ (C-Z, orange line), CrtW and CrtZ in tandem (C-WZ, purple line), CrtZ and CrtW in tandem (C-ZW, violet line). All spectra are normalised at their maximal Qy absorbance (680 nm). (**b**) Comparison of the difference spectra "engineered strain *minus* wild-type" in the 425–605 nm window, colour coding as for the engineered strains in panel (**a**). The main spectral features of the difference spectra are marked by vertical solid lines and the wavelength indicated.

In order to investigate the carotenoids profile of the engineered strains in greater detail, total pigments were extracted and analysed by HPLC. The analysis was focussed on Asx, its precursor β-car and the main intermediates of the biosynthetic pathway (Figure 2), namely echinenone (Ech), Can and Zea.

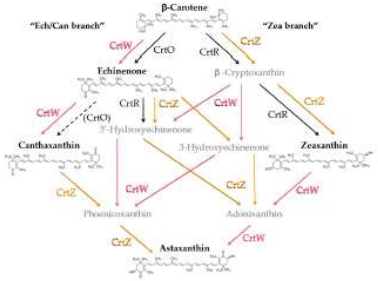
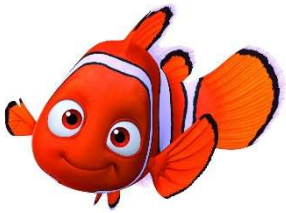


Figure 2. Simplified scheme of the astaxanthin biosynthetic pathway from endogenous β-carotene (β-car) in engineered *Synechocystis* strains, resulting from the heterologous expression of *Brevundimonas* CrtW and CrtZ. The endogenous enzymes CrtO (β-car ketolase) and CrtR (β-car hydroxylase) are shown in black. Parentheses and the dashed arrow indicate weak or possible catalytic function. *Brevundimonas* β-car ketolase (CrtW) and β-car hydroxylase (CrtZ) are indicated in pink and orange letters, respectively. Ech, echinenone; Can, canthaxanthin; Zea, zeaxanthin.



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Thank you for your attention!!

Linneaeus Garde, Uppsala

and please… come to visit me in Turin!!