Engineering of the Plant Abscisic Acid Biosynthetic Pathway in *Escherichia coli*

by

Dominic Ludovice

A thesis submitted in conformity with the requirements for the degree of Master of Science

Cell and Systems Biology
University of Toronto

© Copyright by Dominic Ludovice 2016
Abstract

Abscisic acid (ABA) is a phytohormone involved in many aspects of plant growth and development, including the regulation of seed development, dormancy, germination, and plant responses to environmental stress. *Escherichia coli* is an ideal system in which to engineer the ABA biosynthetic pathway since the early steps have been successfully reproduced in *E. coli* by Misawa and colleagues (1990) using the carotenoid biosynthesis gene cassette from *Pantoea ananatis*. In this study, *Arabidopsis thaliana* genes were used to build the pathway starting from the precursor zeaxanthin. The next biosynthetic enzyme, zeaxanthin epoxidase (ZEP), was successfully expressed in *E. coli*, leading to the effective production of violaxanthin. However, the enzyme responsible for catalyzing the subsequent step has yet to be identified. This system is now being used to identify uncharacterized genes of the pathway.
Acknowledgments

First and foremost, I would like to thank my supervisor, Professor Eiji Nambara, without whom this project would not have been possible. I am grateful for the support and help he has given me throughout this project.

I would also like to thank the current and former members of the Nambara Lab, who treated me like family. Our Post Doctoral Fellow, Dr. Dawei Yan, for helping me in almost all of my lab experiments. Lisza Duermeyer, one of our PhD. students, for keeping the lab in order. Catalina Leoveanu, the other Msc. student, for keeping the office lively. Dr. Masato Otani, our previous Post Doctoral Fellow, for helping me with the HPLC. Ayako Nambara, our lab technician, for keeping the lab from running out of supplies. Ehsan Khodapanahi, one of our PhD. students, for helping fix the automatic pipettor. Yumi Tanaka, our previous lab technician, for the Gibson Assembly mix recipe.

I would also like to extend my thanks to the members of the McCourt Lab, Dr. Shigeo Toh, Dr. Shelly Lumba, Duncan Holbrook-Smith, and Eric Nam for their input during lab meeting, which helped shape this project.

I would like to extend my thanks my committee members, Professor Daphne Goring and Professor Peter McCourt for helping to guide and direct this project.

Finally yet importantly, I would like to thank my friends and family who have kept me sane throughout the two years of this project. I would like to thank my mom, dad and sister for their unconditional love and support throughout this arduous endeavor.
# Table of Contents

Acknowledgments .......................................................................................................................... iii  
Table of Contents........................................................................................................................... iv  
List of Tables ................................................................................................................................. vi  
List of Figures ............................................................................................................................... vii  
Abbreviations ............................................................................................................................... viii  

1 Introduction............................................................................................................................... 1  
  1.1 ABA biosynthesis ............................................................................................................... 3  
  1.2 Engineering the ABA biosynthetic pathway in to *Escherichia coli* .................................... 4  
  1.3 *A. thaliana* genes will supplant the missing steps of the ABA biosynthetic pathway in engineered *E. coli* .............................................................................................................................. 5  
  1.4 The short-term goal is to identify uncharacterized genes of the ABA biosynthetic pathway. .............................................................................................................................. 6  
  1.5 Xanthoxin and beyond ........................................................................................................ 9  

2 Materials and Methods ............................................................................................................. 9  
  2.1 Growing engineered *E. coli* producing carotenoids for HPLC analysis ......................... 9  
  2.2 Harvesting and extraction of carotenoids ......................................................................... 10  
  2.3 HPLC condition ................................................................................................................ 11  
  2.4 Violaxanthin standard ....................................................................................................... 11  
  2.5 Cloning .............................................................................................................................. 11  
     2.5.1 *AtZEP* classical cloning ........................................................................................ 12  
     2.5.2 Cloning of genes of interest .................................................................................. 12  
     2.5.3 *AtNXD1* cloning ................................................................................................. 14  
     2.5.4 *AtNCED3* Cloning ............................................................................................. 14  
     2.5.5 Gibson Assembly of pACHP-Zea(Spec) .............................................................. 14  
     2.5.6 *AtZEP* cloning by Gibson Assembly ................................................................. 15
2.6 Strains of *E. coli* ................................................................................................................ 15
2.7 Plasmids ............................................................................................................................ 15
   2.7.1 Duet Plasmid System ............................................................................................ 15
   2.7.2 Plasmid list ............................................................................................................ 17
2.8 Western Blot ..................................................................................................................... 18
   2.8.1 Growth of *E. coli* harboring pET-AtZEP for protein extraction ...................... 18
   2.8.2 Growth of *E. coli* harboring pCOLA-AtNXD1 for protein extraction .......... 18
   2.8.3 Gel electrophoresis and Western blotting ............................................................. 18
3 Results ...................................................................................................................................... 18
   3.1 Construction and expression of pACHP-Zea(Spec) ......................................................... 18
   3.2 AtZEP expression produces all-trans-violaxanthin ..................................................... 20
   3.3 Expression of carotenoid enzyme-related genes in *E. coli* harboring pACHP(Spec)-AtZEP ................................................................................................................................ 24
   3.4 AtNXD1 expression produces a novel peak ................................................................. 28
   3.5 Expression of AtNCED3 is unable to produce xanthoxin in violaxanthin-producing *E. coli* ..................................................................................................................................... 31
4 Discussion ................................................................................................................................ 33
   4.1 Violaxanthin production in *E. coli* .............................................................................. 33
   4.2 Search for the enzyme that catalyzes violaxanthin isomerization to 9-cis-violaxanthin .. 34
   4.3 AtNXD1 could be the missing neoxanthin synthase .................................................... 36
   4.4 Xanthoxin could not be detected when AtNCED3 was expressed in all-trans-violaxanthin-producing *E. coli* ........................................................................................................ 37
   4.5 Colorimetric assay for screening for the violaxanthin isomerase ................................. 38
References ..................................................................................................................................... 39
Appendix ....................................................................................................................................... 45
List of Tables

Table 1. HPLC conditions for carotenoid detection. .......................................................... 11
Table 2. Genes inserted in pCOLADuet-1 plasmid. .......................................................... 13
Table 3. Novagen Duet plasmids. .................................................................................... 15
Table 4. Plasmids and their descriptions used for this project............................................. 17
List of Figures

Figure 1. The ABA biosynthetic pathway in plants ................................................................. 2

Figure 2. Pantoea ananatis carotenoid gene cassette ............................................................... 5

Figure 3. Isomerization of A) violaxanthin to 9-cis-violaxanthin by an uncharacterized
isomerase, and B) β-carotene to 9-cis-β-carotene by rice D27 enzyme ........................................ 7

Figure 4. The chemical structures of products of the A. thaliana carotenoid biosynthetic pathway
from phytoene to lycopene ............................................................................................................. 8

Figure 5. Streaked colonies of carotenoid producing E. coli ....................................................... 19

Figure 6. HPLC chromatogram of extracted zeaxanthin and violaxanthin from E. coli ............. 20

Figure 7. Western blot of IPTG-induced AtZEP ..................................................................... 22

Figure 8. Violaxanthin levels of IPTG-induced AtZEP and constitutively expressed AtZEP ...... 23

Figure 9. HPLC chromatogram of violaxanthin-producing E. coli harboring the plasmid pCOLA-
PDS ............................................................................................................................ 25

Figure 10. Carotenoid levels of candidate genes expressed in violaxanthin-producing E. coli .. 27

Figure 11. HPLC chromatogram of violaxanthin-producing E. coli containing pCOLA-AtNXD1.
....................................................................................................................................................... 29

Figure 12. Western blot of IPTG-induced BL21 CodonPlus harboring the plasmids pACHP-
Zea(Spec)-AtZEP and pCOLA-AtNXD1 ...................................................................................... 30

Figure 13. Carotenoid levels of violaxanthin-producing E. coli harboring pCOLA-AtNCED3... 32
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AAO3</td>
<td>Abscisic aldehyde oxidase 3</td>
</tr>
<tr>
<td>CCD</td>
<td>Carotenoid cleavage dioxygenase</td>
</tr>
<tr>
<td>CHYB</td>
<td>β-carotene hydroxylase</td>
</tr>
<tr>
<td>CRTISO</td>
<td>Carotenoid isomerase</td>
</tr>
<tr>
<td>DMAPP</td>
<td>Dimethylallyl diphosphate</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl diphosphate</td>
</tr>
<tr>
<td>G3P</td>
<td>Glycerladehyde-3-phosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IDI</td>
<td>Isopentyl diphosphate isomerase</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentyl diphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LCYB</td>
<td>Lycopene β-cyclase</td>
</tr>
<tr>
<td>NEB</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MEP</td>
<td>2-C-methyl-D-erythritol 4-phosphate</td>
</tr>
<tr>
<td>MoCo</td>
<td>Molybdenum cofactor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>MVA</td>
<td>Mevalonate</td>
</tr>
<tr>
<td>NCED</td>
<td>9-cis-epoxycarotenoid dioxygenase</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Absorbance 600 nm</td>
</tr>
<tr>
<td>PDS</td>
<td>Phytoene desaturase</td>
</tr>
<tr>
<td>PSY</td>
<td>Phytoene synthase</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>ZDS</td>
<td>ζ-carotene desaturase</td>
</tr>
<tr>
<td>ZEP</td>
<td>Zeaxanthin epoxidase</td>
</tr>
<tr>
<td>Z-ISO</td>
<td>ζ-carotene isomerase</td>
</tr>
</tbody>
</table>
1 Introduction

Abscisic acid (ABA) is an isoprenoid phytohormone involved in many aspects of plant growth and development. This includes the regulation of seed development, dormancy, germination, and plant responses to environmental stress (Nambara and Marion-Poll, 2005; Ruiz-Sola and Rodríguez-Concepción, 2012). The diversity of ABA’s function is equal to the complexity of its biosynthesis, degradation, transport, signal transduction and perception (Finkelstein, 2013; Xiong and Zhu 2003; Cutler et al. 2010). Understanding how ABA biosynthesis and signaling is regulated will help in finding strategies to make crop plants combat unfavorable environmental factors (Xiong and Zhu 2003).

Since the discovery of ABA in the 1960’s, much effort was devoted to understanding how it was synthesized in plants (Cutler et al. 2010). Through genetics and chemical feeding experiments, most of the enzymes in the plant biosynthetic pathway have been identified and characterized (Xiong and Zhu, 2007). However, there are a few genes that have remained elusive (Nambara and Marion-Poll, 2005).
Figure 1. The ABA biosynthetic pathway in plants. The pathway starts from the basic substrates, pyruvate and glyceraldehyde-3-phosphate (G3P), which are products of glycolysis. Steps enclosed by the trapezoid are part of the MEP pathway. The circle denotes steps that are ABA specific. Steps within the green box are localized in plastids. Steps denoted by question marks, correspond to enzymes or genes that have yet to be identified. IPP (isopentenyl diphosphate), DMAPP (dimethylallyl diphosphate), IDI (isopentenyl diphosphate isomerase), GGPP (geranylgeranyl diphosphate), PSY (phytoene synthase), PDS (phytoene desaturase), Z-ISO (ζ-carotene isomerase), ZDS (ζ-carotene desaturase), CRTISO (carotenoid isomerase), LCYB (lycopene β-cyclase), CHYB (β-carotene hydroxylase), ZEP (zeaxanthin epoxidase), NCED (9-cis-epoxycarotenoid dioxygenase) AAO3 (abscisic aldehyde oxidase 3), MoCo (molybdenum cofactor).

Abcisic Acid

AAO3

MoCo

Abscisic aldehyde

ABA2

Abscisic Acid

Xanthoxin

NCED

9'-cis-neoxanthin

Neoxanthin

9-cis-violaxanthin

Vioalexthnin

ZEP

Zeaxanthin

β-carotene

Lycopene

CRTISO

Z-ISO

9,15,9'-tri-cis-ζ-carotene

PDS

Phytoene

PSY

GGPP

DMAPP

IPP

Pyruvate

G3P

??
1.1 ABA biosynthesis

ABA is synthesized in various organisms (Sakata et al. 2014). The ABA biosynthetic pathway has been studied more extensively in plants. Mutants deficient in ABA have been instrumental in the study of its biosynthesis, exhibiting precocious germination and a wilty phenotype (Cutler et al. 2010). Mutants have been identified in several different plant species, including *Hordeum vulgare, Zea mays, Solanum lycopersicum, Solanum tuberosum, Nicotiana tabacum, Nicotiana plumbaginifolia,* and *Arabidopsis thaliana* (Schwartz et al. 1997; Marin et al. 1996; North et al. 2007; Xiong and Zhu, 2003). Studies using feeding experiments have elucidated the identity of the compounds in the pathway before even identifying the enzymes involved (Nambara and Marion-Poll, 2005).

The ABA biosynthetic pathway begins with the biosynthesis of isoprenoid precursors, which are derived from two different pathways. The first pathway is the 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) pathway, present in bacteria, algae, and plant plastids. The second is the mevalonate (MVA) pathway, present in some bacteria, archea, fungi, plants, and animals (Lohr et al. 2012). The MEP pathway uses pyruvate and glyceraldehyde-3-phosphate while the MVA pathway uses acetate as starting materials. They both produce two 5-carbon isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Holstein, and Hohl, 2004; Lohr et al. 2012). ABA biosynthesis occurs in plastids and therefore utilizes products from the MEP pathway (Ruiz-Sola and Rodriguez-Concepcion, 2012).

DMAPP, a product of the MEP pathway, undergoes three condensation reactions, adding an IPP molecule each time, to form geranylgeranyl pyrophosphate (GGPP). Two GGPPs are then condensed into phytoene (Cunningahm and Gantt, 1998). This is the first committed step in the synthesis of carotenoids. Carotenoids are a class of plant pigments that consist of 40 or more carbons. They produce a range of colours, from red to yellow, due to their numerous, sometimes up to 15, conjugated double bonds (Isaacson et al. 2002). There are two major groups within the carotenoids: carotenes, which are either cyclized at one, both, or none of the ends of the molecule, and xanthophylls, which are oxygen-containing carotenoid derivatives. Carotenoids accumulate in plastids, such as chloroplasts and chromoplasts (Ruiz-Sola and Rodriguez-Concepcion, 2012). Carotenoid biosynthetic enzymes are also localized in the chloroplasts, distributed between the stroma, envelope membrane, and thylakoid membrane (Ruiz-Sola and
Rodriguez-Concepcion, 2012; Nambara and Marion-Poll, 2005). There are a few steps in the pathway both the carotenoid and the ABA biosynthetic pathways, which remains uncharacterized. They are indicated by question marks in Figure 1. These enzymes include a neoxanthin synthase, which uses violaxanthin to make neoxanthin, and isomerase(s), which make 9-cis isomers of violaxanthin and neoxanthin (Nambara and Marion-Poll, 2005). Cleavage of the 9-cis-violaxanthin and 9’-cis-neoxanthin produces a C_{15} compound called xanthoxin, which then undergoes a two-step conversion to finally yield ABA (Xiong and Zhu, 2003).

1.2 Engineering the ABA biosynthetic pathway in \textit{Escherichia coli}

The bacteria \textit{Escherichia coli} are an ideal platform for engineering of the ABA biosynthetic pathway. The carotenoid precursors of ABA have been produced in the non-carotenogenic \textit{E. coli}. A bacterial gene cassette, seen in Figure 2, when expressed in \textit{E. coli} facilitated the production of the carotenoids phytoene, \(\zeta\)-carotene, lycopene, \(\beta\)-carotene and zeaxanthin. This gene cassette was derived from the non-photosynthetic bacteria \textit{Pantoea ananatis}, formerly known as \textit{Erwinia uredovora} (Misawa et al. 1990; Misawa et al. 1995). With the help of this gene cassette, several plant carotenoid enzymes upstream of zeaxanthin production will be bypassed when engineering the pathway in \textit{E. coli}. Only a few attempts have made to produce carotenoids downstream of zeaxanthin in the ABA biosynthetic pathway in bacteria (Zhu et al. 2002; Neuman et al. 2014). The reason was the ABA biosynthetic pathway in other organisms is not as well researched as in plants. Therefore, building the indirect ABA biosynthetic pathway in \textit{E. coli} would require the expression of plant genes.
Figure 2. *Pantoea ananatis* carotenoid gene cassette. When expressed in *E. coli*, it produces zeaxanthin.

1.3 *A. thaliana* genes will supplant the missing steps of the ABA biosynthetic pathway in engineered *E. coli*.

Several of the major ABA biosynthetic mutants, which severely affect ABA levels have been identified in *A. thaliana*. In addition, ABA biosynthetic pathway in angiosperms like *Z. mays*, *N. plumbaginifolia* and *A. thaliana* is highly conserved (Xiong and Zhu 2003). Therefore, starting from zeaxanthin, the genes in ABA biosynthetic pathway that are currently missing in *E. coli* will be filled in using *A. thaliana* genes. The first step in engineering the pathway is to have a functioning zeaxanthin epoxidase (ZEP) enzyme in *E. coli*, capable of producing violaxanthin. AtZEP or ABA1 catalyzes a two-step reaction from zeaxanthin to violaxanthin. Mutants are impaired in ABA biosynthesis (Duckham *et al.*1991). The gene was first isolated in *N. plumbaginifolia* (Marin *et al.* 1996). Its homologue in *Capsicum annuum* was shown to catalyze the reaction from zeaxanthin to violaxanthin in *vitro* (Bouvier *et al.* 1996).
1.4 The short-term goal is to identify uncharacterized genes of the ABA biosynthetic pathway.

There are two pathways to synthesize xanthoxin from all-trans-violaxanthin. One path is through a 9-cis-violaxanthin intermediate and the other path is through neoxanthin and 9’-cis-neoxanthin (Nambara and Marion-Poll, 2005). The enzymes for these steps have yet to be identified, seen as question marks in Figure 1. After expressing zeaxanthin epoxidase in zeaxanthin-producing *E. coli* and confirming the presence of violaxanthin, the next step would be to express these uncharacterized genes.

Several types of plant enzymes are known to catalyze carotenoid isomerization. Candidate genes for the uncharacterized violaxanthin isomerase, which isomerizes all-trans-violaxanthin into 9-cis-violaxanthin, were chosen based on their homology to known genes in the carotenoid pathway and their ability to facilitate similar chemical reactions. The candidates for the violaxanthin isomerase can be separated into two groups. The *A. thaliana* genes belonging to the first group are homologues of the rice gene *DWARF27* (*D27*). Rice D27 has been shown *in vitro* to isomerize all-trans-β-carotene to 9-cis-β-carotene. 9-cis-β-carotene is a precursor of the hormone strigolactone (Lin *et al.* 2009). It is a similar reaction to the isomerization of all-trans-violaxanthin to 9-cis-violaxanthin, seen in Figure 3 (Alder *et al.* 2012; Nambara and Marion-Poll, 2005). Furthermore, β-carotene and 9-cis-β-carotene have similar structures to violaxanthin and 9-cis-violaxanthin, respectively.
Another group of candidate genes is homologues of the carotenoid enzymes PDS, ZDS, and CRTISO. Plant PDS catalyzes a two-step reaction from phytoene to 9,15,9'-cis-ζ-carotene. PDS adds two conjugated double bonds to phytoene and facilitates the isomerization of its 9 and 9’ carbons from trans to cis configurations (Qin et al. 2007; Matthews et al. 2003). The PDS isomerization is similar to the reaction catalyzed by the uncharacterized violaxanthin isomerase. ZDS adds two conjugated double bonds to 9,9'-cis-ζ-carotene and forms prolycopene (Isaacson et al. 2004). CRTISO facilitates the isomerization of prolycopene to lycopene by changing its 9,9’-cis configuration to all-trans (Park et al. 2002; Isaacson et al. 2004).

**Figure 3.** Isomerization of A) violaxanthin to 9-cis-violaxanthin by an uncharacterized isomerase, and B) β-carotene to 9-cis-β-carotene by rice D27 enzyme.
Figure 4. The chemical structures of products of the *A. thaliana* carotenoid biosynthetic pathway from phytoene to lycopene. PDS (phytoene desaturase), Z-ISO (ζ-carotene isomerase), ZDS (ζ-carotene desaturase), CRTISO (carotenoid isomerase).

In the alternate pathway, that synthesizes xanthoxin, two candidate neoxanthin synthase genes that may catalyze the isomerization of violaxanthin to neoxanthin were isolated. These were *ABA4* and *NXD1*. *aba4* plants had reduced levels of ABA under dehydration stress. When ABA4
is constitutively expressed levels of trans-neoxanthin increased (North et al. 2007). A mutation in *neoxanthin deficient 1* (*nxd1*), a *S. lycopersicum* gene, impaired the accumulation of neoxanthin and 9'-cis-neoxanthin in the plant (Neuman et al. 2014). *A. thaliana* mutants of its ortholog, *AtNXD1*, were also impaired in the accumulation of neoxanthin and its isomer. *NXD1* is a highly conserved gene, but no deleterious effects were recorded in mutants. They are still able to synthesize ABA at a similar level to wild type, suggesting that 9-cis-violaxanthin is sufficient for the biosynthesis of ABA in *A. thaliana* (Neuman et al. 2014).

1.5 Xanthoxin and beyond

The next step after producing sufficient amounts of 9-cis-violaxanthin or 9'-cis-neoxanthin is the addition of an *NCED* gene to produce xanthoxin from the cleavage of 9-cis-violaxanthin and 9'-cis-neoxanthin (Figure 1, Schwartz et al. 1997). There are 9 *NCED* related genes, designated as carotenoid cleavage dioxygenase (CCD), found in *A. thaliana* (Nambara and Marion-Poll 2005). Five of these, *AtNCED2*, 3, 5, 6 and 9, are involved in the biosynthesis of ABA and have been shown to catalyze the formation of xanthoxin (Iuchi et al. 2001; Toh et al. 2008, Frey et al. 2012).

In this study, I expressed known carotenoid enzymes in the engineered *E. coli* producing zeaxanthin. I optimized expression of AtZEP/ABA1 by investigating various promoters, growth conditions, and carotenoid extraction methods, developing a novel functional analysis system to identify and characterize unidentified carotenoid genes. By using this system, I expressed homologues of carotenoid enzyme genes and examined if these genes alter the artificial carotenoid biosynthesis pathway in *E. coli*.

2 Materials and Methods

2.1 Growing engineered *E. coli* producing carotenoids for HPLC analysis

*E. coli* were grown overnight at 37°C on plates with the appropriate antibiotics. A single colony was inoculated into 2 mL of LB liquid media with proper antibiotics and was grown in the 37°C
shaker at 220 rpm. Fifty-µL of the overnight culture was inoculated into 5 mL of LB liquid media with the correct antibiotics shaken overnight at 37°C and 220 rpm. The following day, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The *E. coli* was then transferred to the correct temperature for expression. Growing conditions were adapted from Isaacson *et al.* (2004) and Neuman *et al.* (2014).

### 2.2 Harvesting and extraction of carotenoids

One-mL of the IPTG-induced culture was used for the carotenoid extraction. To determine the cell concentration of *E. coli* in the culture, absorbance at 600 nm (OD$_{600}$) was measured for the 1mL samples. Cells were pelleted by centrifuging at 6000 rpm for 3 minutes and removing the supernatant, 800 µl of acetone were added, along with glass beads, to the cell pellet. The cells were lysed using the Qiagen TissueLyser II for 5 min at 30 frequency/s. Lysed cells were centrifuged at 13000 rpm for 1 min. Cell debris formed a pellet at the bottom of the tube. The supernatant, now containing the carotenoids, was pipetted out into a new glass tube. The acetone wash step was repeated another 1-2 times, or until the supernatant became colourless. The supernatant in the glass tube was vacuum dried using a Labconco CentriVap Concentrator for approximately 45 min, or until all the liquid evaporated. The carotenoids remained at the bottom of the tube and were re-dissolved in 1 mL ethyl acetate. The tube was agitated for approximately 30 seconds and 1ml of 1% (v/v) acetic acid dissolved in ddH$_2$O, was added. Samples were then agitated again for another 10 seconds and subsequently centrifuged. The top layer was transferred into a new glass tube. The ethyl acetate wash step was repeated until the top liquid layer became colourless. The top liquid layer was dried using the Labconco CentriVap Concentrator for approximately 1 hour, or until all the liquid had evaporated. The dried sample was dissolved using 49.5% (v/v) methanol, 49.5% (v/v) ddH$_2$O, and 1% acetic acid solution. Afterwards, the samples analyzed using High Pressure Liquid Chromatography (HPLC) or were stored at -20°C for future analysis.
2.3 HPLC condition

Carotenoids were separated by reverse phase HPLC using a C18 column

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Acetonitrile: Isopropanol$^a$</th>
<th>Methanol$^b$</th>
<th>H$_2$O$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-9</td>
<td>60%</td>
<td>10%</td>
<td>30%</td>
</tr>
<tr>
<td>9-12</td>
<td>85%</td>
<td>10%</td>
<td>5%</td>
</tr>
<tr>
<td>12-14</td>
<td>90%</td>
<td>10%</td>
<td>0%</td>
</tr>
<tr>
<td>14-20</td>
<td>60%</td>
<td>10%</td>
<td>30%</td>
</tr>
</tbody>
</table>

HPLC solvents consisted of $^a$94.5% acetonitrile and 5.55% isopropanol, $^b$99.5% methanol and 0.5% acetic acid, and $^c$99.5% ddH$_2$O and 0.5% acetic acid.

Solvent compositions during the HPLC analysis are shown in Table 1. The samples were injected using the Rheodyne 7725i injector, at a flow rate of 1 ml min$^{-1}$. The total run time for one sample injected into the HPLC was 20 min. Light absorption peaks were detected at 254 nm, 380 nm, 450 nm, and 600 nm using an Agilent Technologies 12000 series Photodiode Array Detector G1315D.

2.4 Violaxanthin standard

All-trans-violaxanthin (CAS# 126-29-4) standard was purchased from Chromadex. The purity of the standard was 99.3% by HPLC and the product concentration in a prepared ethanol solution was 0.897 mg/L.

2.5 Cloning

Information for the coding sequences of each gene was obtained from The Arabidopsis Information Resource website (https://www.arabidopsis.org). The protein sequence was entered into the program ChloroP (http://www.cbs.dtu.dk/services/ChloroP) to determine the presences of a transit peptide sequence. This information was then used to make primers from the coding DNA sequence to exclude the potential transit peptide sequence from the sequence that is to be inserted into a plasmid. The transit peptide sequence was removed from the insert because it is cleaved from the mature protein in plants.

New England BioLabs (NEB) Phusion DNA Polymerase was used, according to the manufacturer’s instructions, for all cloning. Restriction enzyme digestion was performed
according to the manufacturer’s instruction (NEB or ThermoFischer Scientific). Ligation reactions were performed using T4 DNA Ligase (NEB, catalog number M0202), in accordance with its protocol.

Gibson assembly was performed using the NEB Gibson Assembly Master Mix, catalog number E2611, and by following relevant protocols (Gibson et al. 2009).

2.5.1 AtZEP classical cloning

AT5G67030 or AtZEP was amplified from dry seed cDNA using the forward primer, GGATCCGGCGGCGACGGCGTTAGTT and reverse primer GGCGCGCCTCAAGCTGTCTGAAAGTAATTTATCGTTG, with a BamHI site and an AscI site, respectively. AtZEP contains a transit peptide excluded by the cloning procedure. The annealing temperature was 66°C and the elongation time was 1 minute. AtZEP was inserted into the first multiple cloning site (MCS) of pETDuet-1 plasmid using the restriction sites, BamHI and AscI (NEB, catalogue numbers for BamHI and AscI are R0136S and R0558S, respectively). Double digests were preformed on both pETDuet-1 plasmid and the AtZEP insert using the CutSmart buffer.

2.5.2 Cloning of genes of interest

In order to express carotenoid enzyme genes and their homologues in violaxanthin-producing E. coli, AT1G03055, AT1G64680, AT4G01995, AT1G57770, AT5G49555, AT4G14210, and AT3G04870 were cloned from plasmids obtained from Arabidopsis Biological Resource Center (ABRC, Table 2). AT1G06820 was cloned from 24-hr-imbibed seed cDNA. Forward primers, reverse primers, annealing temperatures for PCR, and elongation times for PCR, are enumerated in Table 2. The restriction sites for AscI and NotI (NEB, catalog number R3189) were added into the forward and reverse primers of each candidate, respectively. Double digests were performed on both pCOLIDuet-1 plasmid and inserts using the NEB CutSmart buffer. Genes of interest were inserted in the first MCS of pCOLIDuet-1. Plasmids created are listed in Table 4.
Table 2. Genes inserted in pCOLADuet-1 plasmid.

<table>
<thead>
<tr>
<th>AGI Code</th>
<th>ABRC stock code</th>
<th>Predicted Transit Peptide</th>
<th>Forward Primer (AscI)</th>
<th>Reverse Primer (NotI-HF)</th>
<th>Annealing Temperature</th>
<th>Elongation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G03055</td>
<td>G12648</td>
<td>No</td>
<td>GCCGCCGCTGGCTGC AAAAGAGACAGCA</td>
<td>GCCGCCGCTAATGCTTCACACCGTAGC</td>
<td>60</td>
<td>15 sec</td>
</tr>
<tr>
<td>AT1G64680</td>
<td>G61363</td>
<td>Yes</td>
<td>GCCGCCGCTGGATGCC GGCTATGGCTAGTCG</td>
<td>GCCGCCGCTCATGCGCTAGTTTAGGGC</td>
<td>60</td>
<td>15 sec</td>
</tr>
<tr>
<td>AT4G01995</td>
<td>U16633</td>
<td>No</td>
<td>GCCGCCGCTGTCAGAGTGAGCTCCG</td>
<td>GCCGCCGCTTACGGCTAAGGACACTCCC</td>
<td>61</td>
<td>15 sec</td>
</tr>
<tr>
<td>AT1G06820</td>
<td>Cloned from 24hr imbibed seed cDNA</td>
<td>Yes</td>
<td>GCCGCCGCTGGCTAGTGAGCTCCG</td>
<td>GCCGCCGCTTACGGCTAAGGACACTCCC</td>
<td>61</td>
<td>15 sec</td>
</tr>
<tr>
<td>AT1G57770</td>
<td>G18710</td>
<td>No</td>
<td>GCCGCCGCCAGAGCTCCG</td>
<td>GCCGCCGCTACAAGGCACAATGCCATCA</td>
<td>60</td>
<td>1 min</td>
</tr>
<tr>
<td>AT5G49555</td>
<td>W21356</td>
<td>Yes</td>
<td>GCCGCCGCTGGCTAGTGAGCTCCG</td>
<td>GCCGCCGCTTACGGCTAAGGACACTCCC</td>
<td>60</td>
<td>1 min</td>
</tr>
<tr>
<td>AT4G14210</td>
<td>G09151</td>
<td>Yes</td>
<td>GCCGCCGCTGGCTAGTGAGCTCCG</td>
<td>GCCGCCGCTTACGGCTAAGGACACTCCC</td>
<td>62</td>
<td>1 min</td>
</tr>
<tr>
<td>AT3G04870</td>
<td>G19281</td>
<td>Yes</td>
<td>GCCGCCGCTGGAGACAATGAGTTAAACGCTCCG</td>
<td>GCCGCCGCTTACGGCTACAGCCAGGCTTACCATCC</td>
<td>60</td>
<td>1 min</td>
</tr>
</tbody>
</table>

AGI code for each candidate. ABRC stock code of the plasmid used for its cloning unless otherwise specified. Transit peptide prediction was done through the program ChloroP. Forward primers, reverse primers, the annealing temperature and elongation time used for candidate cloning using the PCR amplification using NEB Phusion DNA Polymerase are listed.
2.5.3 AtNXD1 cloning

AT1G28100 or AtNXD1, the candidate for neoxanthin synthase was cloned using 24-hr-imibed seed cDNA using the forward primer, ATTCGAGCTCGGCCTGATGGACGTTGAAAGAAAAGA, and reverse primer, TACTTTCTGTTCGACCTTAAGTTACTTTGAAAGGTAAATTACA. The restriction sites for AscI and AflII (NEB, catalog number R0520) were inserted into the forward and reverse primers, respectively. For PCR, the annealing temperature used was 56°C and the elongation time was 30 seconds. NEB CutSmart buffer was used for digestion of the plasmid and insert. AtNXD1 was inserted into the pCOLADuet-1 first MCS, and named pCOLA-AtNXD1. 

2.5.4 AtNCED3 Cloning

AT3G14440 or AtNCED3, one of the five NCEDs in A. thaliana was cloned using genomic DNA from seedlings. The forward primer used was CATATGGCCAGTCGTGTCACACG with an NdeI restriction site. The reverse primer used was GGTACCCACGACCTGCTTCGC with a KpnI restriction site inserted into it. The PCR annealing temperature was 60°C and elongation time was 1 minute. AtNCED3 and pCOLADuet-1 was digested in NEB CutSmart buffer (NdeI, NEB, catalog number R0111, KpnI, NEB, catalog number R0142). AtNCED3 was inserted into the second MCS of pCOLADuet-1, and was designated pCOLA-AtNCED3. 

2.5.5 Gibson Assembly of pACHP-Zea(Spec)

The spectinomycin resistance gene, together with its ampicillin resistance promoter in pCDFDuet-1, was amplified using the forward primer CGTCTTTTCATTGCCATACGGTTATTTGCCGACTACC, and reverse primer TGAATGCTCATCCGGAATTTTTGTTTATTTTCTAAATACATTC. Annealing temperature for PCR was 70°C and the elongation time was 30 seconds. Using the EcoRI enzyme (ThermoFischer Scientific, catalog number ER0271) and the EcoRI specific buffer, pACHP-Zea was cut in the middle of the chloramphenicol resistance gene and linearized. Gibson assembly was used to insert the spectinomycin resistance gene into pACHP-Zea. The new plasmid was named pACHP-Zea(Spec).
2.5.6 AtZEP cloning by Gibson Assembly

AtZEP was amplified from dry seed cDNA with the forward primer GTTTATAAGGACAGCCCAGAATGGCGGCAGCGGTTAGTT and reverse primer AAATTGCACTGAAATCTAGATCAAGCTGCTGCTGAAGTAATTTATCGTTG. This was done at the annealing temperature of 66°C and elongation time was 15 seconds. The P. ananatis promoter was amplified from plasmid pACHP-Zea with the forward primer TCAGATAAAATTTCTAGAAATTTTCCACCTTTTCCACAAG and reverse primer AACTAACGCGTCGCCACCTCGGCTGTCCTTATAAAC. The annealing temperature used for amplification was 64°C and the elongation time was 1 minute. pACHP-Zea was linearized using the XbaI enzyme (ThermoFischer Scientific, catalog number ER068) and Tango buffer. Gibson assembly was used to insert AtZEP into the pACHP-Zea(Spec) plasmid. It was designated as pACHP-Zea(Spec)-AtZEP.

2.6 Strains of E. coli

For cloning purposes and propagation of plasmids, DH5α was used.

BL21-CodonPlus (DE3)-RIPL (BL21 CodonPlus), catalog number 230280, from Agilent Technologies was used for carotenoid production and for gene expression. DE3 denotes that the strain carries the IPTG inducible T7 RNA polymerase. RIPL CodonPlus means that it carries the rare tRNAs argU (AGA, AGG), ileY (AUA), proL (CCC), leuW (CUA).

2.7 Plasmids

2.7.1 Duet Plasmid System

Table 3. Novagen Duet plasmids.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Catalogue numbers</th>
<th>Antibiotic resistance</th>
<th>Origin of replication</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYCDuet-1</td>
<td>71147-3</td>
<td>Chloramphenicol</td>
<td>P15A</td>
</tr>
<tr>
<td>pCDFDuet-1</td>
<td>71340-3</td>
<td>Spectinomycin</td>
<td>CDF</td>
</tr>
<tr>
<td>pCOLADuet-1</td>
<td>71406-3</td>
<td>Kanamycin</td>
<td>ColA</td>
</tr>
<tr>
<td>pETDuet-1</td>
<td>71146-3</td>
<td>Ampicillin or Carbenicillin</td>
<td>ColE1</td>
</tr>
</tbody>
</table>

The Duet Plasmid system features four different plasmids, each with different origins of replication, and antibiotic resistance, shown in Table 3. Each plasmid contains two MCSs under the control of a T7 promoter, allowing for control over protein expression. The first MCS
contains an N-terminal His\textsubscript{6}-tag, while the second MCS contains a C-terminal Myc-tag (Yan et al. 2008). Only two of the plasmids were used for this project, pETDuet-1 and pCOLADuet-1.
### 2.7.2 Plasmid list

Table 4. Plasmids and their descriptions used for this project.

<table>
<thead>
<tr>
<th>Plasmid$^a$</th>
<th>AGI Code$^b$</th>
<th>Description</th>
<th>Antibiotic</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACHP-Zea</td>
<td>N/A</td>
<td>Carotenoid gene cassette and IDI</td>
<td>Chloramphenicol</td>
<td>Misawa et al. 1995, unpublished data</td>
</tr>
<tr>
<td>pACHP-Zea(Spec)</td>
<td>N/A</td>
<td>Carotenoid gene cassette and IDI. Switched antibiotic resistance for use transformation into BL21 CodonPlus</td>
<td>Spectinomycin</td>
<td>This project</td>
</tr>
<tr>
<td>pACHP-Zea(Spec)-AtZEP</td>
<td>N/A</td>
<td>Inserted AtZEP under a P. ananatis promoter</td>
<td>Spectinomycin</td>
<td>This project</td>
</tr>
<tr>
<td>pET-AtZEP</td>
<td>AT5G67030</td>
<td>AtZEP in the 1st MCS</td>
<td>Ampicillin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-AT1G03055</td>
<td>AT1G03055</td>
<td>D27 homologue in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-AT1G64680</td>
<td>AT1G64680</td>
<td>D27 homologue in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-AT4G01995</td>
<td>AT4G01995</td>
<td>D27 homologue in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-CRTISO</td>
<td>AT1G06820</td>
<td>CRTISO/CCR2 in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-AT1G57770</td>
<td>AT1G57770</td>
<td>CRTISO-like / PDH-like in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-CRTISO</td>
<td>AT5G49555</td>
<td>CRTISO-like /PDS in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-PDS</td>
<td>AT4G14210</td>
<td>PDS3 in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-ZDS</td>
<td>AT3G04870</td>
<td>ZDS in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-AtNXD1</td>
<td>AT1G28100</td>
<td>AtNXD1 in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
<tr>
<td>pCOLA-AtNCED3</td>
<td>AT3G14440</td>
<td>AtNCED3 in the 1st MCS</td>
<td>Kanamycin</td>
<td>This project</td>
</tr>
</tbody>
</table>

$^a$Besides the first 3 all of the plasmids are IPTG inducible  
$^b$AGI codes were included for single gene inserts.
2.8 Western Blot

2.8.1 Growth of *E. coli* harboring pET-AtZEP for protein extraction

BL21 CodonPlus harboring pET-AtZEP was streaked from glycerol stock onto plates containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol and grown overnight at 37°C. A single colony was used to inoculate 5 mL of LB liquid media with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. The liquid culture was grown overnight at 37°C with shaking at 220 rpm. OD was measured at OD$_{600}$ before addition of IPTG. The culture was then transferred to 30°C shaking at 220 rpm. Before IPTG addition, 1 hour, 5 hours, and 10 hours after IPTG addition, 50 mL samples were taken from the culture and the OD$_{600}$ absorbance were taken.

2.8.2 Growth of *E. coli* harboring pCOLA-AtNXD1 for protein extraction

BL21 CodonPlus pACHP-Zea(Spec) pCOLA-AtNXD1 was streaked from glycerol stock onto selection plates (100 µg/ml spectinomycin, 50 µg/ml kanamycin, and 30 µg/ml chloramphenicol) and then grown overnight at 37°C. A single colony was picked and used to inoculate 5 mL of LB media with 100 µg/ml spectinomycin, 50 µg/ml kanamycin, and 30 µg/ml chloramphenicol. The culture was allowed to grow for a day at 37°C. IPTG was added and the culture was incubated for an additional 5 hours at 30°C before harvesting.

2.8.3 Gel electrophoresis and Western blotting

Cells were pelleted and stored in -80°C when not immediately used for western blot. After, the cells were boiled in SDS loading buffer and loaded on the 12% acrylamide gel. The gel was run at 150 volts for 1-2 hours until the dye ran off the gel. The protein was transferred to a PVDF membrane at 100 volts for 1 min/kDa of protein. The concentration of the first antibody, Roche Diagnostics Anti-His$_6$, was 1:4000. The concentration of the secondary antibody, Anti-mouse IgG AP-linked (Cell Signaling Technology catalog number 7056), was 1:20000.

3 Results

3.1 Construction and expression of pACHP-Zea(Spec)

The pACHP-Zea plasmid contains a gene cassette composed of five carotenoid genes from the bacteria *P. ananatis*, shown in Figure 2 (Misawa *et al*. 1995). In addition to those five genes, *IDI*
from maize was also inserted into the plasmid. IDI reversibly isomerizes IPP into DMAPP. These steps are highlighted by the trapezoid in Figure 1. Expression of the maize IDI increases carotenoid production in *E. coli* (Bartley *et al.* 1999, unpublished data).

The host chosen for carotenoid production was the *E. coli* strain BL21 CodonPlus. This strain features extra copies of the genes that encode rare tRNAs, which limit translation of heterologous proteins in *E. coli*. The greater availability of these tRNAs allows for higher levels of expression of protein in the BL21 CodonPlus strains, which are poorly expressed in the conventional BL21 strains (Carstens *et al.* 2002).

DE3 strains contain the IPTG inducible T7 RNA polymerase, which allows for the induction of genes under the control of the T7 promoter (Carstens *et al.* 2002). However, pACHP-Zea contains the chloramphenicol resistance marker, the same resistance marker used in BL21CodonPlus, which prohibited its transformation using pACHP-Zea (Misawa *et al.* 1995; Neuman *et al.* 2014). I introduced a spectinomycin resistance gene from pCDFDuet-1 and disrupted the chloramphenicol resistance gene in pACHP-Zea to solve this problem. The new plasmid, designated as pACHP-Zea(Spec), produces the carotenoid zeaxanthin, when expressed in *E. coli*. Phenotypically, BL21 CodonPlus harboring pACHP-Zea(Spec) shows a change of colour from the normal colour, white (Figure 5A), to yellow-orange (Figure 5B). The colour matches the description for purified zeaxanthin (Sun *et al.* 1996).

**Figure 5.** Streaked colonies of carotenoid producing *E. coli*. A) BL21 CodonPlus B) BL21 CodonPlus pACHP-Zea(Spec) after 1 day at 37°C.
Carotenoids were extracted using two washes; the first using acetone and the second ethyl acetate. After re-dissolving the carotenoids in methanol and water, samples were injected into the HPLC. The zeaxanthin-producing *E. coli* produced a large peak at around 5.3 min from zeaxanthin, shown in Figure 6A.

3.2 *AtZEP* expression produces all-trans-violaxanthin

![HPLC chromatogram of extracted zeaxanthin and violaxanthin from *E. coli*. A) BL21 CodonPlus pACHP-Zea(Spec). The culture was grown for 2 days before harvesting. B) BL21 CodonPlus pACHP-Zea(Spec) pET-AtZEP. It was grown 1 day before IPTG addition and harvested a day later. The arrow indicates the novel peak for violaxanthin that appeared after IPTG induction of BL21 CodonPlus pACHP-Zea(Spec) pET-AtZEP. C) All-trans-violaxanthin standard from Chromadex. Absorbance was measured at 450 nm with the reference wavelength 600 nm.](image-url)
The next step in the pathway is catalyzed by zeaxanthin epoxidase (ZEP), which produces violaxanthin from the precursor zeaxanthin. ZEP/ABA1 catalyzes the first committed step in the biosynthesis of ABA (Nambara and Marion-Poll, 2005).

AtZEP was inserted into the IPTG inducible pETDuet-1 plasmid, designated as pET-AtZEP. This plasmid and the zeaxanthin-producing plasmid, pACHP-Zea(Spec) were co-transformed into BL21 CodonPlus. I experimented with the timing of the addition of IPTG to induce production of violaxanthin at 30°C in 5 mL cultures. I found that the conventional addition of IPTG after growth to OD$_{600}$ in the range of 0.4 to 0.6 was sufficient for protein production to occur 5 hours after addition of IPTG. However, it was not sufficient to produce violaxanthin at detectable levels for HPLC analysis, even after additional growth for 2 or 3 days (data not shown). I found that BL21 CodonPlus E. coli harboring pACHP-Zea(Spec) needs to be grown for at least 24 hours at 30°C in 5 mL cultures. The HPLC chromatogram of E. coli transformed with both pACHP-Zea(Spec) and pET-ZEP IPTG produced a new peak at around 3 min, with the addition of IPTG (Figure 6B). The identity of this product was confirmed with the injection of an all-trans-violaxanthin standard into the HPLC, which produced a peak appearing at, or close to, the same time as the novel peak (Figure 6C). However, violaxanthin production was limited in this condition and a large amount of zeaxanthin remained (Figure 6B).
To investigate whether the problem was with protein expression, I performed a western blot using the BL21 CodonPlus *E. coli* containing the plasmids pET-*AtZEP* and pACHP-Zea(Spec) and analyzed the protein levels from each strain. The *E. coli* was grown overnight in a 5 mL culture at 30°C. IPTG was added to the culture; and it was grown for an additional 5 hours. A band was seen between 70-80 kDa for pET-*AtZEP* after IPTG addition (Figure 7). Its size matches the known size of the AtZEP protein at 72 kDa (Marin et al. 1996).
I tried to resolve the issue of low violaxanthin production by expressing \textit{AtZEP} constitutively. \textit{AtZEP} was inserted into pAHCP-Zea (Spec) plasmid under the control of the \textit{P. ananatis} promoter using the Gibson Assembly method. This promoter also regulates the expression of the carotenoid cluster in pACHP-Zea. The new plasmid was designated as pACHP-Zea(Spec)-\textit{AtZEP} (Misawa \textit{et al}. 1995, Neuman \textit{et al}. 2014).

\textbf{Figure 8.} Violaxanthin levels of IPTG-induced \textit{AtZEP} and constitutively expressed \textit{AtZEP}. The IPTG-induced \textit{AtZEP} strain contains the plasmids pACHP-Zea(Spec) and pET-\textit{AtZEP} (left), while the constitutively expressed \textit{AtZEP} strain contains the plasmid pACHP(Spec)-\textit{AtZEP} (right). The day denotes time of harvest starting from when IPTG was added. The Day 0 sample was collected before IPTG addition. IPTG was added only to the \textit{E. coli} harboring the pET-\textit{AtZEP} plasmid. Each day 1mL of \textit{E. coli} culture was taken from each sample for carotenoid extraction. Error bars represent SE (n=3). Absorbance was measured at 450 nm with the reference wavelength 600 nm.

I performed a time course experiment using the IPTG-induced \textit{AtZEP} and constitutively expressed \textit{AtZEP} in \textit{E. coli} harboring pACHP-Zea(Spec) to elucidate at what time all-\textit{trans}-violaxanthin levels peaked, and to compare how much is produced from each plasmid. Figure 6 shows the level of violaxanthin produced by the induced \textit{AtZEP} strain, which was lower than the constitutive \textit{AtZEP} strain in all of the days of the time course experiment. Violaxanthin accumulation was highest at 24 hours after IPTG addition or the 48 hours of \textit{E. coli} growth for the induced \textit{AtZEP}. For the constitutively expressed \textit{AtZEP}, the peak was reached on the second day of \textit{E. coli} growth. The concentration of violaxanthin then slowly decreased over time after the peak had been reached. \textit{E. coli} harboring \textit{AtZEP} under the control of a constitutive promoter produced more violaxanthin compared to the one under the control of the T7 promoter. Therefore, I decided to use the strain containing the constitutively expressed \textit{AtZEP} as the host to
express the remaining genes for this study. I previously tested levels of carotenoids grown at different temperatures on plates. I found the suitable temperature range for both zeaxanthin and violaxanthin production in *E. coli* was between 16°C-30°C (Appendix Figure A1).

3.3 Expression of carotenoid enzyme-related genes in *E. coli* harboring pACHP(Spec)-AtZEP

Figure 1 illustrates the two pathways that contribute to xanthoxin production. The first is through 9-*cis*-violaxanthin and the second is through neoxanthin and 9'-*cis*-neoxanthin. Both 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin are cleaved by NCED *in vitro*. When comparing the two pathways, violaxanthin conversion to xanthoxin consists of only two steps, while there are three steps if the neoxanthin and 9'-*cis*-neoxanthin are used. It follows that the next step after stably producing violaxanthin is to search for the uncharacterized violaxanthin isomerase. Candidate genes were chosen based on their homology with known genes of the carotenoid biosynthetic enzymes. AT1G03055, AT1G64680, and AT4G01995 are D27 homologues. D27 isomerizes all-*trans*-β-carotene into 9-*cis*-β-carotene, a similar reaction to the isomerization that occurs to all-*trans*-violaxanthin to form 9-*cis*-violaxanthin (Alders *et al.* 2012; Nambara and Marion-Poll, 2005). AT1G57770 and AT5G49555 are homologous to enzymes of the carotenoid biosynthetic pathway, including PDS, ZDS, and CRTISO. The genes of interest (Table 2) were expressed using the plasmid constructs shown in Table 4, in the *E. coli* harboring the plasmid pACHP-Zea(Spec)-AtZEP.

From the previous experiment (Figure 8), violaxanthin production of *E. coli* that constitutively expresses AtZEP, peaks after 2 days of growth. I decided to add the IPTG after 1 day of growth for gene induction to synchronize it with the increase of violaxanthin.
Of the eight constructs tested, only the plasmid containing the gene for PDS, or AT4G14210, showed a new peak with the retention time of approximately 12 min, when compared to the empty vector (Figure 7B). However, I do not think the novel peak is 9-cis-violaxanthin. The novel peak’s identity is most likely 9,15,9'-tri-cis-ζ-carotene. The reason is PDS catalyzes the desaturation of phytoene to 9,15,9'-tri-cis-ζ-carotene (Isaacson et al. 2004). The 9-cis-violaxanthin peak should appear in between the zeaxanthin peak and the violaxanthin peak (Qin et al. 2008).
It is possible the proteins expressed could have an effect on the biosynthesis of violaxanthin and zeaxanthin, since their homologues function upstream of both carotenoid compounds in the biosynthetic pathway. As a result, I performed another time course experiment using BL21 CodonPlus *E. coli* harboring both pACHP-Zea(Spec) and the candidate construct. I measured both violaxanthin and zeaxanthin levels for each construct (Figure 10). PDS lead to a reduction in both carotenoid levels. Some of the candidates showed an increase in the levels of both carotenoids, including AT1G64680, an uncharacterized D27 homologue, and AT1G57770 (a CRTISO homologue), compared to the empty vector. I suspect these two proteins function upstream of both zeaxanthin and violaxanthin biosynthesis. Expression of the D27 ortholog in *A. thaliana*, AT1G03055, showed reduced violaxanthin levels (Figure 8A). Additionally, the zeaxanthin levels remained close to the levels of the empty vector.
Figure 10. Carotenoid levels of candidate genes expressed in violaxanthin-producing *E. coli*. A) Violaxanthin levels B) zeaxanthin levels of genes of interest. Cultures were grown in LB media for 1 day before IPTG was added. The days denote time of harvest starting from when IPTG was added. Each day, 1 mL was taken from each sample for carotenoid extraction. Candidate descriptions are listed in Table 3. EV stands for empty vector. Error bars represent SE (n=3). Absorbance was measured at 450 nm with the reference wavelength 600 nm. V (violaxanthin), Z (zeaxanthin).
Some of the genes, such as AT4G14210 and PDS, caused a decrease in the levels of both carotenoids. Others, including AT1G64680, led to an increase in the amount of violaxanthin and zeaxanthin when compared to the empty vector.

3.4 AtNXD1 expression produces a novel peak

I was unable to identify the enzyme that catalyzes the isomerization of violaxanthin to 9-cis-violaxanthin from the isomerase candidate genes. This prompted me to test the alternative pathway that uses neoxanthin as a precursor for xanthoxin production, since the E. coli was able to stably produce violaxanthin. The first candidate was the newly discovered gene AtNXD1. nxd1 mutants in S. lycopersicum and A. thaliana failed to accumulate neoxanthin and its isomer. However, ABA levels in S. lycopersicum remained the same as in wild type. This suggests plants primarily use 9-cis-violaxanthin as a substrate for ABA biosynthesis (Neuman et al. 2014). Similarly, ABA4, a small protein in A. thaliana, affected production of neoxanthin in planta. aba4 also showed lower levels of ABA under heat stress, suggesting neoxanthin’s importance in the production of ABA when the plant is experiencing heat stress (North et al. 2007). AtNXD1 was cloned into the pCOLADuet-1 plasmid in the first MCS, making pCOLA-AtNXD1. I was unable to clone ABA4.
Figure 11. HPLC chromatogram of violaxanthin-producing *E. coli* containing pCOLA-AtNXD1. 
A) BL21 CodonPlus harboring the plasmid pACHP-Zea(Spec)-AtZEP 
B) BL21 CodonPlus with the plasmids pACHP-Zea(Spec)-AtZEP and pCOLA-AtNXD1. 
Cell cultures were grown for 24 hours before IPTG addition and harvested the next day. 
The arrow indicates the peak predicted to be neoxanthin. 
Absorbance was measured at 450 nm with the reference wavelength 600 nm. 
V (violaxanthin), Z (zeaxanthin).
The HPLC chromatogram of BL21 CodonPlus *E. coli* carrying the plasmids pACHP-Zea(Spec) and pCOLA-AtNXD1 produced a small novel peak at approximately 2.5 min. It appeared before the all-trans-violaxanthin peak, which was at approximately 3 min in the chromatogram (Figure 9). It is possible the novel peak represents neoxanthin. From literature, the neoxanthin peak appears before the violaxanthin peak, similar to the novel peak (Qin *et al.* 2008).

![Western blot](image)

**Figure 12.** Western blot of IPTG-induced BL21 CodonPlus harboring the plasmids pACHP-Zea(Spec)-AtZEP and pCOLA-AtNXD1. The primary antibody used was Anti-His<sub>6</sub> and, the secondary antibody used was Anti-mouse IgG AP-linked. Cells were grown overnight before IPTG addition. Protein expression was induced at 30°C for 5 hours.

To investigate if AtNXD1 was expressed, I performed western blot analysis. A band at approximately 31 kDa was observed, which intensified after the addition of IPTG (Figure 12). This is consistent with the known size of ATNXD1 (Neuman *et al.* 2014). A small amount of protein was detected even before the addition of IPTG.
3.5 Expression of AtNCED3 is unable to produce xanthoxin in violaxanthin-producing *E. coli*

I was unable to find the gene responsible for the isomerization of violaxanthin to 9-cis-violaxanthin. I decided proceed to the next step and express AtNCEDs in all-trans-violaxanthin-producing BL21 CodonPlus *E. coli*. This was to investigate whether all-trans-violaxanthin-producing in *E. coli* without the violaxanthin isomerase, is able to produce xanthoxin if an AtNCED was expressed. AtNCEDs uses both 9-cis-violaxanthin and 9'-cis-neoxanthin as substrates to produce xanthoxin (Schwartz *et al.* 1997). There are 5 NCEDs in *A. thaliana*, which are all capable of producing xanthoxin from 9-cis-violaxanthin and 9'-cis-neoxanthin. I was able to clone AtNCED3 into the pCOLADuet-1 plasmid, making the pCOLA-AtNCED3 plasmid. I then transformed this plasmid into all-trans-violaxanthin-producing *E. coli*. After IPTG induction, no new peak appeared in the chromatograms obtained. Next, I measured levels of zeaxanthin and violaxanthin using a time course experiment to discern if AtNCED3 had any effect.
Figure 13. Carotenoid levels of violaxanthin-producing *E. coli* harboring pCOLA-AtNCED3. A) Violaxanthin levels B) zeaxanthin levels of pACHP-Zea(Spec)-AtZEP pCOLA-AtNCED3. Cultures were grown in LB media for 1 day before IPTG was added. The days denote time of harvest starting from when IPTG was added. The Day 0 sample was collected before IPTG was added. Each day 1 mL was taken from each sample for carotenoid extraction. Absorbance was measured at 450 nm with the reference wavelength 600 nm. Error bars represent SE (n=3).

Violaxanthin levels of the NCED3 expressing *E. coli* were lower while zeaxanthin levels were higher in comparison to the empty vector (Figure 11).
4 Discussion

The ABA deficient mutants in plants have been instrumental in the discovery of the ABA biosynthetic pathway. ABA deficient mutants usually have precocious germination and wilty phenotypes, which helped in their identification. However, some enzymes in the biosynthetic pathway remain elusive. In an effort to characterized genes of the ABA biosynthesis pathway, I introduced several uncharacterized enzymes, in carotenoid-producing \textit{E. coli} (Figure 1). Zeaxanthin, a carotenoid precursor of ABA, has been successfully produced in \textit{E. coli} using a gene cassette from \textit{P. ananatis} (Misawa \textit{et al.} 1990; Misawa \textit{et al.} 1995). Subsequently, many aspects of the carotenoid biosynthetic pathway have been researched extensively in \textit{E. coli}, with some studies focusing on increasing the yield of carotenoids in \textit{E. coli} (Yoon \textit{et al.} 2007; Yoon \textit{et al.} 2009). Others were interested in producing novel carotenoids by introducing different enzymes from plants and bacteria into carotenoid-producing \textit{E. coli} (Cunningham and Gantt 2007). Its usefulness was shown when it was able to predict the presence of two isomerases in plants, which were upstream of the ZEP enzyme, Z-ISO and CRTISO (Bartley \textit{et al.} 1999, Park \textit{et al.} 2002; Chen \textit{et al.} 2010). Both isomerases allow carotenoid biosynthesis to proceed when plants are subjected to dark conditions (Bartley \textit{et al.} 1999). In addition, it has also been used to identify the Arabidopsis gene that encodes for lycopene β and ε cyclase (Cunningham \textit{et al.} 1996). However, few attempts have been made to produce compounds downstream of zeaxanthin. This was because the next step after zeaxanthin was only well characterized in plants (Zhu \textit{et al.} 2002; Neuman \textit{et al.} 2014). The aim of this project was to engineer the ABA biosynthetic pathway in \textit{E. coli}, downstream of zeaxanthin production.

4.1 Violaxanthin production in \textit{E. coli}

In order to produce violaxanthin in bacteria, as has been established by previous researchers, I began by selecting the appropriate strain of \textit{E. coli}. BL21 CodonPlus is suitable for carotenoid gene expression and protein production (Baneyx, 1999). A number of groups have studied the effects of different \textit{E. coli} strains on the yield of carotenoids and have come to the conclusion that K strains are better than B strains for the production of carotenoids (Cunningham and Gantt, 2007; Yoon \textit{et al.} 2009). K strain-derived \textit{E. coli}, such as DH5α and XL1Blue, are most commonly used for plasmid propagation. B strain derived-\textit{E. coli}, such as BL21 CodonPlus, on the other hand, are used and optimized for protein expression. However, these studies were
conducted using bacterial carotenoid genes and not plant genes (Cunningham and Gantt, 2007; Yoon et al. 2009). My experiments involved the expression of plant genes in order to progress the plant ABA biosynthetic pathway in *E. coli*.

The next step was to recapitulate the results of Zhu et al. (2002) and Neuman et al. (2014), who were able to produce violaxanthin in *E. coli*. I began by expressing *AtZEP* under the control of an IPTG inducible promoter. Upon addition of IPTG, production of violaxanthin slightly increased in *E. coli* strains containing the plasmids pACHP-Zea(Spec) and pET-*AtZEP*. This observation is supported by *in vitro* findings from Marin et al. (1996), who succeeded in producing violaxanthin from zeaxanthin, using the ZEP homolog from *N. plumbaginifolia* to catalyze the reaction.

Since violaxanthin production could not be consistently detected using HPLC, I attempted to produce this carotenoid by introducing *AtZEP* under the control of the *P. ananatis* gene cassette promoter. *AtZEP* was inserted into the pACHP-Zea(Spec) plasmid, resulting in the pACHP-Zea(Spec)-*AtZEP* plasmid. This was similar to the approach used by Neuman et al. (2014), whereby *AtZEP* was constitutively expressed. The plasmid pACHP-Zea(Spec)-*AtZEP* increased the violaxanthin yield considerably in comparison to the IPTG inducible pET-*AtZEP* (Figure 8). It could be due to a number of reasons. First, *AtZEP* is present for a longer period of time in *E. coli* harboring the constitutive construct pACHP-Zea(Spec)-*AtZEP*. Second, the T7 promoter used in pET-*AtZEP* is very strong and results in a high level of protein expression (Tabor, 1990; Deuschle et al. 1986). The high level of protein expression could have caused the creation of inclusion bodies. The inclusion bodies are aggregates of improperly folded and mostly non-functional protein (Lilie et al. 1998). Therefore, I decided to continue using the construct that constitutively expressed *AtZEP*, pACHP-Zea(Spec)-*AtZEP*.

4.2 Search for the enzyme that catalyzes violaxanthin isomerization to 9-cis-violaxanthin

Once the production of violaxanthin had been achieved, it was possible to go through two alternate routes towards the production of xanthoxin. The main path is through 9-cis-violaxanthin and the alternate path is through neoxanthin and 9'-cis-neoxanthin (Neuman et al. 2014). To move forward, I chose the shorter path through 9-cis-violaxanthin. I identified several
genes of interest based on their homology to known ABA or carotenoid biosynthetic enzymes and the enzymatic function they perform. All of the genes of interest were inserted under the control of a T7 promoter in an IPTG inducible plasmid pCOLADuet-1. The genes of interest can be separated into two categories. The first category contains the genes AT1G03055, AT1G64680, and AT4G01995, which are homologs of the D27 rice gene. In vitro experiments showed that D27 catalyzed the conversion of β-carotene to 9-cis-β-carotene, similar to the isomerization of violaxanthin to 9-cis-violaxanthin (Lin et al. 2009, Waters et al. 2012). Both β-carotene and violaxanthin, and their 9-cis isomers have similar structures (Figure 3). The second category contained the genes AT1G57770 and AT5G49555, which are homologues of the enzymes in the carotenoid biosynthetic pathway. These are PDS, CRTISO, and ZDS. PDS catalyzes the reaction from phytoene to 9,15,9'-tri-cis-ζ-carotene. It adds two conjugated double bonds and simultaneously isomerizes the product to its 9,9'-cis isomer (Qin et al. 2007, Isaacson et al. 2004). CRTISO catalyzes the reaction from 9,9'-di-cis-ζ-carotene to prolycopene. CRTISO adds two conjugated double bonds and at the same time isomerizes the two cis configurations at the 9 and 9' position to trans (Figure 4, Park et al. 2002). ZDS is a homologue of the gene PDS (Isaacson et al. 2004). However, when I tested them, most of the constructs did not produce new peaks in HPLC analysis. This suggests that they did not produce any new product or that the reactions they catalyze are inefficient. One reason for this might be that they need helper proteins for the reaction to occur, as was the case for the in vitro experiment using ZEP from C. annuum (Bouvier et al. 1996). In the experiment, in order for ZEP to epoxidize zeaxanthin to violaxanthin, a plant ferredoxin and a ferredoxin-oxidoreductase were required. These proteins are used by ZEP for electron transfer for the reaction to occur. However, these results also showed that it was possible to epoxidize zeaxanthin to violaxanthin with the help of a bacterial rubredoxin. This could explain why AtZEP is able to function in E. coli without the help of other proteins.

My attempts at finding the gene responsible for the isomerization of violaxanthin to 9-cis-violaxanthin were unsuccessful. However, I did find that some of the candidate genes effected the production of both zeaxanthin and violaxanthin. The gene candidates AT1G03055, AT1G64680, AT4G01995, AT1G57770, and AT5G49555 were homologues of D27, an isomerase (Lin et al. 2009; Waters et al. 2012). I measured the violaxanthin and zeaxanthin levels in E. coli by expressing the candidate genes to determine if they had an effect. When the
uncharacterized gene, AT1G64680, was expressed, production of both carotenoids increased, suggesting it plays a role in carotenoid biosynthesis, upstream of both zeaxanthin and violaxanthin. This finding illustrates the potential application of this system for studying carotenoid enzymes.

Expression of PDS further supports the use of such a system to investigate the ABA and carotenoid biosynthetic pathway. PDS is a plant enzyme that catalyzes the addition of conjugated double bonds and isomerization of phytoene into 9,15,9'-tri-cis-ζ-carotene (Figure 4, Isaacson et al. 2004; Bartley et al. 1999). When PDS was expressed in the violaxanthin-producing *E. coli*, a novel peak appeared, believed to be that of 9,15,9'-tri-cis-ζ-carotene (Figure 9). In bacteria, the enzyme CrtI is able to catalyze the conversion from phytoene to lycopene (Misawa et al. 1990). In plant systems, however, four enzymes are required for this reaction. The way CrtI, desaturates the phytoene molecule to produces and asymmetric ζ-carotene isomer, 7,8,11,12-tetrahydrolycopene. Plant PDS; on the other hand, desaturates phytoene in a symmetrical manner at carbons 11 and 11'. It produces a symmetrical ζ-carotene isomer, 7,8,7',8'-tetrahydrolycopene (Isaacson et al. 2004). When PDS is expressed, the bacteria are able to produce the intermediate 9,15,9'-tri-cis-ζ-carotene, a molecule that CrtI cannot use as a substrate. As a result, production of the downstream compounds is hindered, reducing the levels of both violaxanthin and zeaxanthin (Figure 10).

4.3 AtNXD1 could be the missing neoxanthin synthase

The alternate pathway for the conversion of neoxanthin to 9'-cis-neoxanthin is currently uncharacterized. The *A. thaliana* mutants of *ABA4* and *AtNXD1* were unable to accumulate neoxanthin and its 9'-cis isomer. I attempted to clone both *ABA4* and *AtNXD1*, but was successful only with *AtNXD1*. *AtNXD1* was under the control of a T7 promoter in the pCOLADuet-1 plasmid, creating the plasmid pCOLA-AtNXD1. The addition of IPTG to *E. coli* harboring pACHP-Zea(Spec)-AtZEP and pCOLA-AtNXD1 produced a new peak in HPLC analysis (Figure 11). This new peak could be that of neoxanthin, since it appeared before violaxanthin, in agreement with literature (Snyder et al. 2004; Neuman et al. 2014). Neuman et al. (2014) expressed AtNXD1 to see if it produced neoxanthin by using a construct similar to the one used in this study and a different strain of *E. coli*, XL1Blue. However, no evidence of neoxanthin production was presented. Nevertheless, the identity of this compound needs to be
confirmed using a neoxanthin standard. If I were able to confirm the identity of the novel peak as neoxanthin, the next step would be to transform the neoxanthin-producing \textit{E. coli} with the candidates for the 9-	extit{cis} isomerization of violaxanthin and test if the failed candidate genes for 9-	extit{cis}-vioalxanthin isomerization are able to perform the 9’-	extit{cis}-neoxanthin isomerization instead.

4.4 Xanthoxin could not be detected when AtNCED3 was expressed in all-	extit{trans}-violaxanthin-producing \textit{E. coli}

Next, I introduced the \textit{A. thaliana} AtNCED3 into violaxanthin-producing \textit{E. coli}. NCED catalyzes the cleavage reaction of the 9-	extit{cis}-violaxanthin and 9’-	extit{cis}-neoxanthin to form xanthoxin (Iuchi \textit{et al.} 2001; Nambara and Marion-Poll, 2005). \textit{Cis-trans} isomerization of carotenoids occurs by both enzymatic reaction and photoreaction (Janik \textit{et al.} 2010; Bartley \textit{et al.} 1999). I expect that all-	extit{trans}-violaxanthin synthesized in the engineered \textit{E. coli} would form \textit{cis}-isomer(s) at some level even without the presence of an isomerase(s). I expressed AtNCED3 in violaxanthin-producing BL21 CodonPlus \textit{E. coli}. No new peak was observed in HPLC analysis after IPTG induction of NCED3. This result does not necessarily rule out the production of xanthoxin. It could mean that xanthoxin was not detected using the current protocol or is being masked by another peak. In addition, the level of violaxanthin decreased, while the level of zeaxanthin increased. This indicates AtNCED3 may have some regulatory function in the production of violaxanthin.

AtNCEDs have a key regulatory function in the biosynthesis of ABA. There are five AtNCEDs (2, 3, 5, 6, and 9) and all five are able to catalyze the cleavage of 9-	extit{cis}-violaxanthin and 9’-	extit{cis}-neoxanthin (Nambara and Marion-Poll, 2005). The NCEDs are targeted to the plastids, but localize in different sites, such as the stroma or the membrane in chloroplasts (Iuchi \textit{et al.} 2001). Furthermore, they are expressed at different times and in different tissues during the growth and developmental stages of the plant (Tan \textit{et al.} 2003). AtNCED3 in particular becomes highly expressed in drought stressed plants (Toh \textit{et al.} 2008). Altogether, this allows for the tight regulation of ABA biosynthesis. If AtNCED3 is indeed able to control or affect the biosynthesis of violaxanthin, this could provide another level of regulation for ABA biosynthesis through the NCEDs.
4.5 Colorimetric assay for screening for the violaxanthin isomerase

The all-trans-violaxanthin-producing *E. coli*, which also expresses AtNCED3, will be used as a screen to find the uncharacterized enzyme in the carotenoid pathway that catalyzes the isomerization of *trans*-violaxanthin to *9-cis*-violaxanthin. Carotenoids produce colours in the spectrum of red to yellow. This phenotype would be useful for identifying genes that affect the carotenoid pathway. This technique was utilized for the discovery of the β-carotene hydroxylase gene, responsible for the conversion of β-carotene to zeaxanthin, in *A. thaliana* (Cunningham *et al.* 1996). β-carotene-producing *E. coli* was a deep orange yellow in colour, while zeaxanthin-producing *E. coli* was yellow. This enabled researchers to distinguish between zeaxanthin-producing *E. coli* from β-carotene-producing *E. coli* using the difference in colours (Sun *et al.* 1996).

Future research could involve transforming an *A. thaliana* cDNA library in *E. coli* that contains both the pACHP-Zea(Spec)-AtZEP and pCOLA-AtNCED3. AtNCED produces the colorless chemical xanthoxin from *9-cis*-violaxanthin, while all-trans-violaxanthin-producing *E. coli* is light yellow in colour. Expression of the correct gene from the cDNA library should lead to a paler or white colony. The production of xanthoxin would then be confirmed through HPLC.

In conclusion, most of the major discoveries in ABA biosynthetic pathway was done through genetics, however; a few steps of the pathway remains uncharacterized. Engineering the pathway in a heterologous system, like *E. coli*, could be the key in finding these uncharacterized genes of the pathway. *E. coli* has been used for decades to understand the carotenoid biosynthetic pathway. This was a convenient starting point as the indirect ABA biosynthetic pathway in plants utilizes carotenoids as substrates. Expression of plant genes in this system was able to show *AtNXD1* as the potential uncharacterized neoxanthin synthase enzyme. This system could also be used to study carotenoid gene homologs and how they affect the biosynthetic pathway.
References


Cunningham, F. X., Pogson, B., Sun, Z., McDonald, K. A., DellaPenna, D., & Gantt, E. (1996). Functional analysis of the beta and epsilon lycopene cyclase enzymes of Arabidopsis...


Appendix

Figure A1. A) Violaxanthin and B) zeaxanthin of carotenoid-producing *E. coli*. *E. coli* were grown overnight in liquid media with appropriate antibiotics. 100µL of the overnight culture was spread on a plate with appropriate antibiotics. The plate is incubated at 37°C for a day. IPTG was added to *E. coli* containing both pACHP-Zea(Spec) and pET-AtZEP plasmids. The plates were then transferred to their designated temperatures and left to grow for another 2 days. After 3 days of plate growth the cells were then harvested and pelleted. Their carotenoids were extracted and injected into the HPLC. Absorbance was measured at 450nm with the reference wavelength 600nm. Error bars represent SE (n=3).
Figure A2. Plasmid constructs used in this study. A) pACHP-Zea B) pACHP-Zea(Spec) C) pACHP-Zea(Spec)-AtZEP D) pET-ZEP E) pCOLADuet-1.

ATGGACGTTGAAGAAAAGAGGGTTCATCTGGTTATGCCAAGGCCTCCATGGATATTCC
AAAGGAAGTGCCTTGTACCAAGATCTCATCTTGTGAAGGCTGACACTCTCTTGCGGTTTCTTCC
TTGCTAGCTACGATGATAGGCCGGCTGCTGCTCTTGATGAGCTTTTGAAGCAG
GGATTGTATGGAACCCACTTCCTACATCATGCGATGGGCTGAGGCTTGGACACATTTGGTTTG
TTTCTTGACACATTTTGGTTTGGGAACACTACTCTCTGCTCTCCTCTGAGAATTGGATG
GTAAGGTTAGTGAGTTGATAGTGCTGCTTTCTACTGATATCTGCAACATCCAATT
AGATCAGACGAGACCACAGTTGGAAACTGGATGGGACCTGCAATCAAAATGGCTCT
CCCAAGTTTTAGTGGCAACATCAACATTAACGCTCAAAACTTGGCTCTGCAAGTACTCTATCGCC
TCTCGACTGCAAGGGGATGGAGATGACAGAGAAGTTTACAGAACAGAATCACACATCAAGAGTCACTTGAAAAT
GAAAGGCAGCTAAGCAAAGCCGTGATGCTATCGAAACCAATCATACCTCTTCAATT
CATAATGGCTTAAACATGGAAGCTCTGGCTGTGTAATTACCCTTC

Figure A3. AtNXD1 insert sequence in the plasmid pCOLA-AtNXD1.