Carotenoid Biosynthesis in Seed of *Arabidopsis thaliana*

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Abstract

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Seed of *Arabidopsis thaliana* accumulate carotenoids during development. These are mainly β-carotene, violaxanthin and lutein. By overexpression of an endogenous *phytoene synthase* (*psy*) under control of the seed-specific napin (*nap*) promoter the carotenoid content was elevated, resulting in increased levels in mainly β-carotene, violaxanthin, lutein and α-carotene. Transgenic seeds were also found to contain increased levels of ABA and displayed a delay in germination. The levels of ABA had a good correlation to the varying increases in lutein and violaxanthin content in seeds from plants hemizygous for the *nap:psy* construct. Seeds from plants overexpressing a *β-carotene ketolase* (*bkt*) from the green algae *Haematococcus pluvialis*, also under control of the napin promoter contained small amounts of ketocarotenoids, mainly 4-keto-lutein. When plants carrying the *nap:bkt* construct were crossed to plants carrying *nap:psy* this resulted in seeds where the total ketocarotenoid content had increased up to 13-fold. In seeds of the aba1-3 mutant of Arabidopsis which is mutated in the *zeaxanthin* epoxididase (*zep*) zeaxanthin levels were increased up to 40-fold and in seed of plants overexpressing an endogenous *β-carotene hydroxylase* (*bch*) it was found that the violaxanthin content had increased up to 20-fold. In seed from plants transgenic for *nap:bch*, neoxanthin and ABA levels were also increased. Just as for plants transgenic for *nap:psy* these seeds displayed a delay in germination. When γ-tocopherol content was analysed it was found that in seeds of *nap:psy* and *nap:bch* transformants it was only half of that found in wild type. In the Arabidopsis mutant abscisic acid-insensitive 3-1 (abi3-1) that is mutated in a seed-specific transcription factor, the seeds were found to have increased levels of β-carotene, violaxanthin, lutein and γ-tocopherol.

Keywords: *Arabidopsis thaliana*, carotenoids, *phytoene synthase*, *napin*, ABA, *β-carotene ketolase*, *β-carotene hydroxylase*, γ-tocopherol.

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Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Lindgren LO, Stalberg KG, Hoglund AS. (2003). Seed-specific overexpression of an endogenous Arabidopsis phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. Plant Physiol. 132(2):779-785.

II. Stålberg KG, Lindgren LO, Ek B, Hoglund AS. (2003). Synthesis of keocarotenoids in the seed of *Arabidopsis thaliana* (Plant J. In Print).

III. Lindgren LO, Stalberg KG, Hoglund AS. Analysis of altered carotenoid synthesis in seed of *Arabidopsis thaliana* and its relation to accumulation of tocopherol and ABA (Manuscript).

IV. Lindgren LO, Stalberg KG, Hoglund AS. Pigment analysis of the seed of the abi3-1 and aba3-1 mutants of Arabidopsis thaliana. (Manuscript).

Abbreviations

IPP	isopentenyl pyrophosphate
GA	gibberellins
ABA	abscisic acid
DOXP	1-deoxy-D-xylulose-5-phosphate
DXS	1-deoxy-D-xylulose-5-phosphate synthase
GGPP	geranylgeranyl pyrophosphate
PSY	phytoene synthase
PDS	phytoene desaturase
ZDS	zeta-carotene desaturase
CRTISO	carotene isomerase
LCY-B	lycopene β-cyclase
LCY-E	lycopene ε-cyclase
lut2	lutein-deficient 2
BCH	β-carotene hydroxylase
ECH	ε-carotene hydroxylase
lut1	lutein-deficient 1
ZEP	zeaxanthin epoxidase
VDE	violaxanthin de-epoxidase
npq1	non-photochemical quenching 1
NXS	neoxanthin synthase
vp14	viviparous 14
nced	9-*cis* epoxy-carotenoid dioxygenase
CCD1	carotenoid cleavage di-oxygenase 1
aba2	aba-deficient 2
aba3	aba-deficient 3
SDR1	short-chain alcohol dehydrogenase/reductase
AAO3	abscisic aldehyde oxidase 3
abi3	abscisic acid-insensetive 3
CCS	capsanthin-capsorubin synthase
ZCD	zeaxanthin cleavage deoxygenase
BKT	β-carotene ketolase
NAP	napin
HPLC	high performance liquid chromatography
ELISA	enzyme-linked immunosorbent assay
MS	mass spectrometry

Introduction

The DOXP pathway of isoprenoid biosynthesis

Carotenoids were for a long time assumed being synthesized by the mevalonate pathway for isoprenoid biosynthesis. This view was prevalent up until the mid-nineties when it was discovered that the carotenoid precursor isopentenyl pyrophosphate (IPP) was synthesized by two independent metabolic pathways in plants (Lichtentaler *et al*., 1997). The first pathway occurs in the cytoplasmic compartment from mevalonic acid and gives rise to compounds such as sterols and cytokinins. The second pathway is responsible for biosynthesis of gibberellins (GA), carotenoids, abscisic acid (ABA) and also contributes to the biosynthesis of tocopherols as well as chlorophyll A and B. This plastidial pathway of isoprenoid biosynthesis is named after its first metabolite 1-deoxyxylulose 5-phosphate (DOXP) and has pyruvate and glyceraldehyde-3-phosphate as precursors. When the cytosolic pathway was blocked by the inhibitor lovastatin, the plastidial pathway was transiently upregulated at the post-translational level (Laule *et al.,* 2003). Recent results have also shown that IPP can move between the plastid and the cytosol, indicating the presence of an IPP transporter localized to the plastidial membranes (Nagata *et al.*, 2002; Bick & Lange, 2003). Considering that ABA, GA, cytokinin and brassinosteroids could have interconnected pathways for their biosynthesis is an intriguing challenge for the interpretation of the regulation of these important plant hormones.

Overexpression and anti-sense constructs of an endogenous *1-deoxyxylulose 5-phosphate synthase* (*dxs*) in leaves of Arabidopsis, under control of the 35S promoter, was shown to have profound effects on the levels of downstream metabolites. In the extreme cases of overexpression in leaf tissue, ABA levels increased up to 4-fold and the α-tocopherol content more than doubled (Estevez *et al.,* 2001).

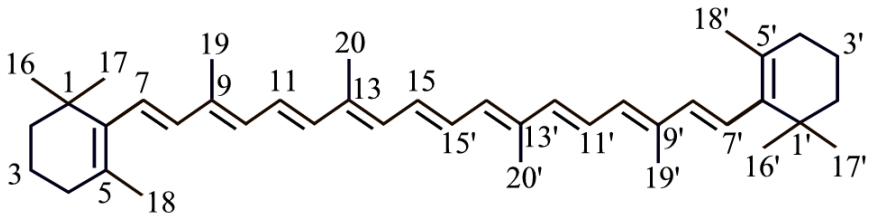

Figure 1. Nomenclature for numbering carbon atoms in carotenoids. The molecule in the figure depicts β-carotene. Some numbers have been omitted for clarity.

The biosynthetic pathway of carotenoids

A proper starting point for the carotenoid biosynthesis is the conversion of geranylgeranyl pyrophosphate (GGPP) to phytoene by the enzyme phytoene syntase (PSY) (Figure 3). This enzyme takes two units of the C-20 compound GGPP and condensates them to yield the C-40 compound phytoene. This enzymatic step has been found to be rate limiting in several different plant species, tissues and developmental states. In higher plants a gene from tomato was cloned and shown to encode for a protein able to complement a bacterial mutant lacking in *psy* activity. Phytoene, not a true pigment in that it is unable to absorb light at visible wavelengths, undergoes four consecutive desaturation steps. In higher plants these reactions are performed by two related desaturases. The first two steps are performed by phytoene desaturase (PDS) and the latter two by ζ-carotene desaturase (ZDS). These reactions yield the red pigment lycopene, the main pigment in red tomato. Lycopene produced by these desaturase reactions in plants and homologously, in cyano-bacteria, is in *cis*-form, also called pro-lycopene (Bartley *et al*., 1999). There is one further enzymatic step necessary to produce the all-*trans*-lycopene that is considered to be the main substrate for down-stream reactions. This is yet another difference from eubacterial and fungal biosynthesis where only one desaturase reaction is required to directly yield all-*trans*-lycopene. The enzyme carotene isomerase (CRTISO) has been found to perform this function and converts lycopene from *cis*- to *trans*-lycopene (Park *et al*., 2002; Isaacson *et al*., 2002).

The next two steps result in a bifurcation of the pathway, where one path is leading to the synthesis of β-carotene by two consecutive cyclization reactions by the enzyme β-carotene cyclase (LCY-B) (Hugueney *et al*., 1995) and the other branch leads to α-carotene where two different cyclases are neccessary. One is again LCY-B and the other enzyme is ε-carotene cyclase (LCY-E) (Cunningham *et al*., 1996). Mutants impaired in the ε-ring cyclization step (lut2) accumulate higher levels of β-carotene and violaxanthin (Pogson *et al*., 1996). α- and β-carotene only differs in the position of a double-bound in one of the end-rings and since not all bounds are part of the conjugated system in α-carotene this leads to a small, but obvious difference in the typical three-band carotenoid spectra between the compounds (Figure 2).

Oxygenated carotenoids are named xanthophylls and two hydroxylation steps convert β-carotene first to cryptoxanthin and then to zeaxanthin by the action of β-carotene hydroxylase (BCH). Alfa-carotene is also twice hydroxylated, but by two different enzymatic reactions. The β-ring is hydroxylated by BCH and the ε-ring by ε-carotene hydroxylase (ECH). This enzyme has not yet been cloned, but a mutant named lutein-deficient 1 (lut1), with a decreased lutein content, has been characterized (Pogson *et al*., 1996; Tian & DellaPenna, 2001). Interestingly, double homozygous knockout-mutants of the two known beta-carotene hydroxylases in Arabidopsis are viable and still have the ability to synthesize hydroxylated beta-rings (Tian *et al*., 2003).

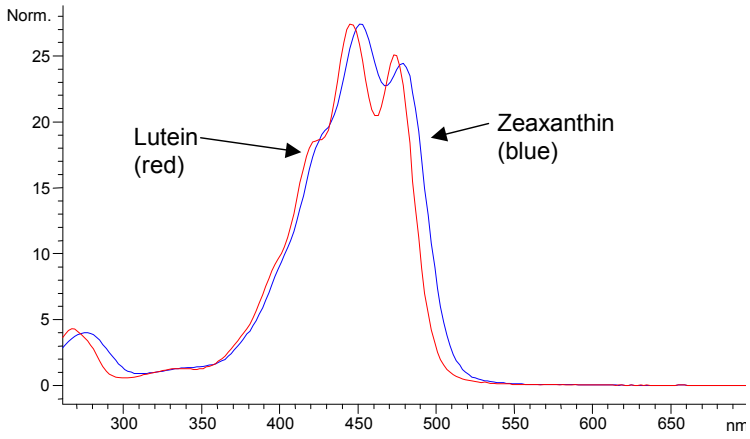

Figure 2. Overlay of absorbance spectra for the xanthophylls lutein and zeaxanthin. Both of these carotenoids display the typical three-band spectrum and it is possible to see that the peaks of lutein have maxima at slightly shorter wavelengths than zeaxanthin.

The hydroxylated beta-rings of zeaxanthin are epoxidated in two steps to yield first antheraxanthin and then violaxanthin. Mutants in Arabidopsis in *zeaxanthin epoxidase* (*zep*) accumulate high levels of zeaxanthin in leaf (Rock & Zeevaart, 1991) and have a wilty phenotype due to their inability to close stomata caused by ABA deficency. During light stress violaxanthin can be converted back to antheraxanthin and zeaxanthin by the enzyme violaxanthin de-epoxidase (VDE). This flux between these three compounds, governed by light intensity, constitutes the xanthophyll cycle. A mutant of *vde* in Arabidopsis (npq1) had reduced non-photochemical quenching and exhibited more photo-inhibition in leaf than wildtype, when exposed to high illumination (Niyogi *et al*., 1998).

The last of the ubiquitous carotenoids in higher plants is neoxanthin, synthesized from violaxanthin by the enzyme neoxanthin synthase (NXS). *nxs* has been cloned from tomato and potato (Bouvier *et al*., 2000; Al-Babili *et al*., 2000), but not from Arabidopsis. Neoxanthin synthases are related to the β-carotene cyclases and one of the two *lcy-b* identified in tomato has an identical nucleotide sequence to that of *nxs*, suggesting that neoxanthin synthases in Arabidopsis could also be members of this family, as has been suggested in a review by Cunningham; (2002). On the other hand, color complementation experiments in *Esherischia coli* with a plasmid carrying the cDNA of the enzyme with the proposed BCH/NXS synthase was unable to convert lycopene into beta-carotene (Bouvier *et al*., 2000). However, null-mutants in tomato of this gene (Ronen *et al*., 2000) were still able to synthesize neoxanthin, which is indicating the presence of additional neoxanthin synthase activity in tomato.

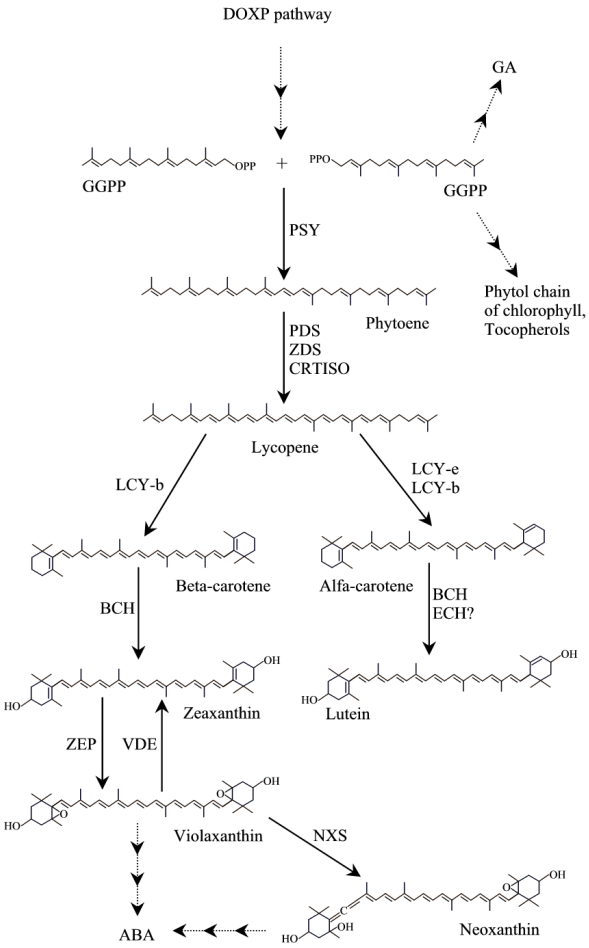

Figure 3. Simplified overwiev of the carotenoid methabolic pathway. 1-deoxy-D-xylulose-5-phosphate pathway (DOXP), Geranylgeranyl Pyrophosphate (GGPP), Phytoene Synthase (PSY), Phytoene Desaturase (PDS), Zeta-Carotene Desaturase (ZDS), Carotene Isomerase (CRTISO), Lycopene β-Cyclase (LCY-b), Lycopene ε-Cyclase (LCY-e), β-Carotene Hydroxylase (BCH), ε-Carotene Hydroxylase (ECH), Zeaxanthin Epoxidase (ZEP), Violaxanthin De-epoxidase (VDE), Neoxanthin Synthase (NXS), Abscisic Acid (ABA).

ABA biosynthesis

The first committed step in the ABA biosynthesis pathway was originally identified by a mutation in mays, named viviparous 14 (vp14). This mutant was found to have reduced levels of ABA during embryonic development (Tan *et al*., 1997). *Vp14* is homologous to the 9-*cis*-epoxy-carotenoid dioxygenases (*nced*s) in Arabidopsis and as the name implies, this enzyme cleaves 9-*cis*-epoxy-forms of violaxanthin and neoxanthin at the 11,12 position (Figure 1) to produce the ABA-precursor xanthoxin (Figure 4). Since *trans*-violaxanthin has to be converted to its 9-*cis*-form to become a substrate for NCED, the question remains whether there is an enzyme responsible for this action that has not yet been characterized. When a *vp14* homologue from tomato was overexpressed in tobacco, the ABA content increased up to 10-fold, showing that this enzymatic step is rate-limiting for ABA biosynthesis in leaf (Thompson *et al*., 2000).

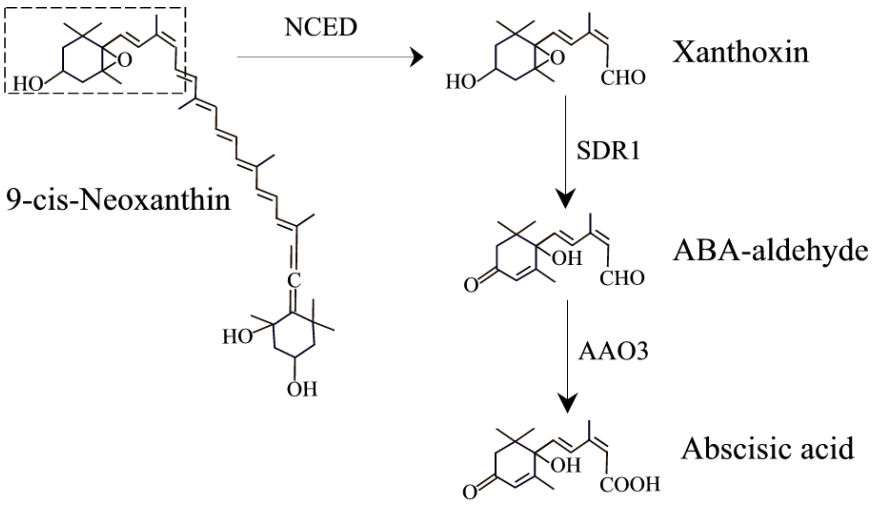

Figure 4. The ABA biosynthetic pathway. 9-*cis*-isomers of violaxanthin and neoxanthin are cleaved into the C-15 compound xanthoxin. Note that the last step where ABA-aldehyde is converted into ABA has two different kinds of mutants. One is in *aao3* itself and the second one is in the synthesis of a molybdenum cofactor to this enzyme. NCED, 9-*cis*-epoxy-carotenoid dioxygenase; SDR1, Shortchain dehydrogenase/reductase 1; AAO3, ABA-aldehyde oxidase 3. (Adapted from Tan et al., 1997)

The *nced*s of Arabidopsis can be divided into two groups with 4 and 5 members respectively. Five *nced*s that were more closely related to *vp14*, have been tested for transcriptional activity and by GUS-staining (Tan *et al*., 2003). In this study it was reported that *nced 3*,*5*,*6* and *9* were expressed during seed development. *Atnced3* is upregulated during drought stress and plants overexpressing this enzyme had increased levels of ABA in leaf as well as an increased resistance to drought (Iuchi *et al*., 2001). The first characterized member of this group,

Arabidopsis thaliana carotenoid cleavage di-oxygenase 1 (AtCCD1), can utilize a number of carotenoids as substrates (Schwartz *et al*., 2001). This enzyme did not synthesize xanthoxin from carotenoid precursors and it seems therefore likely that at least some of these NCED homologues are involved in activities apart from ABA biosynthesis.

ABA biosynthesis has been characterized in the aba-deficient mutants aba2 and aba3 in Arabidopsis (Schwartz *et al*., 1997). The aba2 mutant could not convert xanthoxin to ABA-aldehyde and aba3 was unable to make ABA out of ABA-aldehyde in cell-free extracts of leaves of Arabidopsis. The aba3 mutation was found to be in an enzyme for synthesis of a molybdenum co-factor (MoCo) to an abscisic acid aldehyde oxidase. In Arabidopsis the enzyme impaired in the aba2 mutant encodes for a short chain dehydrogenase/reductase (SDR1) that is constitutively expressed in roots, stems, siliques and leaves (Gonzalez-Guzman *et al*., 2002). Abscisic aldehyde oxidase 3 (AAO3) was found to be able to convert ABA-aldehyde into ABA and was also transcriptionally upregulated in leaves during water-stress (Seo *et al*., 2000). It is therefore a likely candidate for the last reaction of ABA biosynthesis in Arabidopsis. Analysis of targeting-peptides suggests that SDR1 and AAO3 are both localised to the cytosol (data not shown).

ABA and seed dormancy

Seed of higher plants undergo double fertilization of the egg-cell and the central cell. The fertilized egg-cell then develops into the diploid embryo while the triploid central cell develops into the endosperm. The endosperm will then provide energy and nutrients for the seedling at germination or for the developing embryo. In Arabidopsis as well as in other dicotelydoneous plants, the embryo undergo several developmental stages that ends with a fully developed, dormant embryo consisting of protoderm, vascular tissue and ground meristem (Goldberg *et al*., 1994). Upon maturation the seed enter a quiescent state of dormancy that has been defined as the failure of an intact viable seed to complete germination under favourable conditions (Bewley, 1997).

Several aba-mutants from maize, tobacco and Arabidopsis have been found to have precocious germination and ABA has therefore been implicated as a necessary hormonal signal for the seed to enter the dormancy state. In Arabidopsis the aba1, aba2 and aba3 mutants were first isolated through their ability to germinate in the presence of inhibitors to the germination-promoting hormone GA (Koornneef *et al*., 1982; Leon-Kloosterziel *et al*., 1996). Mutants able to germinate in presence of inhibiting levels of ABA in the growth medium have also been found. These are the ABA insensitive mutants, abi1-abi5 (Koornneef *et al*., 1984; Finkelstein 1994). A severe mutant allele of *abi3* was found to produce desiccation intolerant greenish seeds that accumulated decreased amounts of storage proteins (Nambara *et al*., 1992). When the *abi3* transcription factor was overexpressed with the 35S promoter it was found that leaves accumulated seed-specific transcripts upon ABA induction (Parcy *et al*., 1994).

Constitutive overexpression of *zep* in tobacco was found to increase dormancy and mature seed had increased levels of ABA (Frey *et al*., 1999). Increased dormancy was also the result when an endogenous *nced* was overexpressed in tomato (Thompson *et al*., 2000) and taken together with the previous results of precocious germination found in ABA mutants, this underlines the importance of ABA in induction of seed dormancy.

ABA has been implicated to have many important roles in regulation during seed development. For extensive reviews on this fascinating topic, please see McCarty (1995), Bewley (1997) and Bentsink & Koornneef, (2002).

Carotenoid secondary metabolites

Apocarotenoids

Several specific pathways for carotenoid biosynthesis have evolved in eukaryotes by evolution of novel enzymatic activities. In the pepper family capsanthin-capsorubin synthase (CCS) is able to convert the epoxy-carotenoids antheraxanthin and violaxanthin into capsanthin and capsorubin (Bouvier *et al*, 1994). In this study yellow pepper was found to be accumulating violaxanthin and *ccs* could not be found either as a transcript or with antibodies. It is therefore likely to be a fruit-specific mutant of this enzyme. *ccs* is closely related to *lcy-b* and *nxs* and has probably evolved by gene duplication from the latter (Bouvier *et al*., 2000). Other examples of secondary metabolites of the carotenoid pathway are bixin derived from lycopene and crocetin from zeaxanthin, in *Bixa orellana* and crocus respectively. The first committed step in crocetin biosynthesis is performed by zeaxanthin cleavage deoxygenase (ZCD) which cleave zeaxanthin at the 7,8 and 7',8' positions. This enzyme is related to the carotenoid cleavage dioxygenase 1 (AtCCD1) of Arabidopsis which cleave carotenoids at the 9,10 and 9',10' positions. As earlier mentioned this shows that NCED-related enzymes are by no means restricted to nine-*cis*-epoxy forms of violaxanthin and neoxanthin for substrates and that at least some members of this family are involved in production of the relatively unknown apo-carotenoids (Bouvier *et al*., 2003a, Bouvier *et al*., 2003b; Schwartz *et al*., 2001). Apocarotenoid biosynthesis might occur in the Tasmanian flower *Boronia megastigma* that was reported to contain C-13 norisoprenoids and C-27 apocarotenids probably derived from cleavage of C-40 carotenoid precursors (Cooper *et al*., 2003). Another example of tissues containing carotenoid derivatives is found in arbuscular roots where C-14 and and C-13 isoprenoids accumulate during the process of mycorrhization in wildtype *Zea mays*, but not in mutants deficient in carotenoid biosynthesis (Fester *et al*., 2002).

One of the most interesting discoveries regarding carotenoid cleavage dioxygenases was done in *Drosophila melanogaster* where an enzyme was cloned by mutational analysis and homology to *vp14*. This enzyme is part of the vitamin A biosynthesis in animals and cleaves β-carotene into the C-15 compound retinal (von Lintig & Vogt; 2000). Further insights into apocarotenoid biosynthesis could undoubtedly open up new areas of genetical engineering.

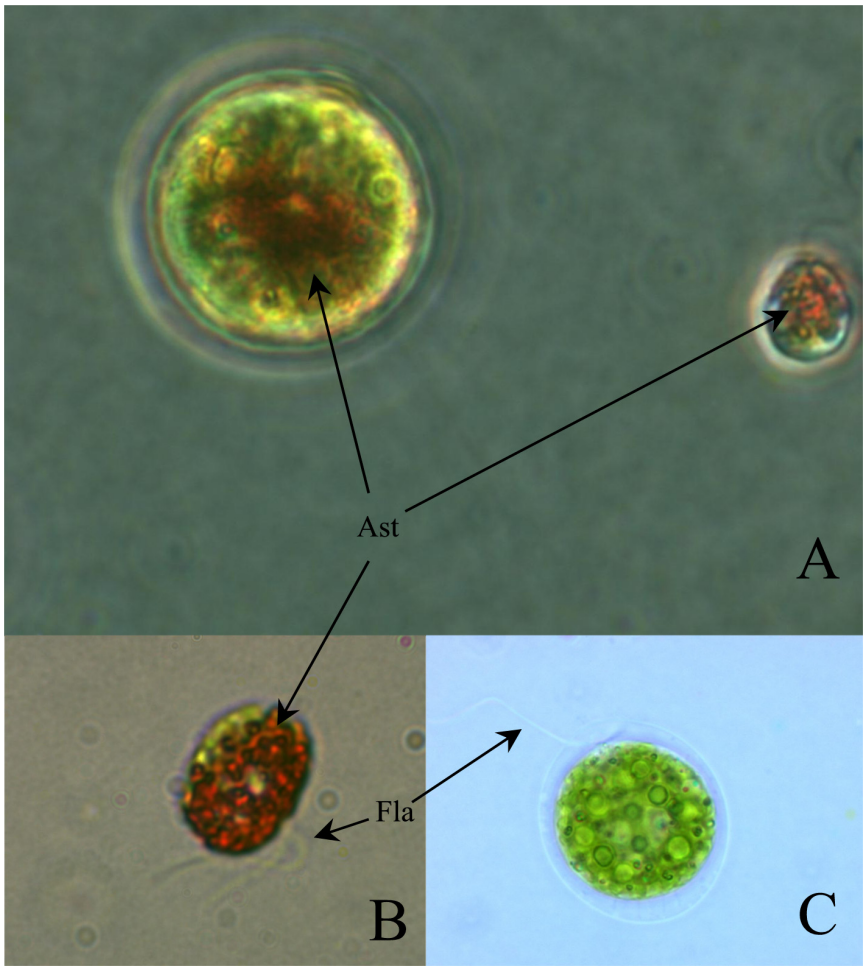

Figure 5. The monocellular green algae *H. pluvalis* in different developmental states.
A) *H. pluvialis* in a vegetative state. On the right is what could possibly be a gamete of this species.
B) Possible gamete with cytoplasmic astaxanthin vesicles.
C) *H. pluvialis* in normal vegetative state.
Ast, Astaxanthin; Fla, Flagella.

Ketocarotenoids

Astaxanthin is a ketocarotenoid synthesized in bacteria, fungi, algae and in certain plant species. In *Adonis aestivalis* of the *Ranunculaceae* family astaxanthin accumulates mainly as di-esters in chromoplasts and the two most common fatty acids found were oleic acid (C18:1) and palmitic acid (C16:0) (Kamata & Simpson, 1987). No enzyme with ketolase activity has been cloned from higher plants and perhaps astaxanthin biosynthetic enzymes have evolved several times independently of each other during the course of evolution, making cloning based on homology more difficult.

Ketocarotenoids contribute to the coloration found in several species of fish and bird. In white stork (*Ciconia ciconia*) that is feeding on crayfish containing astaxanthin, the pigment was found to accumulate in skin, bill and tarsi (Negro & Garrido-Fernandez, 2000).

In the green alga *Haematococcus pluvialis* astaxanthin accumulates in cytosolic vesicles during environmental stress and nutrient starvation (Figure 5). The β-carotene ketolase (*bkt*) of *H. pluvialis* was the first enzyme to be cloned by the method of color complementation (Figure 6) in *E. coli* (Kajiwara *et al*., 1995; Lotan & Hirshberg, 1995). This enzyme introduces a ketogroup at the 4 and 4' positions in the beta-rings of β -carotene to yield canthaxanthin (Figure 7). In presence of β-carotene hydroxylase activity the rings of canthaxanthin can then be hydroxylated at the 3 and 3' positions to yield astaxanthin. Some data support that the hydroxylase reaction must take place after the introduction of a keto-group (Breitenbach *et al*., 1996), but BKT and bacterial ketolases analyzed experimentally *in vitro* have been shown to produce 4-ketozeaxanthin from zeaxanthin as well (Fraser *et al*., 1997).

The compartment of astaxanthin synthesis in *H. pluvialis* is still not conclusively determined. Immunogold labelling of the BKT protein gave positive signals both in plastids and the cytosolic vesicles, originating from the endoplasmic reticulum, but activity assays implicated that astaxanthin was only synthesized in the vesicles (Grunewald *et al*., 2001). This would suggest that astaxanthin precursors are transported out of the plastid to the vesicles where the last steps of the biosynthesis take place. However, since at least one strain of *H. pluvialis* is able to synthesize 4-keto-lutein (Stalberg *et al*., unpublished data), this transport mechanism does either not discriminate between α- and β-carotenes or at least some BKT activity is also found in the plastid.

Biotechnological applications

Due to their widespread use, economical importance and their potential uses in medicine, carotenoid biosynthesis has been extensively studied within the field of biotechnology. Introducing carotenoid biosynthesis genes into *E. coli* has been of great use in elucidating their enzymatic functions and production in bacteria has also been suggested for industrial purposes (Sandmann *et al*., 1999; Misawa and Shimada, 1997). By combining a number of different genes in *E coli* it was found that the bacteria were able to synthesize novel kinds of carotenoids with altered properties (Albrecht *et al*., 2000). Some of these novel compounds were reported to have improved antioxidative properties as compared to lycopene. A study where desaturases from several organisms were introduced together in *E. coli*, lead to the finding of bacteria producing 3,4,3',4'-tetradehydrolycopene, a derivative with two more double-bounds than lycopene, further extending the conjugated system (Schmidt-Dannert *et al*., 2000).

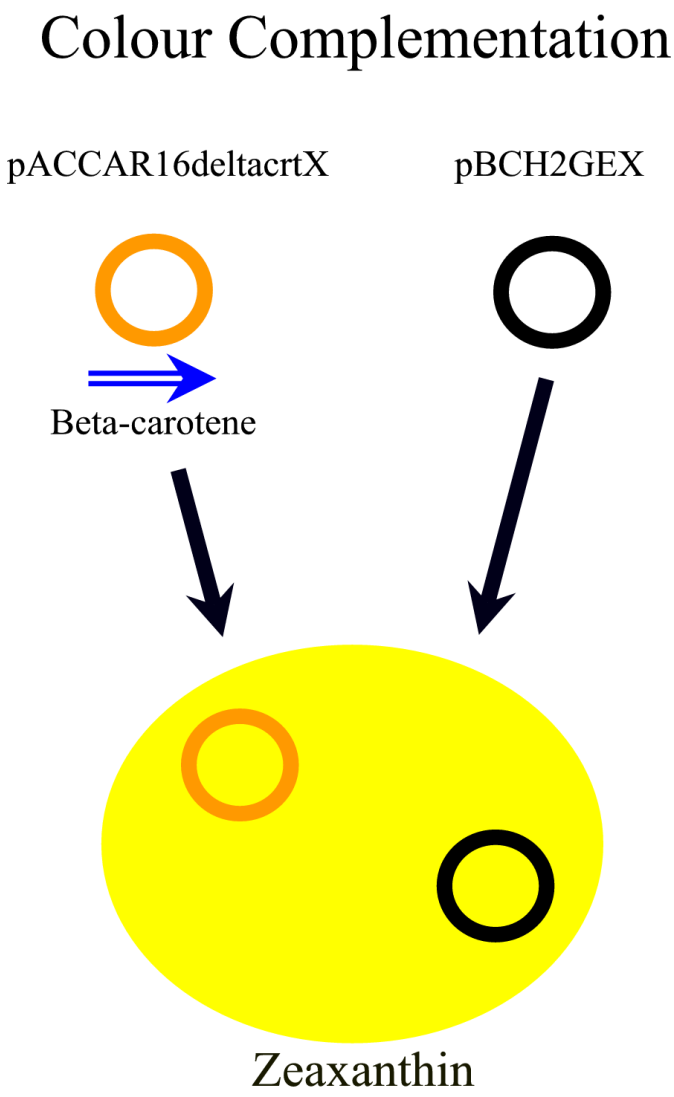

Figure 5. The method of Colour Complementation. The plasmid pACCARdeltacrtX carries all the enzymes necessary for synthesis of β-carotene in *E. coli* and a chloramphenicol resistance gene. The plasmid pBCH2GEX carries a potential β-carotene hydroxylase gene, in this case the second *bch* from *Arabidopsis thaliana* and an ampicillin resistance gene. When both plasmids are introduced into *E. coli* it starts producing hydroxylated β, β-carotenes, *i.e.* β-cryptoxanthin and zeaxanthin. This method has successfully been used in conjunction with cDNA expression libraries to directly clone and confirm the function of novel genes. Since introduction of enzymes that can utilize carotenoids for substrate will give rise to new pigments it is possible to screen bacterial colonies for colour changes, hence the name Colour Complementation.

The tomato-fruit has been used extensively in experiments aiming to increase and alter the amount of pigments. Introduction of a bacterial phytoene desaturase in tomato had the unexpected result to increase the β-carotene content up to 3-fold (Römer *et al*., 2000) and overexpression of a bacterial phytoene synthase using a tomato *polygalacturonase* promoter, increased the total carotenoid content up to 4-fold (Fraser *et al*., 2002). When the Arabidopsis *lcy-b* was overexpressed under control of a *pds* promoter it was found that the β-carotene content increased up to 6-fold and also that the total carotenoid content had increased (Rosati *et al*., 2000). When the Arabidopsis *lcy-b* was simultaneously overexpressed together with a pepper *bch*, this lead to increased levels of the xanthophylls β-cryptoxanthin and zeaxanthin as well (Dharmapuri *et al*., 2002).

The most famous example of altered carotenoid biosynthesis in higher plants is probably the Golden Rice. The rice-seed endosperm does not contain any carotenoids, but by introducing a phytoene synthase from *Narcissus pseudonarcissus* and a bacterial phytoene desaturase the rice-seed was able to synthesize α-carotene, β-carotene, zeaxanthin and lutein (Ye *et al*., 2000). This accumulation pattern also indicated that *lcy-b*, *lcy-e*, *bch* and *ech* are either already expressed in the endosperm during rice seed development or induced by accumulation of upstream carotenoid precursors.

In photosynthesizing tissues carotenoids are among other things protecting the photosystems against free radicals that are produced under strong illumination when the photochemical quenching is limited. This stress leads to photoinhibition and eventually to degradation of the photosystems. Zeaxanthin has been implicated in protection against this harmful condition since its synthesis from violaxanthin by VDE is induced by strong illumination. By overexpressing of an endogenous *bch* in Arabidopsis the xanthophyll cycle elements (zeaxanthin, antheraxanthin, violaxanthin) were increased when the plants were grown under low and moderate light. The transgenic plants also produced far less of anthocyanins, a pigment typically synthesized in leaves during stress, when they were grown under strong light (Davison *et al*., 2002). Somewhat surprisingly, constitutive overexpression of a bacterial β-carotene hydroxylase in tobacco did not have this increase in xanthophylls at low light, but had higher levels of photosynthetic pigments after being subjected to high illumination and UV irradiation (Götz *et al*., 2002).

A possible role for keto-carotenoids in UV-protection has been found in transgenics of the cyanobacteria Synechocystis. Overexpression of *bkt from H. pluvialis* resulted in production of mainly canthaxanthin, but also other ketocarotenoids. The transgenic strain was found to have an increased resistance against photoinhibition at high light and against UV-B irradiation (Albrecht *et al*., 2001).

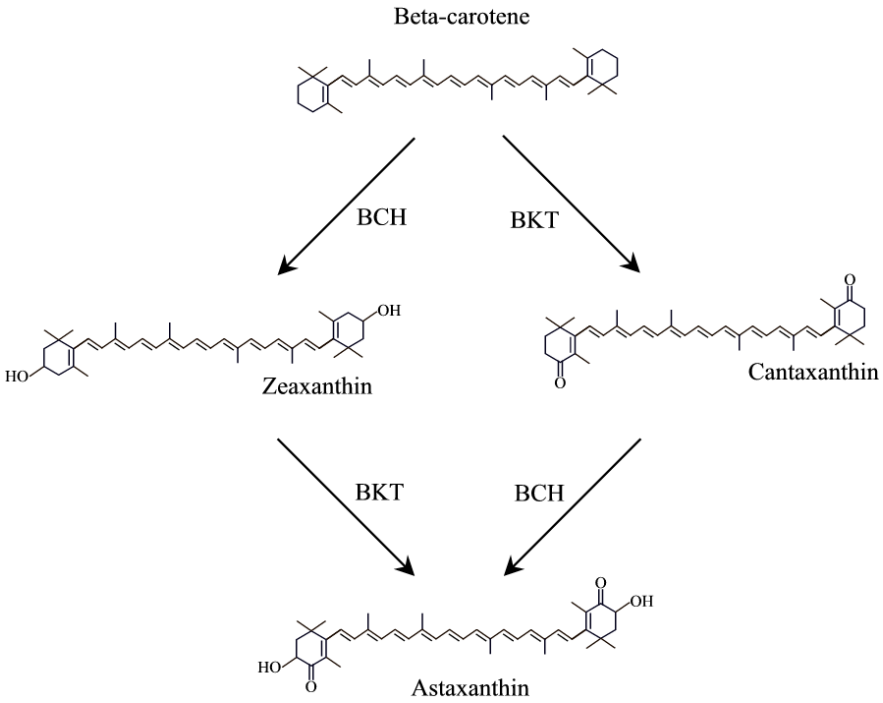

Figure 7. Possible routes for astaxanthin biosynthesis in *H. pluvialis*. BCH, β-carotene hydroxylase; BKT, β-carotene ketolase.

Aims of the Project

By seed-specific overexpression of carotenoid biosyntheis genes we wanted to see whether it was possible to increase and alter the composition in seed of Arabidopsis.

Since the plastidial pathway of isoprenoid biosyntheis is involved in processes important and unique to plants we were interested to find out more about physiological effects, especially regarding ABA-induction.

Results and Discussion

I

The gene encoding phytoene synthase has previously been found rate limiting for carotenoid biosynthesis in the plant seed on two occasions: Overexpression of a phytoene synthase from *N. pseudonarcissus* in rice seed (Ye *et al*., 2000) and overexpression of a bacterial phytoene synthase from *Erwinia uredovora* in oil seed rape (canola) (Shewmaker *et al*., 1999). In rice seed introduction of two enzymes were necessary to achieve carotenoid accumulation in the endosperm. The bacterial phytoene desaturase used, also from *Erwinia uredovora*, is able to perform two desaturation steps of phytoene whereas in plants two different enzymes (PDS, ZDS) are necessary. In canola the introduction of the bacterial phytoene synthase was sufficient to dramatically increase the carotenoid content of the seed.

In our first paper we show that seed specific overexpression of an endogenous *psy* leads to much increased accumulation of carotenoids with the highest absolute increases found in lutein and ß-carotene content, but also to significant increases in lycopene, α-carotene, violaxanthin and neoxanthin. Thus, at the onset of napin transcription enzymes responsible for the activities of PDS, ZDS, CRTISO, LCY-B, LCY-E, BCH, ECH, ZEP and NXS are either present or upregulated at the transcriptional or post-transcriptional level in seed of Arabidopsis.

In this study we also show that the same holds true for the activities of NCED, SDR1, AAO3 and a hypothetical trans to cis isomerase, acting on violaxanthin and neoxanthin, since the ABA levels were also increased in the these transformants. The most striking difference between seed specific overexpression of a bacterial phytoene synthase in canola and of an endogenous phytoene synthase in Arabidopsis is found in the accumulation of α-carotenoids. In the mature canola seed α-carotene accumulates to high levels, but the lutein level show a comparatively small increase. In the Arabidopsis seed we found that lutein had the highest absolute increase whereas the level of α-carotene was smaller. Apparently, the activity of BCH and ECH must be either higher in the Arabidopsis seed at this stage of seed development or the differences in accumulation pattern can be attributed to differences of a bacterial and an endogenous phytoene synthase in providing substrate for down-stream enzymatic reactions. Since the plants used were heterozygous for *nap*:*psy* and were expected to have different copy numbers of the construct as well as different insertion sites which also would affect transcriptional activity, seeds from individual plants were displaying a huge range in total carotenoid content. By selecting 10 transformants and one wild type that were ranging from low to high in carotenoid content we examined how different carotenoids correlated to the ABA level and germination frequency. When the correlation was tested between carotenoids and germination frequency we found the highest value for lutein (-0.92), although the one for the ABA-precursor

violaxanthin was of similar magnitude (-0.88). Lutein is not considered to be a precursor for ABA and the lut1 and lut2 mutants do not exhibit wilty phenotypes or any other traits commonly associated with ABA deficiency (Pogson *et al*., 1996), so one explanation for the high correlation could be that lutein is synthesized in proportional amounts to the ABA that induces the state of seed dormancy, which then would be proportional to the germination frequency itself. The correlation between ABA and lutein was not as high as the one for germination and lutein, but this can be explained by the fact that the method used for ABA quantification, ELISA, is not as exact as quantification by HPLC.

II

In higher plants, the *bkt* from *H. pluvialis* has been overexpressed in tobacco nectaries under control of the PDS promoter from tomato (Mann *et al*., 2000). This tissue that normally accumulate mainly ß-carotene and violaxanthin, but not lutein, now accumulated ketocarotenoids such as canthaxanthin, adonixanthin, adonirubin and astaxanthin. A significant proportion of the ketocarotenoids was reported to be esterified in this study.

In our second paper we report of the results of overexpression of a *bkt* from *H. pluvialis*, under control of the *Brassica napus* napin promoter. Transgenic plants were found to accumulate smaller amounts of ketocarotenoids and also a compound with a unique absorbance spectrum similar to those of ketocarotenoids. Identification of this novel compound, 4-keto-lutein, was done by a combination of HPLC chromatography of chemically modified compounds and MS.

Several peaks in the HPLC chromatogram of transgenic plants, with ketocarotenoid spectra, were found in the hydrophobic regions, indicating modifications had occurred, since most of these are rather hydrophilic in nature. After treatment with a cholesterol esterase from *Pseudomonas*, novel, more hydrophilic peaks could be found with identical absorbance spectra, suggesting that fatty acids had been removed. Esterified carotenoids are not uncommon in chromoplasts of higher plants and have been found in flowers of *Tagetes erecta* (Moehs *et al*., 2001), *A. astevalis* (Kamata & Simpson, 1987) and yellow varieties of the ornamental *Gerbera hybrida* (Lindgren, unpublished data). In the primary *bkt* transformants it was found that a higher proportion of ketoxanthophylls were esterified than of the other xanthophylls (lutein). This would suggest that ketoxanthophylls are either more readily esterified or that oxygenation by BKT has a preference for esterified xanthophylls.

By crossing the *bkt* transformants to plants overexpressing an endogenous *psy* described in **I** the yield of ketocarotenoids in the transformants was increased up to 13-fold. This increase in yield was obviously achieved by providing more substrate for BKT to act upon, but the exact reaction sequence for the compounds have yet to be determined. Canthaxanthin is likely to have been synthesized directly from ß-carotene, but 4-keto-lutein could either have been synthesized directly from lutein, or from α-carotenoids with one or no hydroxy-groups.

Crossings between *bkt* transformants and transformants overexpressing an endogenous *bch* (**III**) did not result in any impressive increase of the ketocarotenoid yield (data not shown), suggesting that an increased ß-carotene hydroxylase activity does not have a positive effect on ketocarotenoid accumulation.

III

An Arabidopsis *bch* and a bacterial ß-carotene hydroxylase have previously been constitutively overexpressed in Arabidopsis (Davison *et al*., 2002) and tobacco (Götz *et al*., 2002) respectively. Overexpression in Arabidopsis lead to an accumulation of mainly violaxanthin that increased up to 2.5-fold in leaves grown at moderate light intensities. The antheraxanthin level also increased while those of the other carotenoids measured in this study (ß-carotene, lutein, neoxanthin) did not significantly differ between transformants and wild type.

In the third paper we report of the results of seed-specific overexpression of an endogenous *bch* in *Arabidopsis thaliana* of ecotype Wassilevskija. When the accumulation pattern of carotenoids was analyzed we found increases in violaxanthin, lutein and neoxanthin with the biggest increase in violaxanthin that increased up to 20-fold. The total carotenoid content was found to increase on average 2.7 times in six selected transformants and the ABA content more than doubled. When germination frequencies were analysed it was found that these transformants had a delay in germination as compared to wild type, but after 66 hours there was no longer any significant difference. In the aba1-3 mutant of ecotype Landsberg erecta that is a leaky mutant of *zep* (Rock & Zeevaart, 1991) we found significant increases in seed of ß-carotene, zeaxanthin and lutein. The biggest increase was found in accumulation of zeaxanthin with an average more than 43 times that of wild type and the total carotenoid content in this mutant was increased 3.8-fold. Seed-specific overexpression of an endogenous *lcy-b* had the unexpected result to lower the lutein content in seed by more than 50%. No other difference between wild type and transformant could be found in carotenoid levels, ABA content or germination frequency and the result is difficult to interpret. If this was a case of gene silencing then one would expect decreases in other carotenoids as well, but the level of for example violaxanthin shows no significant difference. One interpretation would be that at this specific developmental state, only lutein is synthesized and therefor affected by gene silencing of the *lcy-b*. However, the results from overexpression of *psy* in paper **I** speak against this interpretation since a number of other carotenoids apparently are synthesized in those transformants. Northern blotting on mRNA from embryonic tissue could possibly resolve this problem and another option could be to cross the *lcy-b* transformants to *psy* transformants. This crossing could result in accumulation of rare precursors such as mono-hydroxylated carotenoids and lycopene that might give an idea where the pathway is inhibited. A third option could be that products of the pathway are participating in up- and down-regulation of specific enzymatic reactions as has been suggested in the cases of PSY by ß-carotene (Fraser *et al*., 2000) and ZEP by ABA (Xiong *et al*., 2002). Such a

regulation could explain increases and decreases in carotenoids not directly associated with the transgenically affected enzyme.

γ-tocopherol content was analysed by HPLC in the *bch*-, *lcy-b*- and *psy*-transformants as well as in the aba1-3 mutant in order to find out how this pathway was affected by increased and decreased accumulation of carotenoids. We found that γ-tocopherol levels were about halved in the *psy* and *bch* transformants and that there was a small increase in the aba1-3 mutant as compared to wild type. These results implicate that there is a competition for substrate between these pathways, but since the *psy* transformant had about twice as high total carotenoid content as the *bch* transformant while the γ-tocopherol content was similar, the interplay between tocopherol/carotenoid biosynthesis must be affected by other factors as well.

IV

In an attempt to find factors involved in regulation of seed carotenoid biosynthesis in Arabidopsis the ABA-insensitive mutants abi3-abi5 were analysed by HPLC. Included in this study was also the aba3-1 mutant (Leon-Klosterziel et al., 1996) that is impaired in the conversion from ABA-aldehyde to ABA. Of the abi mutants it was only abi3-1 (Koornneef *et al*., 1984) of ecotype Landsberg erecta that had significant increases in the carotenoids measured, with more than twice as high total carotenoid content. The highest absolute increases were found in ß-carotene (4-fold), lutein (80% more) and violaxanthin (about 5-fold). In this mutant the γ-tocopherol content was also increased with about 60%. The abi3 seed has been reported to be greenish, indicating increased chlorophyll content and did indeed have higher levels of chlorophyll A and B. The general distribution and accumulation of carotenoids and chlorophyll was however far more similar to that of seed than to leaf. The aba3-1 mutant of ecotype Columbia was found to have seeds with a similar pigment accumulation and distribution as the abi3-1 mutant

Conclusions

- PSY is rate limiting for carotenoid biosynthesis during seed development in Arabidopsis and overexpression by the napin promoter causes accumulation of a number of metabolites, most prominently lutein, α-carotene, β-carotene and violaxanthin. Due to increased synthesis of precursors the ABA level is also increased and this is most likely coupled to an increased dormancy of the mature seed. Indeed, there is a strong correlation between increases in carotenoid levels of the mature seed (lutein an violaxanthin) and germination frequency.

- The Arabidosis seed is able to produce ketocarotenoids and introduction of *bkt* causes accumulation of mainly α-carotenoid derivatives, but also significant amounts of canthaxanthin in crossings between *psy* and *bkt* transformants. Apparently, crossings between these two hugely increased the amount of ketocarotenoids since this provided the BKT with more substrate to act upon.

- Seed-specific overexpression of *bch* resulted in an accumulation of violaxanthin, and evidently, ZEP must be active enough *in vivo* to convert all the available zeaxanthin into violaxanthin, since this compound does not accumulate.

- The seeds of the aba1-3 mutant have an increased total carotenoid content with zeaxanthin as the most common xanthophyll.

- Since lutein is accumulating to a higher extent in the abi3-1 mutant of Arabidopsis seed than in leaf of this mutant it can be concluded that regulation of carotenoid biosynthesis is different in these tissues.

- Increased accumulation of carotenoids and γ-tocopherol is found in the abi3-1 mutant, but not in the abi4-1 and abi5-1 mutants.

Future Perspectives

- Analysis of carotenoid secondary metabolites would undoubtedly give insight into novel physiological processes.

- The results in Arabidosis are to be used for achieving high-yield synthesis of carotenoids and ketocarotenoids in seed of oil-seed rape.

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