J Plant Growth Regul (2005) 24:253–273
DOI: 10.1007/s00344-005-0070-6

JPGR
Journal of Plant Growth Regulation

© 2005 Springer Science+Business Media, Inc.

Regulation and Manipulation of the Biosynthesis of Abscisic Acid, Including the Supply of Xanthophyll Precursors

Ian B. Taylor,[1]* Tineke Sonneveld,[1] Timothy D. H. Bugg,[2] and Andrew J. Thompson[3]

[1]*Plant Sciences Division, School of Biosciences, The University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, UK;* [2]*Department of Chemistry, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK;* [3]*Warwick HRI, University of Warwick, Wellesbourne, Warwick, CV35 9EF, UK*

Abstract

Mutant plants deficient in the phytohormone abscisic acid (ABA) are typically unable to control their stomatal behavior appropriately in response to water stress, leading to a "wilty" phenotype. In plant species showing strong seed dormancy, ABA deficiency of the seed results in a second clearly recognizable phenotype, that is, early germination. Mutants selected by means of this latter character are often collectively termed "viviparous." These two broad classes include mutants that are defective in their ability to synthesize ABA. A number of these genetic lesions have been assigned to specific steps in ABA biosynthesis and have been invaluable in elucidating many important features of the pathway. Most of the genes encoding ABA biosynthetic enzymes have now been cloned and their expression has been studied and manipulated. Genetically modified plants constitutively overexpressing ABA biosynthesis genes have been produced and analyzed over the last 6 years. In some cases these plants have been found to have elevated ABA concentrations, leading to altered stomatal behavior and increased seed dormancy. Genetic manipulation of ABA synthesis in photosynthetic tissues has been most effectively achieved through overexpression of the key rate-limiting biosynthetic enzyme 9-*cis*-epoxycarotenoid dioxygenase, and downregulation of the major catabolic enzyme ABA 8′-hydroxylase. However in non-photosynthetic tissue manipulation of ABA synthesis is a more complex task because of the limiting supply of xanthophyll precursors. The recent cloning of genes encoding enzymes controlling important pathways of ABA catabolism has been reviewed elsewhere, and so only information relevant to the regulation and manipulation of ABA synthesis, including supply of xanthophyll precursors, is discussed in this review.

Key words: ABA synthesis; Regulation; Genetic manipulation; Water-use efficiency; Seed dormancy

Received: 8 June 2005; accepted: 9 June 2005; Online publication: 12 December 2005
**Corresponding author; e-mail:* ian.taylor@nottingham.ac.uk

Introduction

The phytohormone known as abscisic acid (ABA) is important in the control of plant responses to abiotic stress, notably water deficit. In seeds, ABA content is developmentally regulated, peaking in the maturation phase, in preparation for desiccation, and falling to low levels in the dry seed. After imbibition, ABA accumulation once again becomes an important factor in determining whether the seed will germinate or remain dormant (reviewed in Nambara and Marion-Poll 2003). In vegetative tissues (shoots and roots) ABA levels are also subject to developmental control, but they can be more dramatically increased in response to adverse environmental conditions, particularly drought stress.

Although ABA is very unevenly distributed between different tissues, cells, and sub-cellular compartments, the overall ABA content of a plant is determined by the relative rates of synthesis and breakdown/catabolism—that is, by ABA metabolism. The regulation of these two processes and the prospects for manipulating them to artificially elevate ABA concentrations is important. But the main focus of this review is ABA synthesis, because the latest advances in ABA catabolism have been reviewed very recently (Nambara and Marion-Poll 2005). Because ABA synthesis is likely to be limited by production of xanthophylls in non-photosynthetic tissues, we have included a review of the regulation and manipulation of the pathway leading to xanthophyll biosynthesis.

ABA Synthesis

Direct versus Indirect Pathways

Abscisic acid is a 15-carbon sesquiterpene that is assembled from isomeric 5-carbon isoprenoid building blocks known as isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Beyond this, it is difficult to make a universal statement about ABA synthesis that is applicable to all of the organisms in which it has been detected. Two fungal genera, *Cercospora* and *Botrytis*, contain species known to accumulate substantial amounts of ABA. In these fungi, ABA is derived from IPP and DMAPP molecules that have themselves been synthesized via the well-established mevalonate (MVA) pathway (Hirai and others 2000). Two IPP and one DMAPP precursor molecules are used to synthesize the important 15-carbon compound known as farnesyl diphosphate (FPP). The precise sequence of intermediates between FPP and ABA has been found to vary in different fungal species (see Nambara and Marion-Poll 2005 for a recent review); because they are all 15-carbon compounds, the fungal route of ABA biosynthesis has often been referred to as the "direct pathway."

The overall route of ABA synthesis in plants is more complex than in fungi and has become known as the "indirect pathway." This is so because 40-carbon oxygenated carotenoid (or xanthophyll) precursor molecules are oxidatively cleaved to form the first 15-carbon intermediate in ABA biosynthesis, as originally proposed in the 1960s (Taylor and Smith 1967). The substantial body of evidence demonstrating that plants use an indirect pathway of ABA biosynthesis via xanthophyll precursors has been summarized in several reviews (Liotenberg and others 1999; Zeevaart 1999). More recent comparative genomic approaches have provided support for the idea that the ABA pathway in land plants has evolved with few changes from that used by green algae, such as *Chlamydomonas reinhardtii* (Yoshida and others 2003), and by "primitive" plants such as the "model" moss species *Physcomitrella patens* (Knight and others 1995). Evidence relating to the evolution of ABA metabolism in different taxonomic groups has been recently reviewed (Nambara and Marion-Poll 2005) and is not detailed in this article.

Plants synthesize many terpenoid compounds from IPP and DMAPP molecules that have been formed in the cytosol via the MVA pathway; but ABA is not one of them. Instead, the xanthophyll precursors of ABA in plants are assembled from IPP and DMAPP building blocks formed from pyruvate and D-glyceraldehyde 3-phosphate in plastids, by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Arigoni and others 1997; Lichtenthaler and others 1997). It is therefore important to note that the ultimate precursor of ABA is not MVA, as originally assumed (Milborrow 1974). The early steps in the "plastidial/MEP pathway" are potentially important in maintaining adequate supplies of the precursors needed to sustain high levels of ABA accumulation and they are therefore considered in detail in the paragraphs that follow.

The Plastidial/MEP Pathway (Leading to all-*trans*-lycopene)

Abscisic acid biosynthesis in plants is perhaps best considered a minor (but important) branch of carotenoid synthesis in plastids. In specialized "carotenogenic" organs, such as ripening tomato fruit, the red pigment known as all-*trans*-lycopene can be the major end product of carotenoid synthesis tak-

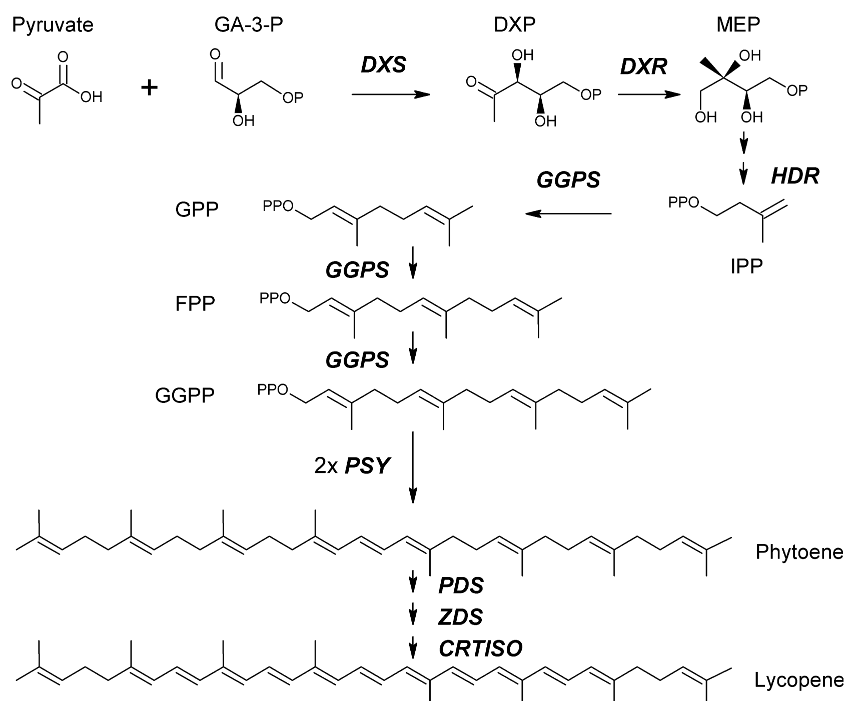

Figure 1. Outline of the plastidial/ MEP pathway leading to all-*trans*-lycopene. Abbreviations of key enzyme names are given in bold italics: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDR, hydroxymethylbutenyl diphosphate reductase; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase. Note that GGPS performs three successive condensation reactions: IPP is first condensed with its isomer DMAPP (not shown) forming GPP, before successively condensing each product with IPP to form FPP, and finally GGPP.

ing place in the chromoplasts, using the ''plastidial/ MEP'' pathway. In immature green tomato fruit and more general photosynthetic tissues such as leaves, lycopene is synthesized in the chloroplasts and is required as an intermediate in the synthesis of more complex carotenoids, including the xanthophylls that can be used as precursors of ABA.

DXS to HDR: Enzymes involved in the formation of IPP and DMAPP. A number of important steps in the early parts of the plastidial pathway, leading to the formation of the simple linear carotenoid all-*trans*-lycopene, are illustrated in Figure 1. The first reaction involves the condensation of pyruvate and D-glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DXP), catalyzed by the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS). This condensation reaction is likely to represent one key control point at the start of the pathway. However, it should be noted that DXP is an important precursor of thiamine diphosphate and pyridoxal phosphate, as well as being essential for the synthesis of the universal isoprene building blocks IPP and DMAPP. It has been pointed out that the conversion of the DXP substrate to 2-C-methyl-D-erythritol 4-phosphate (MEP) by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), thereby diverting DXP away from the thiamine/ pyridoxal route, represents an important branch point and is arguably the first step specific to isoprenoid synthesis within the plastidial pathway (Carretero-Paulet and others 2002).

In *Arabidopsis thaliana* there appears to be only a single *DXS* gene, which is impaired in a mutant known as *cla1*. This mutant has an albino phenotype associated with very low levels of chlorophyll and carotenoid pigments. Phenotypic reversion of the mutant has been achieved by feeding the DXS enzyme product, that is, DXP (Estevez and others 2001). The *Arabidopsis* genome may be atypical in having only one *DXS* gene. Evidence has been obtained for the presence of two *DXS* genes in a number of monocotyledonous and dicotyledonous species, including maize and tomato (Walter and others 2002).

Transgenic *Arabidopsis* plants constitutively overexpressing the *CLA1* gene (Estevez and others 2001) were found to have increased amounts of the DXS enzyme. This increase was correlated with increases in total chlorophyll and carotenoid content in 15-day-old seedlings. In the context of a review on ABA synthesis, it is interesting to note that a DXS overexpressing line contained 4 times more ABA than the wild type. The only clear phenotypic effect of the elevated ABA reported was in delaying seed germination (Estevez and others 2001). This suggests that in both photosynthetic (that is, young seedlings) and non-photosynthetic (that is, seed) tissue, the first step in the MEP pathway, catalyzed by DXS, can be a rate limiting step in ABA biosynthesis.

Initial studies on the relative importance of DXS and DXR in carotenoid synthesis in tomato fruit indicated that *DXR* was constitutively expressed and

therefore "non-limiting," whereas *DXS* transcript levels were strongly correlated with increases in all-*trans*-lycopene during the ripening process (Lois and others 2000; Rodriguez-Concepcion and others 2001). In contrast, *Arabidopsis DXR* expression appeared to be modulated throughout development and was found to closely parallel that of *DXS*. Both were highly expressed in young photosynthetic tissues following induction by light; but they were also detected in non-photosynthetic tissues, such as adult roots. It was suggested that both *DXS* and *DXR* genes "participate" in regulating the plastidial/MEP pathway (Carretero-Paulet and others 2002).

Several plant-encoded enzymes are involved in the conversion of MEP to the universal isoprene intermediates IPP and DMAPP. All of the genes encoding these "early" MEP pathway enzymes in bacteria and in plants have been identified (reviewed by Rodriguez-Concepcion and Boronat 2002). The final step of this part of the pathway involves an enzyme termed "hydroxymethylbutenyl diphosphate (HMBPP) reductase (HDR)," which controls a branch point that results in the independent formation of IPP and its isomer DMAPP from HMBPP (Hoeffler and others 2002). Downregulation of the HDR gene in plants results in an albino phenotype (Page and others 2004).

The tomato *LeHDR* gene was substantially upregulated throughout carotenoid formation during fruit ripening. After illumination of dark-grown *Arabidopsis* seedlings, *AtHDR* transcript levels doubled at 1 hour and increased 10-fold after 6 hours in the light (Botella-Pavia and others 2004). Dark-grown *Arabidopsis* seedlings from a line (LH13) transformed with a 35S::*LeHDR* construct showed no significant differences in carotenoid levels in comparison to untransformed control plants; whereas in the light they produced around 50% more of each of the major carotenoids/xanthophylls—that is, β-carotene, lutein, and violaxanthin (Botella-Pavia and others 2004). The same study also revealed that the imbibed seeds of this transgenic line (LH13) germinated more slowly than the untransformed control, suggesting that increased xanthophyll precursor pools are "channeled to the biosynthesis of ABA in transgenic seeds." The authors also point out that "future attempts to efficiently increase metabolic flux through the MEP pathway to carotenoids and other plastidial isoprenoids should take into account the requirement to target multiple steps simultaneously, including HDR."

GGPP synthase. Enzymes catalyzing condensation reactions in terpenoid biosynthesis have attracted attention as potential key control points. In this context, geranylgeranyl diphosphate synthase (GGPS) enzymes appear to be of central importance to the operation of both the MVA (cytosolic) and non-MVA (plastidial/MEP) pathways. These enzymes are responsible for three reactions in which the 5-carbon molecule IPP is successively condensed with its isomer DMAPP to form 10, 15, and 20-carbon intermediates, known as geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), respectively.

It has been noted that there are 12 *GGPS*-like genes within the *Arabidopsis* genome (Hirschberg 2001). *In vitro* assays have shown that five GGPS enzymes, encoded by the *AtGGPS* genes known as *GGPS1, GGPS2, GGPS3, GGPS4*, and *GGPS6*, have detectable activity (Okada and others 2000). Only two of these gene products, GGPS1 and GGPS3, have transit peptide sequences that indicate that they would be targeted to chloroplasts and could therefore be directly involved in the plastidial/MEP pathway of terpenoid biosynthesis. From a number of localization/expression studies, Okada and others (2000) concluded that "the GGPS1 and GGPS3 proteins supply GGPP for the biosynthesis of chlorophyll, carotenoids, and *ent*-kaurene, precursor of gibberellin, in the plastids." These authors also noted that *GGPS1* promoter activity was high in upper plant parts, but that there was almost no activity in roots. This contrasted with the expression pattern for GGPS3 which appears to be mainly root-specific (Okada and others 2000).

Prior to this, it had been demonstrated in the highly carotenogenic fruit of bell pepper plants (*Capsicum annuum*) that the chloroplast-to-chromoplast transition was associated with a strong induction of a *CaGGPS* transcript. This transition was correlated with enzyme activity, except at the end of the ripening process, when the mRNA levels fell and yet GGPS polypeptide content remained high (Kuntz and others 1992).

Phytoene synthase and the subsequent formation of all-trans-lycopene. The 20-carbon compound known as GGPP is a precursor for the biosynthesis of gibberellins, the phytol tail of chlorophylls, phylloquinones, and tocopherols. These pathways are potentially in competition with carotenoid biosynthesis for the supply of GGPP available in plastids. The condensation of two GGPP molecules to form the colorless 40-carbon compound known as phytoene represents the first committed step in carotenoid biosynthesis. It is therefore not surprising that over the last 10 years genes encoding phytoene synthase have represented a major target of a

succession of attempts to increase the carotenoid content of a variety of plant tissues/organs by genetic manipulation.

Pioneering work in this area followed from the characterization of the *yellow flesh* (or *r*) mutant of tomato, which has an almost carotenoid-free pericarp in ripe fruit and pale yellow petals in the mature corolla (Fray and Grierson 1993). The *R* gene encodes a chromoplast-specific phytoene synthase (*PSY1*) (Fray and Grierson 1993). A chloroplast-specific phytoene synthase (*PSY2*) was detected in leaves and green tomato fruit (Bartley and Scolnik 1993). Transcripts encoding the PSY2 enzyme have been detected by reverse transcriptase polymerase chain reaction (RT-PCR), although not by northern analysis, in *r* mutant and wild-type tomato fruit throughout ripening (Fraser and others 1999). It was demonstrated that extracts from ripe *r* mutant fruit could synthesize phytoene *in vitro*, but they were unable to synthesize carotenoid *in vivo*. Fraser and others (1999) concluded that although the mutant has an enzyme activity theoretically able to synthesize phytoene in ripe fruit, the PSY2 enzyme is in reality unable to compensate for the absence of PSY1.

Partial purification of phytoene synthase from tomato chloroplasts has revealed that it can form part of a larger protein complex, together with other carotenoid biosynthesis enzymes, for example, GGPS (Fraser and others 2000). It is very likely that differential multi-enzyme complex assembly is an important feature of carotenoid synthesis in the chloroplasts, chromoplasts, and etioplasts of various plant cells, and this probably represents one factor that has complicated the many attempts to engineer plants with increased carotenoid contents. Many of these compounds are also involved in the ABA biosynthetic pathway. Therefore, information gained from attempts to manipulate carotenoid pool sizes in transgenic plants is highly relevant to the problem of providing adequate levels of the xanthophyll precursors needed to sustain high rates of ABA accumulation in shoots, roots, and seeds.

Early attempts to consitutively overexpress the endogenous *PSY1* gene in tomato plants resulted in transformants exhibiting a number of abnormal phenotypes (Fray and others 1995). Most notably, the 35S::*PSY1* mRNA levels were inversely correlated with internode length. Plants exhibiting extreme dwarf phenotypes arose from seedlings that had shown novel pigment patterns in the hypocotyl and cotyledons. In the most severe examples young leaves were pale pink, with reduced levels of chlorophyll (Fray and others 1995). Vegetative tissues from an extreme dwarf transgenic line contained substantial amounts of phytoene, phytoene epoxide, and lycopene, all of which were at undetectable levels in untransformed control plants. The presence of the fruit chromoplast-specific phytoene synthase enzyme (PSY1) in stems and leaves appeared to result in the conversion of excessive amounts of its substrate, GGPP, to its product, phytoene. Diversion of GGPP to phytoene and away from gibberellin (GA) and phytol biosynthesis provides a probable explanation for the GA and chlorophyll deficiencies that were observed; both of them were thought to have contributed to the extreme dwarf phenotype. Another phenotype noted in constitutive *Psy1* overexpressing tomato plants was an increase in their root pigmentation (R. G. Fray, personal communication), suggesting that phytoene synthase catalyzes an important rate-determining step in carotenoid biosynthesis in roots. Fray and others (1995) reported that there was ''little evidence of elevated ABA levels in the dwarfs.''

More recent approaches involving tissue-specific overexpression of phytoene synthase constructs in tomato fruit (Fraser and others 2002), *Brassica napus* seeds (Shewmaker and others 1999) and *Arabidopsis* seeds (Lindgren and others 2003), resulted in an increased carotenoid content, whilst avoiding creating GA deficiency and dwarfism in the vegetative parts of the plant. Overexpression in seeds resulted, in both cases, in decreased germination rates.

A pattern should be noted here, in that the overexpression of *DXS*, *HDR*, and *PSY* in transgenic seed has often resulted in increased ABA levels, accompanied by decreased germination rates. It would appear that it is relatively easy to upregulate the ABA biosynthetic pathway in seeds enough to have a detectable effect on seed germination rates. A slightly different picture emerged recently for potato tubers. Tuber-specific overexpression of phytoene synthase using *crtB* constructs could increase total carotenoid content of tubers, without any detectable elevation of ABA content (Ducreux and others 2005).

Ducreux and others (2005) also reported a consistent two- to threefold increase in expression levels of the gene encoding the next enzyme in the pathway, phytoene desaturase (*PDS*), from quantitative RT-PCR analysis of RNA extracted from their *crtB* over-expressing developing tubers; but there was no clear evidence suggesting a similar upregulation of the ζ-carotene desaturase (*ZDS*) gene or any of the genes involved in later steps in carotenoid/xanthophyll biosynthesis that were analyzed.

Both PDS and ZDS enzymes in plants catalyze two desaturation reactions that successively extend

the chromophore of the colorless carotenoid phytoene to form the red pigment lycopene. These two enzymes carry out similar dehydrogenation reactions; each of them contains FAD and both of them require plastoquinone (Norris and others 1995) and a plastidic terminal oxidase as electron acceptor (Carol and Kuntz 2001). Phytoene desaturase carries out the first two symmetrical desaturation steps to form ζ-carotene via phytofluene; ZDS then uses ζ-carotene as a substrate to form lycopene via neurosporene (see Figure 1).

The bacterial phytoene desaturase known as CRTI, catalyzes all four of the desaturation steps required to form all-*trans*-lycopene directly from phytoene. For this reason it has often been used, together with phytoene synthase, in constructs designed to increase carotenoid content (see, for example, Paine and others 2005). The plant pathway is more complex, not only because two different desaturase enzymes are used but also because an isomerase is involved. *Arabidopsis* PDS and ZDS expressed in *Escherichia coli* have been shown to convert phytoene to a tetra-*cis* isomer of lycopene (known as pro-lycopene) rather than to all-*trans*-lycopene (Bartley and others 1999). It has been proposed that ZDS may form a complex with an isomerase enzyme termed CRTISO to be able to carry out the conversion of the poly-*cis* isomers of ζ-carotene to all-*trans*-lycopene in plant plastids in the dark (Isaacson and others 2002).

Isaacson and others (2002) used a map-based approach to clone the *CRTISO* gene of tomato (also known as the *tangerine* locus). They characterized a mild *tangerine* mutant allele and a severe ''null'' mutant allele leading to the complete loss of functional CRTISO enzyme. When tomato seedlings homozygous for the null-mutant allele were grown in the dark for 7 days, the major carotenoid in their leaves was prolycopene, and there was no detectable β-carotene, violaxanthin, or lutein. In complete contrast, the leaves of wild-type seedlings, grown under identical conditions, contained 4% β-carotene, 19.8% violaxanthin, and 76.2% lutein, with no detectable lycopene. These differences in percentage carotenoid composition were much less pronounced when the seedlings were grown for 10 days in the light after germination. There was 33.2% β-carotene, 22.5% violaxanthin, and 29.2% lutein in the mutant leaves; versus 28.5% β-carotene, 3.3% violaxanthin, and 59.6% lutein in the corresponding wild-type leaves (Isaacson and others 2002). These authors concluded that ''in view of the possibility of substituting the activity of carotenoid isomerase for light in green cells, it is hypothesized that the function of carotenoid isomerase in plants is to enable carotenoid biosynthesis in non-photosynthetic tissues or in the dark. The isomerase must be essential in germinating seedlings and roots, as shown by Park and others (2002), and also in chromoplasts in the absence of chlorophyll sensitization.''

Genuinely root-sourced ABA biosynthesis will therefore depend on functional CRTISO enzyme to form the all-*trans*-lycopene required as a precursor of ABA (see Figures 1 and 2). Photoisomerization can more or less compensate for the lack of isomerase activity in green leaves of the *tangerine* null-mutant and in the outer layers of unripe fruit. However there is no compensation in the chromoplasts of petals and ripe fruit, or the deeper layers of immature *tangerine* fruit, where light fails to penetrate sufficiently (Isaacson and others 2002).

Cyclization and Hydroxylation: the α- and β-Branch Points in the Pathway

In terms of the genetic manipulation of ABA biosynthesis, it is essential to be aware that two alternative routes for the cyclization of lycopene represent a major branch point in the carotenoid pathway in plants. These routes can be termed α- or β-branches of carotenoid synthesis, and it should be noted that only the latter leads to ABA (Figure 2). Therefore the supply of xanthophyll precursors needed to sustain artificially elevated rates of ABA synthesis could potentially be maintained by increasing flux through the β-branch; perhaps partly at the expense of the α-branch. This approach could be carried out by manipulating one or more of a number of genes encoding enzymes involved in the cyclization of lycopene or in the subsequent hydroxylation to form the first xanthophylls.

It has been hypothesized that homodimeric β,β-cyclase complexes catalyze the formation of the symmetrical molecule β-carotene from lycopene. Heterodimeric β,ε-cyclase complexes are thought to catalyze the conversion of lycopene to the asymmetric bicyclic compound, α-carotene (Cunningham and Gantt 1998). Park and others (2002) have suggested that the CRTISO protein may interact directly with these complexes and that its absence may preferentially destabilize β,ε-cyclase complexes, favoring the β-branch at the expense of the α-branch in *crtiso* mutant leaves.

Lycopene β-cyclase (LCY-B) enzymes catalyze the formation of a β-ionone ring at each end of lycopene to form the bicyclic compound β-carotene (Figure 2). In contrast, lycopene ε-cyclase (LCY-E)

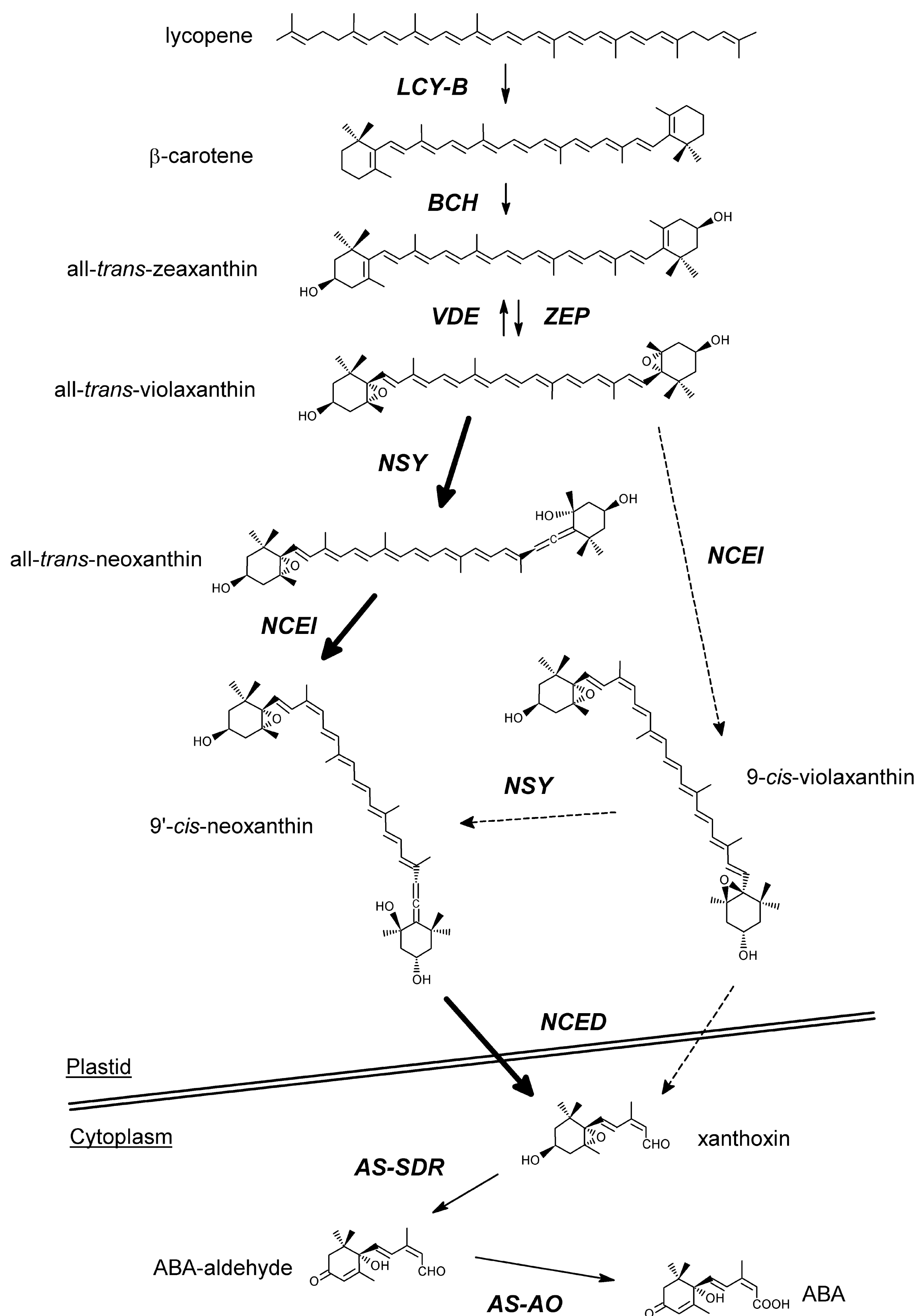

Figure 2. Abscisic acid (ABA) biosynthesis from lycopene to ABA. Abbreviations of enzyme names are given in bold italics: LCY-B, lycopene-*β*-cyclase; BCH, *β*-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violoxanthin deep-oxidase; NSY, neoxanthin synthase; NCEI, 9-*cis*-epoxycarotenoid-forming isomerase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; AS-SDR, ABA-specific short-chain dehydrogenase/reductase; AS-AO, ABA-specific aldehyde oxidase.

normally forms a single epsilon ring on only one end of lycopene to synthesize the monocyclic compound known as δ-carotene. Genes encoding these enzymes are usually downregulated in ripening tomatoes, allowing the fruit to turn red following the accumulation of large amounts of lycopene.

A dominant allele, *Beta* (B), originating from a wild green-fruited *Lycopersicon* species, was found to encode a chromoplast-specific β-cyclase enzyme, LCY-B (CYC-B). The *B* allele is upregulated during ripening, and the presence of the LCY-B (CYC-B) enzyme converts lycopene to β-carotene, changing the tomato fruit color from red to orange (Ronen and others 2000). The ''normal'' *L. esculentum* orthologue/allele at the *Beta* gene locus is not expressed in ripe fruit, but it is strongly expressed in mature flower chromoplasts, in association with the synthesis of bright-yellow xanthophyll pigments in the petals. The tomato mutant known as *old-gold* was originally named after its flower color phenotype, which is due to the accumulation of significant amounts of lycopene and reduced quantities of a number of different β-xanthophylls in the chromoplasts of the mature petals. Ronen and others (2000) characterized two *old-gold* tomato mutants and revealed that they were in fact null-mutant alleles of the *B* locus, impaired in the function of the LCY-B (CYC-B) enzyme in chromoplasts.

Pogson and Rissler (2000) commented, in relation to *Arabidopsis*, that genetic lesions of lycopene β-cyclase are lethal, ''indicating a requirement for bicyclic carotenoid for plant viability.'' It is likely that equivalent tomato mutants impairing the chloroplast-specific LCY-B (CRTL-B) enzyme could also be lethal, in contrast to the *old-gold* mutants affecting the chromoplast-specific LCY-B (CYC-B), which are perfectly viable. On the other hand, many mutations have been identified that disrupt the activity of lycopene ϵ-cyclase without affecting plant viability. *Arabidopsis lut2* mutations result in lutein, the major product of the α-branch of the pathway in leaves, being replaced by equivalent amounts of various β-xanthophylls, particularly violaxanthin (Pogson and Rissler 2000). In plants, elimination of lutein appears to have no obvious detectable effect on growth and development in normal environments, presumably because of the functional complementation of this major α-xanthophyll by equimolar amounts of xanthophylls synthesized via the β-branch (Pogson and others 1996).

Carotenoid synthesis via the α- and β-branches appears to be tightly regulated, balanced, and compensatory. Environmental, as well as genetic, factors can alter the balance between the two branches. It has been reported that there was a fivefold increase in the ratio of *LCY-B* (*CRTL-B*) mRNA to *LCY-E* (*CRTL-E*) mRNA when leaves were subjected to strong light instead of low light intensities. This effect of light was similar in both *Arabidopsis* and tomato plants, resulting in an increase in total xanthophyll content under strong light and a decrease in the ratio of α-carotene–derived (lutein) and β-carotene–derived (zeaxanthin + antheraxanthin + violaxanthin) xanthophylls (Hirschberg 2001).

β-carotene itself represents a major fraction of total carotenoid content in photosynthetic tissues and should not simply be regarded as a transiently formed precursor of the β-xanthophylls. The existence of this large pool indicates that the enzyme(s) responsible for the conversion of β-carotene to zeaxanthin is (are) closely controlled in chloroplasts.

Genetic lesions of *Arabidopsis* affecting epsilon ring hydroxylase activity of the ERH (CRTR-E) protein (*lut1* mutant alleles) are well known, and the *LUT1* gene was recently characterized by map-based cloning (Tian and others 2004). In contrast, there are two genes encoding β-carotene hydroxylase, BCH (CRTR-B) in *Arabidopsis* (Sun and others 1996; Tian and DellaPenna 2001). The presence of two *BCH* genes in Solanaceous species, for example, pepper (Bouvier and others 1998) and tomato (Hirschberg 2001), has also been reported. The facts that the two *Arabidopsis BCH* genes are 79% identical at the nucleic acid level and that they are both expressed in leaves apparently limited the efficiency of downregulation of the two genes using one antisense construct (Rissler and Pogson 2001). In spite of this difficulty, Rissler and Pogson (2001) reported that ''the antisense β-carotene hydroxylase transgene resulted in a maximal reduction in violaxanthin of 64% and a maximal reduction in neoxanthin of 41%. This reduction was reflected in a 22% increase in β-carotene and a reduction in the total carotenoid pool, whereas lutein levels were relatively unaltered.'' T-DNA knockout mutants of the two *BCH* genes in *Arabidopsis* were combined to produce double mutant plants with 20% of the wild-type levels of β-carotene–derived xanthophylls. In these plants there was a compensatory increase in lutein concentration, whereas the β-carotene content remained unchanged (Tian and others 2003).

Conservation of β-carotene pool size in photosynthetic tissues also seems to be a notable feature of attempts to increase the synthesis of β-xanthophylls by upregulation of *BCH* genes in transgenic

plants. Davison and others (2002) obtained a twofold increase in xanthophylls, mainly violaxanthin, in leaves of transgenic *Arabidopsis* lines constitutively overexpressing an endogenous *BCH* gene. Similar results have been obtained in our laboratory using 35S-driven *LeCrtR-b2* (*BCH*) constructs to produce tomato transformants with a twofold increase in all-*trans*-violaxanthin concentration in leaves; the other xanthophylls were not significantly affected (R. Symonds and others, unpublished data). In both of these studies on transformants constitutively overexpressing *BCH* genes, substantial violaxanthin increases occurred without a corresponding decrease in β-carotene, the substrate of the BCH (CRTR-B) enzyme. This may be due to a feedback mechanism, preventing the depletion of the β-carotene pool in chloroplasts despite the extra demand caused by excessive BCH (CRTR-B) enzyme activity. These balancing feedback mechanisms may take place over a period of several days. A 24-hour period of tetracycline induction of *BCH* mRNA in tobacco transformed with a triple-op inducible promoter *LeCrtR-b2* (*BCH*) construct, resulted in a one third increase in β-xanthophyll levels at the expense of the β-carotene pool in leaf tissue (P. Royle and others, unpublished data).

Overexpression of a *BCH* (CRTR-B) gene from *Capsicum annuum*, together with an *Arabidopsis LCY-B (CRTL-B)* gene, under the control of a fruit ripening-specific promoter, resulted in significant increases in β-carotene, β-cryptoxanthin, and zeaxanthin concentrations in ripe transgenic tomatoes (Dharmapuri and others 2002). Different mechanisms for the control and manipulation of carotenoid pool sizes are applicable to different plant tissues. Attempts to manipulate the supply of ABA biosynthetic precursors in chloroplasts, chromoplasts, and etioplasts will have to be based on different strategies, designed to take pre-existing mechanisms that control carotenoid pool sizes into account.

The Xanthophyll (or Violaxanthin) Cycle

ABA biosynthesis (see Figure 2) is inextricably associated with the xanthophyll (or violaxanthin) cycle, but a detailed introduction to the involvement of various carotenoids/xanthophylls in light harvesting and photoprotection is beyond the scope of this review (instead, see Niyogi and others 1997). In brief, the xanthophyll cycle features three closely related β-xanthophylls, zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V). Lutein is the α-branch equivalent of zeaxanthin, and it has been noted that excess amounts of ''free'' lutein can more or less effectively substitute for zeaxanthin in photoprotection. In the absence of both zeaxanthin and lutein, mature *Arabidopsis* leaves become severely photobleached when exposed to high light intensities; but there is much less extensive bleaching if zeaxanthin alone is eliminated (for a review see Pogson and Rissler 2000).

Zeaxanthin is initially formed from β-carotene by BCH (CRTR-B) enzymes, as discussed above (see ''Cyclization and Hydroxylation: the α- and β-Branch Points in the Pathway''). It can also be synthesized from violaxanthin by violaxanthin de-epoxidase (VDE), an enzyme known to require low pH in the lumen and relatively high light intensities to become fully active. This activation of pre-existing VDE is part of a predominantly post-translational mechanism to enable plants to undergo an ''emergency response'' to sudden increases in light levels, by rapid elevation of zeaxanthin content to prevent photobleaching. High light intensities have been found to decrease *VDE* mRNA levels and to increase BCH (CRTR-B) mRNA levels (Rossel and others 2002). This may indicate that increasing the rate of zeaxanthin synthesis using β-carotene as substrate, rather than violaxanthin, represents a more appropriate longer-term response to conditions where the light energy absorbed by chlorophyll is in excess of that used for photosynthesis. This response would seem to have the added advantage of increasing the supply of intermediates needed to sustain ABA biosynthesis, whereas the activation of VDE has precisely the opposite effect.

The epoxidation of zeaxanthin to form violaxanthin, via the mono-epoxy intermediate antheraxanthin, catalyzed by zeaxanthin epoxidase (ZEP) represents half of the xanthophyll cycle, but is also required for ABA biosynthesis. The cloning of a *ZEP* gene from *Nicotiana plumbaginifolia* was the first major breakthrough in the molecular genetics of ABA biosynthesis (Marin and others 1996). Mutants impaired at the step catalyzed by ZEP accumulated high levels of zeaxanthin instead of epoxycarotenoids—for example, violaxanthin—and they have a clear ''wilty'' ABA-deficient phenotype due to abnormal stomatal behavior (Duckham and others 1991; Rock and Zeevaart 1991).

Gene expression studies in *Nicotiana* (Audran and others 1998) and *Lycopersicon* (Thompson and others 2000a), confirmed by microarray studies in *Arabidopsis* (Schaffer and others 2001), have revealed that steady state *ZEP* mRNA levels in leaves are under the control of a circadian oscillator. The transcript peaks during the day and then falls in anticipation of the dark period, during which it remains at a low

level before starting to increase again about an hour before dawn. More recently, Woitsch and Romer (2003) detected a close correlation between the pattern of *ZEP* and *BCH* gene expression in tobacco seedlings, predicting that in photosynthetic tissues *BCH*, as well as *ZEP*, gene expression is ''under circadian clock control.'' Preliminary results in our laboratory (R. Symonds and others, unpublished data) indicate that *LeCrtR-b2* gene expression can be altered by water stress. We have also confirmed earlier findings that the *LeCrtR-b2* gene is highly upregulated in mature tomato flowers, associated with xanthophyll accumulation in the petals; but our results with leaves appear to indicate that this tomato *BCH* gene is not ''exclusively expressed in the flower'' (Hirschberg 2001).

Circadian control of *ZEP* gene expression in photosynthetic tissues seems to result in an adequate supply of violaxanthin to sustain ABA accumulation following drought stress, without the need for further upregulation. The situation is different in roots, which have much lower carotenoid levels than leaves (Parry and Horgan 1992). It was reported that steady-state *NpZEP* mRNA levels in roots increased between three- and sevenfold after an 8-hour period of water stress (Audran and others 1998). A more gradually imposed stress treatment caused by withholding water from intact tomato plants resulted in a fourfold increase in *LeZEP* mRNA levels in the roots, with no corresponding increase in the shoot tissues (Thompson and others 2000a).

Transgenic *N. plumbaginifolia* plants constitutively expressing a 35S::*NpZEP* construct had no obvious phenotype in the leaves, indicating that *ZEP* mRNA levels are not limiting in photosynthetic tissues (Frey and others 1999). However, increased steady-state *NpZEP* mRNA levels were present during seed development, together with increased ABA levels in mature seed and a delay of up to 3 days in germination. It appears that the control of *ZEP* gene expression is one of the rate-determining steps in ABA biosynthesis in non-photosynthetic tissues such as seeds.

When developing underground, potato tubers are also composed of non-photosynthetic tissues. The downregulation of endogenous *StZEP* gene expression, following transformation with a tuber-specific antisense construct, resulted in the accumulation of zeaxanthin rather than violaxanthin in developing transgenic tubers (Romer and others 2002). This result was predictable, but Romer and others (2002) also reported a fivefold increase in total carotenoid content of tubers in some of these *ZEP* antisense transformed lines. In an expression profiling experiment, an inverse relationship was reported between *ZEP* mRNA levels and total carotenoid content of tubers in a range of germplasm (Morris and others 2004). It would be interesting to understand the regulatory mechanisms that are responsible for increasing flux through the carotenoid pathway in tubers, following the downregulation of *ZEP* gene expression. This appears to be an effective way of increasing the zeaxanthin content of tubers for nutritional reasons, but it would obviously be counter-productive in attempts to increase ABA biosynthesis in this tissue.

Nine *cis*-Epoxycarotenoid Synthesis

After the synthesis of all-*trans*-violaxanthin by ZEP, there is some ambiguity as to the exact route of ABA biosynthesis, prior to oxidative cleavage to form xanthoxin (Figure 2). The alternatives were listed in detail in a previous review (Taylor and others 2000), but it is still not possible to choose between them with certainty. Because the enzymes known as nine-*cis*-epoxycarotenoid dioxygenases, or NCEDs (see the section below titled ''Nine *cis* Epoxycarotenoid Cleavage''), are specific to 9-*cis* isomers and do not cleave any all-*trans*-epoxycarotenoids, there are two obvious candidate substrates present in plant tissues, the 9-*cis* isomers of violaxanthin and of neoxanthin.

Schwartz and others (2003a) pointed out that ''circumstantial evidence favors neoxanthin as the primary precursor of ABA'' but that ''definitive evidence of the endogenous substrate would require identification of the C25 by-product *in planta*.'' If 9-*cis*-violaxanthin is the *in vivo* substrate, then C25-epoxy-apo-aldehyde should be formed initially as a by-product. Alternatively, if the allenic epoxycarotenoid 9′-*cis*-neoxanthin is the *in vivo* substrate, then the C25-allenic-apo-aldehyde would be formed as a by-product instead. Neither of these two potential by-products has been found in leaves that are actively synthesizing ABA, and it was therefore proposed that there is a rapid further breakdown (Parry and Horgan 1991). It is tempting to speculate that there could be further oxidative cleavage of the immediate C25 apo-aldehyde by-product, possibly by a carotenoid cleavage dioxygenase (CCD), which is directly or indirectly involved in by-product metabolism, to form smaller apo-aldehyde molecules.

Linforth and others (1987a) reported that leaf tissue from the *notabilis* mutant of tomato, now known to be impaired in the *LeNCED1* gene (see the section below titled ''Nine *cis* Epoxycarotenoid Cleavage''), had threefold lower levels of 2,7-di-

methyl-2,4-octadienedioic acid (ODA) than the wild type. This could be explained by assuming that the ODA detected in these leaf samples arose from the breakdown of a labile C25 by-product formed in the reaction catalyzed by NCED during ABA biosynthesis. It should be noted that ODA is virtually absent in fresh leaf tissue subjected to GC-MS analysis within 8 hours of removal from the plant. However, the threefold difference in ODA concentration between *notabilis* and the wild type can be repeatedly observed in frozen leaf tissue stored for 2 months at −15°C (R. Linforth, unpublished data). This C10 dicarboxylic acid was only formed by oxidation during storage, probably from a C10 aldehyde, which was most likely derived by the oxidative cleavage of the initial C25 apo-aldehyde by-product. Establishing the fate of the by-product would make it clear whether 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin is the *in vivo* substrate of NCED.

In light-grown tomato leaves all-*trans*-violaxanthin was found to represent almost half (49.9%) of the total epoxyxanthophyll content, with 9′-*cis*-neoxanthin making up most of the other half (46.6%). The all-*trans* isomer of neoxanthin and 9-*cis*-violaxanthin represented only 0.85% and 0.34%, respectively (Parry and others 1990). Two enzymes are probably responsible for synthesizing 9′-*cis*-neoxanthin from this large pool of all-*trans*-violaxanthin: neoxanthin synthase (NSY) and a putative nine-*cis* epoxycarotenoid-forming isomerase (NCEI). Genes encoding these enzymes have not yet been cloned, although *Arabidopsis* (H. North and A. Marion-Poll, unpublished data) and tomato (J. Hirschberg, unpublished data) mutants lacking neoxanthin isomers have been reported in a recent review (Nambara and Marion-Poll 2005). These authors have mentioned that the *Arabidopsis* gene has been cloned and that ''further biochemical analysis will indicate whether the encoded protein exhibits NSY activity and produces only all-*trans*-neoxanthin or both neoxanthin isomers.''

Earlier (see the section titled ''Cyclization and Hydroxylation: The α- and β-Branch Points in the Pathway'') it was mentioned that Ronen and others (2000) characterized two alleles of the *B* locus of tomato, originally termed *old-gold* because of their flower color, which were shown to be impaired in the chromoplast-specific lycopene β-cyclase enzyme LCY-B (CYC-B). Because there was some doubt about the reaction catalyzed by this enzyme, we have recently confirmed the function of this gene *in vivo* by showing that its downregulation in RNAi transgenic tomato plants resulted in a large increase in lycopene accumulation in the mature petals, reducing their β-xanthophyll content (R. Symonds and others, unpublished data). Neither the mutant nor the RNAi plants showed any sign of increasing the all-*trans*-violaxanthin content of their leaves at the expense of the neoxanthin isomers. This would have been expected if this enzyme functioned as a neoxanthin synthase *in vivo*, as has been suggested (Al-Babili and others 2000; Bouvier and others 2000). The proposed *NSY* of tomato (Bouvier and others 2000) was identical in DNA sequence to the gene encoding LCY-B (CYC-B) and the ortholog from potato (Al-Babili and others 2000) was very similar. *In vitro* assays indicated neoxanthin synthase rather than lycopene β-cyclase activity (Bouvier and others 2000), but there is no evidence suggesting that LCY-B (CYC-B) functions as a neoxanthin synthase *in vivo*.

Isomerization of all-*trans*-neoxanthin to 9′-*cis*-neoxanthin is likely to be an important step in the ABA biosynthetic pathway. It is probable that an enzyme, rather than light, is mainly responsible for catalyzing this isomerization in leaves, and that the putative NCEI must be important in ABA synthesis in non-photosynthetic tissues, such as roots. If a gene encoding NCEI is cloned, it will be interesting to see whether it resembles the CRTISO enzyme, which catalyzes a *cis* to *trans* isomerization step earlier in the pathway (see the above section titled ''Phytoene Synthase and the Subsequent Formation of all-*trans*-Lycopene''), or NCED-related enzymes that have been implicated in apocarotenoid isomerization (see below).

Nine *cis* Epoxycarotenoid Cleavage

Discovery and enzymology of carotenoid cleavage dioxygenases. Study of a viviparous, ABA-deficient, transposon-tagged maize mutant *vp14* led to the discovery of the first carotenoid cleavage enzyme (Schwartz and others 1997b). It has more recently emerged that there is a large family of related enzymes now classified as carotenoid cleavage dioxygenases (CCDs) that cleave at several different positions in the carotenoid polyene chain (Giuliano and others 2003; Schwartz and others 2003a).

VP14 is a non-heme iron (II)-dependent dioxygenase that cleaves only 9-*cis* isomers of epoxycarotenoids at the 11–12 position (Schwartz and others 1997b) and the activity was originally named neoxanthin cleavage enzyme (Burbidge and others 1997), but was subsequently renamed nine-*cis*-epoxycarotenoid dioxygenase (NCED) at a meeting in Aussois, France, in 1998. Recombinant VP14 is able to cleave both of the natural 9-*cis*-epoxycarotenoids, 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin *in vitro*, however, the *in planta* substrate specificity remains

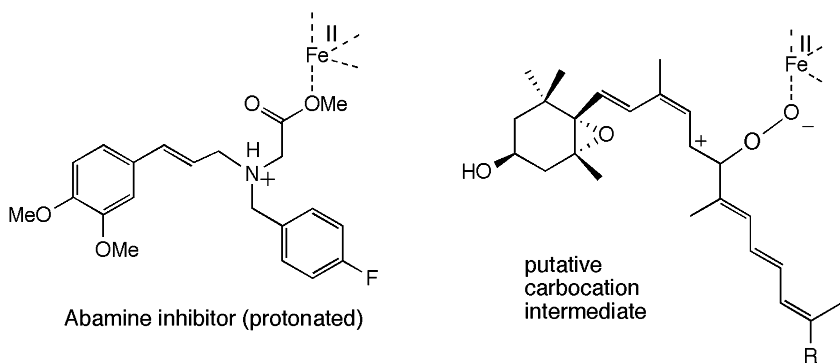

Figure 3. Structural similarity between the NCED inhibitor abamine and the putative NCED carbocation reaction intermediate.

to be demonstrated experimentally, as discussed above (see the section titled ''Nine *cis*-Epoxycarotenoid Synthesis'').

Availability of recombinant NCED has allowed the development of abamine (Figure 3), a synthetic inhibitor of NCED (Han and others 2004a, 2004b). Previously, many researchers have used fluridone and norflurazon, inhibitors of phytoene desaturase, to block carotenoid and therefore ABA synthesis. However, using this approach it is not possible to distinguish ABA effects from the consequences of depleting all carotenoids and apocarotenoids. More specific inhibitors are likely to be valuable in evaluating the role of ABA in plant growth and stress response in systems where genetic analysis is not possible. New functions of abscisic acid in legume nodulation have already been demonstrated using abamine (Suzuki and others 2004).

Recently the crystal structure of apocarotenoid-15,15′-oxygenase (ACO), a *Synechocystis* enzyme that cleaves *β*-apo-carotenals to produce retinal (Ruch and others 2005), has been solved at 2.4 Å resolution (Kloer and others 2005). This structure is the first from the CCD family and contains a seven-bladed *β*-propeller chain fold covered by a dome formed by six large loops, and the active center contains a mononuclear Fe^{2+} coordinated to four histidines. In the CCD family the four histidines are absolutely conserved, and the histidine environments are well conserved; it thus seems likely that all members, including NCED, have a common *β*-propeller fold and that substrate specificity could be determined largely by the dome of loops (Kloer and others 2005).

Earlier, $^{18}O_2$-labeling suggested that NCED is a dioxygenase because nearly 100% of ABA molecules contained a carboxyl ^{18}O (Zeevaart and others 1989). The ACO structure is compatible with the dioxygenase classification because the distance between the Fe^{2+} center and the C15/C15′ of the substrate is around 5.0–5.5 Å (Kloer and others 2005), large enough to accommodate a bound O_2. However, a member of the CCD family from vertebrates, *β*,*β*-carotenoid 15,15′ monooxygenase, gave only 50% incorporation of ^{17}O from $^{17}O_2$ into each aldehyde product; hence the designation as a monooxygenase (Woggon 2002). The molecular mechanism of the NCED-catalyzed cleavage reaction is not known; however, by analogy with the non-heme iron (II)-dependent extradiol catechol dioxygenases that catalyze the oxidative cleavage of aromatic catechol substrates (Bugg 2003), it seems likely that dioxygen activation occurs via a single electron transfer mechanism, and that the polyene substrate would allow the formation of a stable substrate radical intermediate (Figure 4). Formation of a C–O bond at either C-11 or C-12 could then take place, to give a substrate hydroperoxide, which might either form a dioxetane ring or undergo a Criegee rearrangement, as shown in Figure 4. One feature of the latter mechanism is the trapping of a carbocation intermediate by water, which might explain the reduced level of ^{17}O incorporation observed in the mammalian enzyme (Woggon 2002), and it remains possible that all members of the CCD family are dioxygenases sharing a common enzyme mechanism. The existence of a carbo-cation intermediate also provides an explanation for the potent inhibition of NCED by abamine (Han and others 2004a, 2004b), because the protonated form of abamine might resemble the putative carbo-cation intermediate (see Figure 3).

The binding of an all-*trans*-apo-*β*-carotenol substrate to ACO results in a *trans*-*cis* isomerization at the 13,14, and 13′,14′ bonds prior to cleavage, and it has been suggested that related proteins might be isomerases rather than cleavage enzymes (Kloer and others 2005). One intriguing possibility is that an NCED-related enzyme may be responsible for the all-*trans* to 9-*cis* isomerization required for ABA biosynthesis.

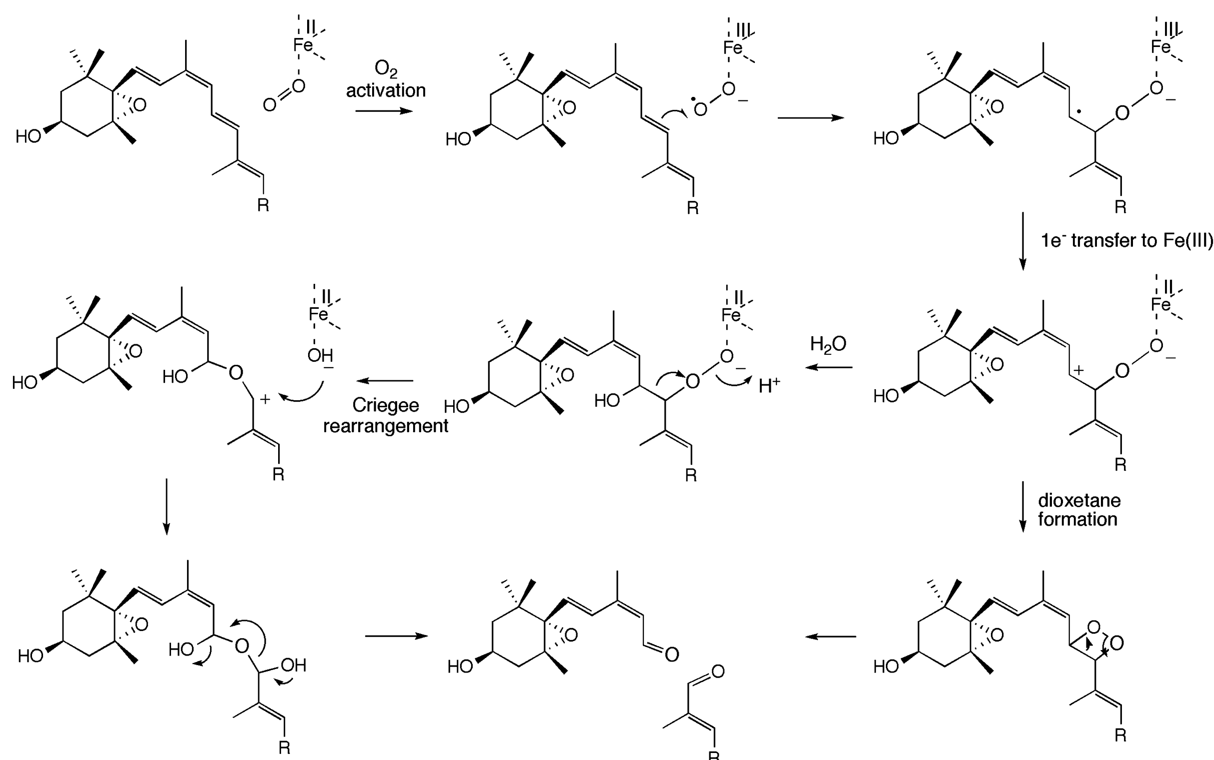

Figure 4. Proposed reaction mechanism for NCED.

NCED genes and gene families. The tomato gene *LeNCED1* was cloned based on homology to *VP14* (Burbidge and others 1997). Mapping (Burbidge and others 1999) and complementation studies (Thompson and others 2004) demonstrated that the ABA-deficient mutant *notabilis* is a null allele of *LeNCED1* containing a single A/T base pair deletion. Other NCED genes have also been characterized: *PvNCED1* from bean (Qin and Zeevaart 1999), *PaNCED1* and *PaNCED3* from avocado (Chernys and Zeevaart 2000), and *VuNCED1* from cowpea (Iuchi and others 2000). The *Arabidopsis* genome contains nine genes with sequence similarity to *VP14* and these have been classified as either *AtNCED* or *AtCCD* genes (Tan and others 2003). *AtNCED 2, 3, 5, 6* and *9* appear to encode NCED activity and are likely to be involved in ABA synthesis (Iuchi and others 2001; Tan and others 2003). *AtCCD1, 7* and *8* have functions unrelated to ABA biosynthesis, whereas the activity of *AtCCD4* is unknown. *AtCCD1* gene product cleaves both the 9,10 and 9′,10′ double bonds of several carotenoid substrates, to produce β-ionone and a C_{14}-apocarotenoid di-aldehyde (Schwartz and others 2001). Both *AtCCD7* and *AtCCD8* appear to act together to produce an unknown apocarotenoid required for suppression of branching (Booker and others 2004; Sorefan and others 2003; Schwartz and others 2004).

Water stress-induced NCED expression. Water stress-induced ABA accumulation is believed to be triggered by loss of turgor (Creelman and Zeevaart 1985; Pierce and Raschke 1980), and before any ABA biosynthesis genes were discovered, it was suggested that cleavage of a C40 epoxycarotenoid intermediate was the key regulatory step in water-stress–induced ABA biosynthesis. This conclusion was based on the abundance of xanthophyll substrates in green tissues, the need for *de novo* translation, and the observation that non-stressed leaves were readily able to convert xanthoxin to ABA (Sindhu and Walton 1987). Higher plant species each appear to have at least one NCED gene that responds strongly and rapidly to leaf dehydration, for example, the probable orthologs *VP14* (Tan and others 1997), *PvNCED1* (Qin and Zeevaart 1999), *LeNCED1* (Thompson and others 2000a), *AtNCED3* (Iuchi and others 2001; Tan and others 2003), *VuNCED1* (Iuchi and others 2000), and *PaNCED1* (Chernys and Zeevaart 2000). Root dehydration also results in increased NCED gene expression (Qin and Zeevaart 1999; Thompson and others 2000a), and protein levels reflect the increase in mRNA in bean leaves and roots (Qin and Zeevaart 1999). This provides good correlative evidence that increased expression of NCED regulates water-stress–induced ABA accumulation.

In *Arabidopsis*, two further NCED genes, *AtNCED9* and *AtNCED5*, appear to have a weak response to dehydration stress in leaves (6-hour treatment), whereas only *AtNCED3* was able to respond over a 35-minute time course (Tan and others 2003). It has been noted, where genomic sequences are available, that the rapidly responding *AtNCED3*, *LeNCED1*, *PvNCED1*, and *VP14* genes have a conserved lack of introns (Tan and others 2003; Thompson and others 2004). This may enhance speed of response, or it may facilitate rapid expression under stress.

Diurnal and hormonal regulation of NCED. *LeNCED1* has a diurnal cycle of expression in non-stressed plants (Thompson and others 2000a), with expression peaking at the end of the light period, and with no circadian component (constant low expression under continuous darkness and no anticipation of the light period). This is consistent with either a direct response to light, or to the small drop in water status associated with the imbalance between water uptake and transpiration during the light period (Kramer and Boyer 1995).

LeNCED1 and *VuNCED1* expression did not respond to application of ABA (Iuchi and others 2000; Thompson and others 2000a), and *LeNCED1* expression pattern was not altered by the low ABA content in an ABA-deficient mutant plant (Thompson and others 2000a). In *Arabidopsis*, *AtNCED3* mRNA increased in response to ABA in ecotype Landsberg *erecta* (Cheng and others 2002; Xiong and others 2001b), but for ecotype C24 the response was seen in one experiment but not in another (Xiong and others 2001b, 2002). Based on these data in *Arabidopsis*, it was suggested that thresholds of ABA-induced NCED expression may vary between ecotypes, and that there may be a weak positive feedback loop to allow activation of NCED expression by ABA (Xiong and others 2002; Xiong and Zhu 2003). However, given the weak response and ecotype differences in *Arabidopsis*, and the lack of response in tomato and cowpea as described above, it seems that there is not an evolutionarily conserved positive or negative feedback of NCED transcription by ABA. In has been suggested that ethylene can trigger ABA biosynthesis (Grossmann and Hansen 2001), but the authors are unaware of any reports confirming regulation of ABA biosynthetic genes by ethylene.

Subcellular localization. Known NCEDs have proven or predicted N-terminal stroma-targeting domains, for example VP14 and PvNCED1 (Qin and Zeevaart 1999; Tan and others 2001), and they exist in two forms, a soluble form in the stroma and a form bound to the stromal face of thylakoid membranes. In *Arabidopsis*, the five NCED proteins differ in their fractionation between stroma and thylakoids (Tan and others 2003), and thus access to membrane bound substrate may provide a regulatory mechanism for enzyme action, and competition experiments suggested a specific binding interaction between VP14 and an unknown membrane component (Tan and others 2001).

Tissue specificity and role in seed biology. ABA is known to accumulate in fruit pericarp during development and ripening—for example, in tomato (Mcglasson and Idato 1976) and avocado (Adato and others 1976)—and two genes encoding NCED activity, *PaNCED1* and *PaNCED3* have been identified in avocado that increase their mRNA levels during fruit ripening, supporting the role of NCED in regulating developmental accumulation of ABA in fruit. *PaNCED1* is also responsive to water deficit in leaves, but *PaNCED3* was not detected in leaves even under stress and may be fruit-specific (Chernys and Zeevaart 2000). Tomato expressed sequence tags (EST) databases reveal that *LeNCED1*, the stress-responsive gene, is highly represented in fruit libraries (17/22 ESTs are from fruit tissues: www.sgn.cornell.edu).

The complex tissue specificity and developmental regulation of five *Arabidopsis* NCED genes have been described (Tan and others 2003). *AtNCED3* and *2* were the major genes expressed in roots, whereas *AtNCED5, 6*, and *2* predominated in flowers. A role for internode tissue in modulating the intensity of the ABA stress signal in the xylem has been described (Sauter and Hartung 2002), and so it is interesting that the stress-responsive *AtNCED3* was also highly expressed constitutively in stems (Tan and others 2003).

In developing siliques, *AtNCED6* was the most highly expressed, but as siliques matured, *AtNCED6* mRNA declined (although it was still the most highly expressed NCED gene), and *AtNCED3* and *AtNCED5* mRNAs increased. This suggests a role in seed development for the latter two genes. *AtNCED9* was highly expressed at both silique stages. Promoter::GUS fusions showed that *AtNCED5* and *6* promoters were active in embryo and endosperm during seed development, whereas the water-deficit–inducible *AtNCED3* promoter was active in the basal region of the seed during development, and it was active in the tissue connecting the seed to the placenta (funiculus) in mature siliques (Tan and others 2003).

Null mutants of water-deficit–responsive NCED genes have been described in three species, *VP14*

(Tan and others 1997), *LeNCED1/notabilis* (Burbidge and others 1999), and *AtNCED3* (Iuchi and others 2001; Ruggiero and others 2004). Studies with these mutants show that *AtNCED3* is required to maintain dormancy when seeds are imbibed at high osmoticum (Ruggiero and others 2004), and the viviparous *VP14* is required to maintain dormancy on the maize cob (Tan and others 1997). The funiculus-specific expression of *AtNCED3* suggests that supplying ABA from fruit to seed might provide a mechanism to maintain dormancy in the fruit, but to allow germination upon dispersal; however, the tissue-specificity of VP14 has not been reported. The lack of observable vivipary or germination effects in the *notabilis* mutant may simply reflect the different fruit environment and lack of primary dormancy in cultivated tomato.

Studies with inhibitors of carotenoid synthesis show that *in situ* ABA synthesis is required to maintain dormancy in imbibed seeds (Grappin and others 2000). *Arabidopsis* ecotype Cvi provides a useful model as it displays marked seed dormancy, and alterations in seed dormancy can be explained by the ability of the seed to synthesize ABA once imbibed (Ali-Rachedi and others 2004). Non-dormant Cvi seed, and seed in states of maintained primary dormancy and induced secondary dormancy were analyzed using microarray analysis. It was observed that, compared to non-dormant seed, both *AtNCED6* and *AtNCED9* mRNAs were more than 10-fold higher under primary dormancy and over fivefold higher under secondary dormancy (B. Finch-Savage, University of Warwick, pers. comm.). In contrast, in the same study, there were no dormancy-related differences in the expression of genes for other steps of the ABA biosynthetic pathway (for example, AtZEP). Thus it appears that various combinations of *AtNCED3, 5, 6*, and *9* may be important in regulating synthesis of ABA during seed development and dormancy.

Genetic manipulation. That NCED is a key regulatory step was predicted (Sindhu and Walton 1987), and correlative studies on gene expression supported this prediction (Qin and Zeevaart 1999). A series of overexpression studies have provided definitive evidence that NCED is rate-limiting: when *LeNCED1* was overexpressed in tobacco leaves using a tetracycline-inducible system, a 10-fold increase in ABA was observed and, when the same gene was constitutively overexpressed in tomato, ABA content increased in leaves (Thompson and others 2000b) and also in roots and xylem sap (A. Thompson and others, unpublished data). Similarly, DEX-inducible and constitutive overexpression of *PvNCED1* in tobacco caused an increase in leaf ABA content, as well as an accumulation of the catabolite phaseic acid (Qin and Zeevaart 2002). Although phaseic acid accumulated, it is clear that any activation of catabolism caused by elevated ABA was insufficient to counterbalance the artificially increased rate of synthesis. Constitutive overexpression of *AtNCED3* also caused a doubling of plant ABA content in *Arabidopsis* (Iuchi and others 2001). Overexpression of NCED was associated with physiological changes, including increased seed dormancy (Qin and Zeevaart 2002; Thompson and others 2000b), reduced stomatal conductance, and an increased tendency to guttate at high humidity, presumably because of ABA-enhanced root pressure (Thompson and others 2000b). Enhanced drought tolerance was reported based on the appearance of pot-grown plants after withholding of water (Iuchi and others 2001; Qin and Zeevaart 2002), and improvements in water use efficiency have been found (A. Thompson and others and also I. Taylor and others, unpublished data).

C15 Level of the Pathway (Conversion of Xanthoxin to ABA)

ABA-specific short-chain dehydrogenase/reductase (AS-SDR). Mutants at the *aba2* locus were selected based on their ability to germinate in the presence of inhibitors of gibberellin biosynthesis (Leon-Kloosterziel and others 1996; Nambara and others 1998), and they were found, using a biochemical approach, to be blocked in the conversion of xanthoxin to abscisic-aldehyde (Schwartz and others 1997a). Subsequently, *aba2* mutant alleles (named *gin1/sis4/isi4/sre1/sañ3*) were isolated independently by four groups through their ability to germinate and develop on high concentrations of glucose, sucrose, or NaCl (Cheng and others 2002; Gonzalez-Guzman and others 2002; Laby and others 2000; Rook and others 2001), or by screening for high transpiration by thermal imaging (Merlot and others 2002). The DNA sequences of 11 alleles have been reported, and at least three are null mutations (Cheng and others 2002). The *aba2* gene was identified as a member of the short-chain dehydrogenase/reductase (SDR) gene family (Rook and others 2001), and it was then demonstrated to be a cytosol-localized NAD-dependent oxidoreductase with xanthoxin oxidase activity (Cheng and others 2002; Gonzalez-Guzman and others 2002). In two examples, pairs of mutant alleles showed intragenic complementation, suggesting complementation

through interaction of the subunits of a multimeric SDR enzyme (Merlot and others 2002; Rook and others 2001). There are 56 putative SDR genes in the *Arabidopsis* genome, and the most highly related gene (*AtSDR2*) has only a 62% similarity to *aba2*; in turn, *AtSDR2* is 73% similar to TASSELSEED2 (Cheng and others 2002), a maize gene that is required for stage-specific pistil abortion (Delong and others 1993). The possibility that other *Arabidopsis* SDRs encode xanthoxin oxidases has not been ruled out, nor has the possibility that TASSELSEED2 is essential for pistil abortion through a tissue-specific synthesis of ABA. The detection of ABA in non-stressed seedlings of an *aba2* null mutant (*gin1-3*) at around one third of wild-type levels (Cheng and others 2002) requires a second *Arabidopsis* xanthoxin oxidase gene, or synthesis of ABA through a second pathway.

Expression of *aba2* was not increased by osmotic stress or ABA, and it was similar in roots, stems, leaves, and siliques (Gonzalez-Guzman and others 2002). These findings are in agreement with reports of the constitutive ability of plants to convert xanthoxin to ABA (Sindhu and Walton 1987) and suggest that *aba2* is not a major regulatory step. However, induction of *aba2* by glucose was indicated by northern and promoter::GUS analysis, suggesting a regulation of ABA biosynthesis by sugars (Cheng and others 2002). The same authors report preferential *aba2* promoter activity in vascular tissue, although alternative approaches are required to confirm that this is not an artifact of the promoter::GUS approach (Martin and others 1992).

ABA-specific aldehyde oxidase (AS-AO). Two wilty mutants of tomato, known as *sitiens* and *flacca*, were the first genetic lesions shown to be impaired at the last step in ABA biosynthesis (Sindhu and Walton 1988; Taylor and others 1988). The final precursor, abscisic aldehyde (ABAld), is oxidized to ABA in wild-type plants, but *sitiens* and *flacca* mutants reduce and isomerize ABAld to form mainly the *trans* isomer of abscisic alcohol (*t*ABAlc), which is biologically inactive (Linforth and others 1987b).

The enzyme that oxidizes ABAld to ABA is an aldehyde oxidase (AO), and four AO-encoding genes from *Arabidopsis* have been investigated in detail (Sekimoto and others 1998). Only one of these, the aldehyde oxidase 3 (*AAO3*) gene encodes an ABA-specific aldehyde oxidase (AS-AO). The active enzyme is a homodimer, known as the AOδ isoform, which is highly active in producing ABA from ABAld (Seo and others 2000). *Arabidopsis* plants homozygous for a mutant allele at this gene locus, *aao3*, have a wilty phenotype due to ABA deficiency caused by defective AAO3 (Seo and others 2000). Recently, two new *aao3* mutant alleles have been identified (Gonzalez-Guzman and others 2004), and it has been noted that *aao3* mutants have only slightly reduced seed dormancy compared with other ABA-deficient mutants of *Arabidopsis* (Seo and others 2004). It appears certain that AAO3 is the major AO catalyzing ABA synthesis in leaves in response to water stress, but there may be other AO enzymes contributing to the oxidation of ABAld to ABA in seed (Seo and others 2004).

Constructs based on the *AAO3* gene of *Arabidopsis* have been reported to complement the ABA-deficient tomato mutant *sitiens* (Okamoto and others 2002), indicating that this tomato gene encodes an ABA-specific AO (AS-AO), as proposed earlier (Marin and Marion-Poll 1997). *Sitiens* may well be an ortholog of *Arabidopsis AAO3*, but this is difficult to prove because *AAO3* resembles the other *Arabidopsis AO* genes more closely than any of the tomato *AO* (*TAO*) gene family members (Min and others 2000). None of the five *TAO* genes co-map with *sitiens* (J. Okyere and others, unpublished data), and, as far as the authors are aware, it has not been possible to characterize the DNA sequence changes associated with any of the known *sitiens* mutant alleles. The only published AS-AO gene sequence is that of the *AAO3* gene from *Arabidopsis*.

In contrast, a *flacca* mutant allele of tomato has now been fully characterized and was shown to be due to a ''6bp out-of-frame deletion'' in a conserved C-terminal region of a molybdenum cofactor (MoCo) sulfurase (Sagi and others 2002). The *aba3* mutant of *Arabidopsis* resembles *flacca* in that it too is impaired in MoCo sulfurase function (Bittner and others 2001; Xiong and others 2001a). These enzymes are required to produce a mono-oxo MoCo, which has a terminal S-atom instead of a second O-atom on the cofactor. Aldehyde oxidases are molybdoenzymes that require mono-oxo MoCo for activity (Mendel and Schwarz 1999). This explains why *flacca* and *aba3* mutants functionally resemble *sitiens* and *aao3* in terms of their failure to complete the last step of ABA biosynthesis by catalyzing the oxidation of ABAld to ABA.

As mentioned in the previous section, it has been shown that cell free extracts prepared from water stressed and unstressed leaves have the same level of enzyme activity, in terms of oxidation of xanthoxin to ABA (Sindhu and Walton 1988). This finding appeared to suggest that neither of the last two steps in the pathway represent key control points for drought-induced ABA accumulation. Koiwai and others (2004) have recently reported that AAO3 transcript levels in guard cells increased substantially in response to drought stress, as did the

bulk leaf AAO3 mRNA content overall. However, these authors were unable to detect corresponding increases in AAO3 protein levels in guard cells or in bulk leaf tissues after dehydration. Possible control mechanisms operating at the cytosolic/C15 level of the ABA pathway are intriguing, particularly if they involve oxidation of the aldehydes (xanthoxin and ABAld) around the vascular tissues during transport to potential target sites. However, it must be concluded that regulation of this part of ABA biosynthesis has not yet been fully elucidated.

Concluding Remarks: Prospects for Increasing ABA Biosynthesis through Genetic Manipulation

In photosynthetic tissues, correlative gene expression studies and overexpression experiments have conclusively shown that NCED is the major rate-limiting step in ABA biosynthesis, and overexpression of this single enzyme is sufficient to obtain plants with higher ABA content. In some circumstances it might also be necessary to engineer conversion of part of the large pool of β-carotene to violaxanthin to prevent depletion of xanthophylls, vital components in photoprotection. Downregulation of enzymes of ABA catabolism potentially provides an additional route to ABA overaccumulation in non-stressed green tissues (Kushiro and others 2004).

In non-photosynthetic tissues it is likely that supply of NCED precursors will become rate limiting when ABA synthesis increases. Overexpression of several genes at key points in the pathway from pyruvate to 9-*cis*-epoxycarotenoids, combined with overexpression of NCED to commit xanthophylls to ABA production, may be required to achieve high ABA synthesis rates. Aspects of pathway regulation that are not fully understood may mean that overexpression of enzymes at some early steps in the pathway could result in activation of later steps through feed-forward regulation.

Engineering or breeding of ''high ABA'' plants, with ABA signaling pathways that are activated under non-stressed conditions, is likely to cause complex and subtle changes in whole plant physiology through the involvement of ABA signaling in a wide range of processes, as well as the cross-talk with other hormones. Study of plants that develop and grow with a long-term ''high ABA'' signal will provide new insights into ABA action. In environments where water availability is limited there may be agronomic advantages associated with high ABA accumulation; however, it will be important to determine whether the benefits of improved water-use efficiency and pre-adaptation to stress outweigh any effects on crop productivity. Tissue-specific engineering of ABA synthesis offers the prospect of fine-tuning to suit different agronomic needs.

REFERENCES

Adato I, Gazit S, Blumenfeld A. 1976. Relationship between changes in abscisic acid and ethylene production during ripening of avocado fruits. Aust J Plant Physiol 3:555–558.

Al-Babili S, Hugueney P, Schledz M, Welsch R, Frohnmeyer H, and others. 2000. Identification of a novel gene coding for neoxanthin synthase from Solanum tuberosum. FEBS Lett 485:168–172.

Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, and others. 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of Arabidopsis thaliana. Planta 219:479–488.

Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A, and others. 1997. Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. Proc Natl Acad Sci USA 94:10600–10605.

Audran C, Borel C, Frey A, Sotta B, Meyer C, and others. 1998. Expression studies of the zeaxanthin epoxidase gene in Nicotiana plumbaginifolia. Plant Physiol 118:1021–1028.

Bartley GE, Scolnik PA. 1993. cDNA cloning, expression during development, and genome mapping of psy2, a 2nd tomato gene encoding phytoene synthase. J Biol Chem 268:25718–25721.

Bartley GE, Scolnik PA, Beyer P. 1999. Two Arabidopsis thaliana carotene desaturases, phytoene desaturase and ζ-carotene desaturase, expressed in Escherichia coli, catalyze a poly-cis pathway to yield pro-lycopene. Eur J Biochem 259:396–403.

Bittner F, Oreb M, Mendel RR. 2001. ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in Arabidopsis thaliana. J Biol Chem 276:40381–40384.

Booker J, Auldridge M, Wills S, McCarty D, Klee H, and others. 2004. MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. Curr Biol 14:1232–1238.

Botella-Pavia P, Besumbes O, Phillips MA, Carretero-Paulet L, Boronat A, and others. 2004. Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. Plant J 40:188–199.

Bouvier F, Keller Y, D'Harlingue A, Camara B. 1998. Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (Capsicum annuum L.). Biochim Biophys Acta—Lipids Lipid Metab 1391:320–328.

Bouvier F, D'Harlingue A, Backhaus RA, Kumagai MH, Camara B. 2000. Identification of neoxanthin synthase as a carotenoid cyclase paralog. Eur J Biochem 267:6346–6352.

Bugg TDH. 2003. Dioxygenase enzymes: catalytic mechanisms and chemical models. Tetrahedron 59:7075–7101.

Burbidge A, Grieve T, Jackson A, Thompson A, Taylor I. 1997. Structure and expression of a cDNA encoding a putative neoxanthin cleavage enzyme (NCE), isolated from a wilt-related tomato (Lycopersicon esculentum Mill.) library. J Exp Bot 48:2111–2112.

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, and others. 1999. Characterization of the ABA-deficient tomato mutant notabilis and its relationship with maize Vp14. Plant J 17:427–431.

Carol P, Kuntz M. 2001. A plastid terminal oxidase comes to light: implications for carotenoid biosynthesis and chlororespiration. Trends Plant Sci 6:31–36.

Carretero-Paulet L, Ahumada I, Cunillera N, Rodriguez-Concepcion M, Ferrer A, and others. 2002. Expression and molecular analysis of the Arabidopsis DXR gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-C-methyl-D-erythritol 4- phosphate pathway. Plant Physiol 129:1581–1591.

Cheng WH, Endo A, Zhou L, Penney J, Chen HC, and others. 2002. A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 14:2723–2743.

Chernys JT, Zeevaart JAD. 2000. Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. Plant Physiol 124:343–353.

Creelman RA, Zeevaart JAD. 1985. Abscisic acid accumulation in spinach leaf slices in the presence of penetrating and non-penetrating solutes. Plant Physiol 77:25–28.

Cunningham FX, Gantt E. 1998. Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49:557–583.

Davison PA, Hunter CN, Horton P. 2002. Overexpression of β-carotene hydroxylase enhances stress tolerance in Arabidopsis. Nature 418:203–206.

Delong A, Calderon-Urrea A, Dellaporta SL. 1993. Sex determination gene Tasselseed2 of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. Cell 74:757–768.

Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, and others. 2002. Metabolic engineering of xanthophyll content in tomato fruits. FEBS Lett 519:30–34.

Duckham SC, Linforth RST, Taylor IB. 1991. Abscisic acid-deficient mutants at the aba gene locus of Arabidopsis thaliana are impaired in the epoxidation of zeaxanthin. Plant Cell Environ 14:601–606.

Ducreux LJM, Morris WL, Hedley PE, Shepherd T, Davies HV, and others. 2005. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. J Exp Bot 56:81–89.

Estevez JM, Cantero A, Reindl A, Reichler S, Leon P. 2001. 1-deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem 276:22901–22909.

Fraser PD, Kiano JW, Truesdale MR, Schuch W, Bramley PM. 1999. Phytoene synthase-2 enzyme activity in tomato does not contribute to carotenoid synthesis in ripening fruit. Plant Mol Biol 40:687–698.

Fraser PD, Schuch W, Bramley PM. 2000. Phytoene synthase from tomato (Lycopersicon esculentum) chloroplasts—partial purification and biochemical properties. Planta 211:361–369.

Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, and others. 2002. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. Proc Natl Acad Sci USA 99:1092–1097.

Fray RG, Grierson D. 1993. Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. Plant Mol Biol 22:589–602.

Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, and others. 1995. Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J 8:693–701.

Frey A, Audran C, Marin E, Sotta B, Marion-Poll A. 1999. Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. Plant Mol Biol 39:1267–1274.

Giuliano G, Al-Babili S, von Lintig J. 2003. Carotenoid oxygenases: cleave it or leave it. Trends Plant Sci 8:145–149.

Gonzalez-Guzman M, Apostolova N, Belles JM, Barrero JM, Piqueras P, and others. 2002. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. Plant Cell 14:1833–1846.

Gonzalez-Guzman M, Abia D, Salinas J, Serrano R, Rodriguez PL. 2004. Two new alleles of the abscisic aldehyde oxidase 3 gene reveal its role in abscisic acid biosynthesis in seeds. Plant Physiol 135:325–333.

Grappin P, Bouinot D, Sotta B, Miginiac E, Jullien M. 2000. Control of seed dormancy in Nicotiana plumbaginifolia: post-imbibition abscisic acid synthesis imposes dormancy maintenance. Planta 210:279–285.

Grossmann K, Hansen H. 2001. Ethylene-triggered abscisic acid: a principle in plant growth regulation? Physiol Plant 113:9–14.

Han SY, Kitahata N, Saito T, Kobayashi M, Shinozaki K, and others. 2004a. A new lead compound for abscisic acid biosynthesis inhibitors targeting 9-cis-epoxycarotenoid dioxygenase. Bioorg Med Chem Lett 14:3033–3036.

Han SY, Kitahata N, Sekimata K, Saito T, Kobayashi M, and others. 2004b. A novel inhibitor of 9-cis-epoxycarotenoid dioxygenase in abscisic acid biosynthesis in higher plants. Plant Physiol 135:1574–1582.

Hirai N, Yoshida R, Todoroki Y, Ohigashi H. 2000. Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. Biosci Biotechnol Biochem 64:1448–1458.

Hirschberg J. 2001. Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol 4:210–218.

Hoeffler JF, Hemmerlin A, Grosdemange-Billiard C, Bach TJ, Rohmer M. 2002. Isoprenoid biosynthesis in higher plants and in Escherichia coli: on the branching in the methylerythritol phosphate pathway and the independent biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate. Biochem J 366:573–583.

Isaacson T, Ronen G, Zamir D, Hirschberg J. 2002. Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of β-carotene and xanthophylls in plants. Plant Cell 14:333–342.

Iuchi S, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K. 2000. A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. Plant Physiol 123:553.

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, and others. 2001. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J 27:325–333.

Kloer DP, Ruch S, Al-Babili S, Beyer P, Schulz GE. 2005. The structure of a retinal-forming carotenoid oxygenase. Science 308:267–269.

Knight CD, Sehgal A, Atwal K, Wallace JC, Cove DJ, and others. 1995. Molecular responses to abscisic acid and stress are conserved between moss and cereals. Plant Cell 7:499–506.

Koiwai H, Nakaminami K, Seo M, Mitsuhashi W, Toyomasu T, and others. 2004. Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in Arabidopsis. Plant Physiol 134:1697–1707.

Kramer PJ, Boyer JS. 1995. *Water Relations of Plants and Soils* San Diego, CA, USA: Academic Press.

Kuntz M, Romer S, Suire C, Hugueney P, Weil JH, and others. 1992. Identification of a cDNA for the plastid located geranylgeranyl pyrophosphate synthase from Capsicum annuum—correlative increase in enzyme activity and transcript level during fruit ripening. Plant J 2:25–34.

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, and others. 2004. The Arabidopsis cytochrome P450CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. EMBO J 23:1647–1656.

Laby RJ, Kincaid MS, Kim DG, Gibson SI. 2000. The Arabidopsis sugar-insensitive mutants sis4 and sis5 are defective in abscisic acid synthesis and response. Plant J 23:587–596.

Leon-Kloosterziel KM, Gil MA, Ruijs GJ, Jacobsen SE, Olszewski NE, and others. 1996. Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. Plant J 10:655–661.

Lichtenthaler HK, Schwender J, Disch A, Rohmer M. 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett 400:271–274.

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 132:779–785.

Linforth RST, Taylor IB, Hedden P. 1987a. Abscisic acid and C10 dicarboxylic acids in wilty tomato mutants. J Exp Bot 38:1734–1740.

Linforth RST, Bowman WR, Griffin DA, Marples BA, Taylor IB. 1987b. 2-trans-ABA alcohol accumulation in the wilty tomato mutants flacca and sitiens. Plant Cell Environ 10:599–606.

Liotenberg S, North H, Marion-Poll A. 1999. Molecular biology and regulation of abscisic acid biosynthesis in plants. Plant Physiol Biochem 37:341–350.

Lois LM, Rodriguez-Concepcion M, Gallego F, Campos N, Boronat A. 2000. Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J 22:503–513.

Marin E, Marion-Poll A. 1997. Tomato flacca mutant is impaired in ABA aldehyde oxidase and xanthine dehydrogenase activities. Plant Physiol Biochem 35:369–372.

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, and others. 1996. Molecular identification of zeaxanthin epoxidase of Nicotiana plumbaginifolia, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of Arabidopsis thaliana. EMBO J 15:2331–2342.

Martin T, Wohner R-V, Hummel S, Willmitzer L, Frommer WB. 1992. ''The GUS reporter system as a tool to study plant gene expression'' In: Gallagher SR, editor. GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. San Diego, CA, USA: Academic Press, Inc., pp 23–43.

Mcglasson WB, Idato I. 1976. Changes in the concentrations of abscisic acid in fruits of normal and Nr, rin and nor mutant tomatoes during growth, maturation and senescence. Aust J Plant Physiol 3:809–817.

Mendel RR, Schwarz G. 1999. Molybdoenzymes and molybdenum cofactor in plants. Crit Rev Plant Sci 18:33–69.

Merlot S, Mustilli AC, Genty B, North H, Lefebvre V, and others. 2002. Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. Plant J 30:601–609.

Milborrow B. 1974. The chemistry and physiology of abscisic acid. Annu Rev Plant Physiol 25:259–307.

Min XJ, Okada K, Brockmann B, Koshiba T, Kamiya Y. 2000. Molecular cloning and expression patterns of three putative functional aldehyde oxidase genes and isolation of two aldehyde oxidase pseudogenes in tomato. Biochim Biophys Acta—Gene Struct Expression 1493:337–341.

Morris WL, Ducreux L, Griffiths DW, Stewart D, Davies HV, and others. 2004. Carotenogenesis during tuber development and storage in potato. J Exp Bot 55:975–982.

Nambara E, Marion-Poll A. 2003. ABA action and interactions in seeds. Trends Plant Sci 8:213–217.

Nambara E, Marion-Poll A. 2005. Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56:165–185.

Nambara E, Kawaide H, Kamiya Y, Naito S. 1998. Characterization of an Arabidopsis thaliana mutant that has a defect in ABA accumulation: ABA-dependent and ABA-independent accumulation of free amino acids during dehydration. Plant Cell Physiol 39:853–858.

Niyogi KK, Bjorkman O, Grossman AR. 1997. The roles of specific xanthophylls in photoprotection. Proc Natl Acad Sci USA 94:14162–14167.

Norris SR, Barrette TR, Della-Penna D. 1995. Genetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation. Plant Cell 7:2139–2149.

Okada K, Saito T, Nakagawa T, Kawamukai M, Kamiya Y. 2000. Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in Arabidopsis. Plant Physiol 122:1045–1056.

Okamoto M, Min X, Seo M, Nakabayashi K, Kamiya Y. and others. 2002. Complementation of a tomato ABA-deficient sitiens mutant by an Arabidopsis aldehyde oxidase gene, AAO3. Plant Cell Physiol 43:S42 .

Page JE, Hause G, Raschke M, Gao WY, Schmidt J, and others. 2004. Functional analysis of the final steps of the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway to isoprenoids in plants using virus-induced gene silencing. Plant Physiol 134:1401–1413.

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, and others. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23:482–487.

Park H, Kreunen SS, Cuttriss AJ, DellaPenna D, Pogson BJ. 2002. Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis. Plant Cell 14:321–332.

Parry AD, Horgan R. 1991. Carotenoid metabolism and the biosynthesis of abscisic acid. Phytochemistry 30:815–821.

Parry AD, Horgan R. 1992. Abscisic acid biosynthesis in roots I. The identification of potential abscisic acid precursors, and other carotenoids. Planta 187:185–191.

Parry AD, Babiano MJ, Horgan R. 1990. The role of cis-carotenoids in abscisic acid biosynthesis. Planta 182:118–128.

Pierce M, Raschke K. 1980. Correlation between loss of turgor and accumulation of abscisic acid in detached leaves. Planta 148:174–182.

Pogson BJ, Rissler HM. 2000. Genetic manipulation of carotenoid biosynthesis and photoprotection. Philos Trans R Soc Lond Ser B Biol Sci 355:1395–1403.

Pogson B, McDonald KA, Truong M, Britton G, DellaPenna D. 1996. Arabidopsis carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. Plant Cell 8:1627–1639.

Qin X, Zeevaart JAD. 1999. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc Natl Acad Sci USA 96:15354–15361.

Qin X, Zeevaart JAD. 2002. Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in Nicotiana plumbaginifolia increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol 128:544–551.

Rissler HM, Pogson BJ. 2001. Antisense inhibition of the β-carotene hydroxylase enzyme in Arabidopsis and the implications for carotenoid accumulation, photoprotection and antenna assembly. Photosynth Res 67:127–137.

Rock CD, Zeevaart JAD. 1991. The ABA mutant of Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci USA 88:7496–7499.

Rodriguez-Concepcion M, Ahumada I, Diez-Juez E, Sauret-Gueto S, Lois LM, and others. 2001. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening. Plant J 27:213–222.

Rodriguez-Concepcion M, Boronat A. 2002. Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol 130:1079–1089.

Romer S, Lubeck J, Kauder F, Steiger S, Adomat C, and others. 2002. Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. Metab Eng 4:263–272.

Ronen G, Carmel-Goren L, Zamir D, Hirschberg J. 2000. An alternative pathway to β-carotene formation in plant chromoplasts discovered by map-based cloning of Beta and old-gold color mutations in tomato. Proc Natl Acad Sci USA 97:11102–11107.

Rook F, Corke F, Card R, Munz G, Smith C, and others. 2001. Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signalling. Plant J 26:421–433.

Rossel JB, Wilson IW, Pogson BJ. 2002. Global changes in gene expression in response to high light in Arabidopsis. Plant Physiol 130:1109–1120.

Ruch S, Beyer P, Ernst H, Al-Babili S. 2005. Retinal biosynthesis in Eubacteria: in vitro characterization of a novel carotenoid oxygenase from Synechocystis sp. PCC 6803. Mol Microbiol 55:1015–1024.

Ruggiero B, Koiwa H, Manabe Y, Quist TM, Inan G, and others. 2004. Uncoupling the effects of abscisic acid on plant growth and water relations. Analysis of sto1/nced3, an abscisic acid-deficient but salt stress-tolerant mutant in Arabidopsis. Plant Physiol 136:3134–3147.

Sagi M, Scazzocchio C, Fluhr R. 2002. The absence of molybdenum cofactor sulfuration is the primary cause of the flacca phenotype in tomato plants. Plant J 31:305–317.

Sauter A, Hartung W. 2002. The contribution of internode and mesocotyl tissues to root-to-shoot signalling of abscisic acid. J Exp Bot 53:297–302.

Schaffer R, Landgraf J, Accerbi M, Simon V, Larson M, and others. 2001. Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. Plant Cell 13:113–123.

Schwartz SH, Leon-Kloosterziel KM, Koornneef M, Zeevaart JAD. 1997a. Biochemical characterization of the aba2 and aba3 mutants in Arabidopsis thaliana. Plant Physiol 114:161–166.

Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR. 1997b. Specific oxidative cleavage of carotenoids by VP14 of maize. Science 276:1872–1874.

Schwartz SH, Qin X, Zeevaart JAD. 2001. Characterization of a novel carotenoid cleavage dioxygenase from plants. J Biol Chem 276:25208–25211.

Schwartz SH, Qin XQ, Zeevaart JAD. 2003. Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. Plant Physiol 131:1591–1601.

Schwartz SH, Qin XQ, Loewen MC. 2004. The biochemical characterization of two carotenoid cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound inhibits lateral branching. J Biol Chem 279:46940–46945.

Sekimoto H, Seo M, Kawakami N, Komano T, Desloire S, and others. 1998. Molecular cloning and characterization of aldehyde oxidases in Arabidopsis thaliana. Plant Cell Physiol 39:433–442.

Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll A, and others. 2000. The Arabidopsis aldehyde oxidase 3 (AA03) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc Natl Acad Sci USA 97:12908–12913.

Seo M, Aoki H, Koiwai H, Kamiya Y, Nambara E, and others. 2004. Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant Cell Physiol 45:1694–1703.

Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY. 1999. Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412.

Sindhu RK, Walton DC. 1987. Conversion of xanthoxin to abscisic acid by cell-free preparations from bean leaves. Plant Physiol 85:916–921.

Sindhu RK, Walton DC. 1988. Xanthoxin metabolism in cell-free preparations from wild-type and wilty mutants of tomato. Plant Physiol 88:178–182.

Sorefan K, Booker J, Haurogne K, Goussot M, Bainbridge K, and others. 2003. MAX4 and RMS1 are orthologous dioxygenase-like genes that regulate shoot branching in Arabidopsis and pea. Genes Dev 17:1469–1474.

Sun ZR, Gantt E, Cunningham FX. 1996. Cloning and functional analysis of the β-carotene hydroxylase of Arabidopsis thaliana. J Biol Chem 271:24349–24352.

Suzuki A, Akune M, Kogiso M, Imagama Y, Osuki K, and others. 2004. Control of nodule number by the phytohormone abscisic acid in the roots of two leguminous species. Plant Cell Physiol 45:914–922.

Tan BC, Schwartz SH, Zeevaart JAD, McCarty DR. 1997. Genetic control of abscisic acid biosynthesis in maize. Proc Natl Acad Sci USA 94:12235–12240.

Tan BC, Cline K, McCarty DR. 2001. Localization and targeting of the VP14 epoxy-carotenoid dioxygenase to chloroplast membranes. Plant J 27:373–382.

Tan BC, Joseph LM, Deng WT, Liu LJ, Li QB, and others. 2003. Molecular characterization of the Arabidopsis 9-cis-epoxycarotenoid dioxygenase gene family. Plant J 35:44–56.

Taylor HF, Smith TA. 1967. Production of plant growth inhibitors from xanthophylls: a possible source of dormin. Nature 215:1513–1514.

Taylor IB, Linforth RST, Alnaieb RJ, Bowman WR, Marples BA. 1988. The wilty tomato mutants flacca and sitiens are impaired in the oxidation of ABA-aldehyde to ABA. Plant Cell Environ 11:739–745.

Taylor IB, Burbidge A, Thompson AJ. 2000. Control of abscisic acid synthesis. J Exp Bot 51:1563–1574.

Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burbidge A, and others. 2000a. Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. Plant Mol Biol 42:833–845.

Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell AR, and others. 2000b. Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. Plant J 23:363–374.

Thompson AJ, Thorne ET, Burbidge A, Jackson AC, Sharp RE, and others. 2004. Complementation of notabilis, an abscisic

acid-deficient mutant of tomato: importance of sequence context and utility of partial complementation. Plant Cell Environ 27:459–471.

Tian L, DellaPenna D. 2001. Characterization of a second carotenoid β-hydroxylase gene from Arabidopsis and its relationship to the LUT1 locus. Plant Mol Biol 47:379–388.

Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D. 2003. Functional analysis of β- and ϵ-ring carotenoid hydroxylases in Arabidopsis. Plant Cell 15:1320–1332.

Tian L, Musetti V, Kim JY, Magallanes-Lundback M, DellaPenna D. 2004. The Arabidopsis LUT1 locus encodes a member of the cytochrome P450 family that is required for carotenoid ϵ-ring hydroxylation activity. Proc Natl Acad Sci USA 101: 402–407.

Walter MH, Hans J, Strack D. 2002. Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J 31:243–254.

Woggon WD. 2002. Oxidative cleavage of carotenoids catalyzed by enzyme models and beta-carotene 15,15′-monooxygenase. Pure Appl Chem 74:1397–1408.

Woitsch S, Romer S. 2003. Expression of xanthophyll biosynthetic genes during light-dependent chloroplast differentiation. Plant Physiol 132:1508–1517.

Xiong LM, Zhu JK. 2003. Regulation of abscisic acid biosynthesis. Plant Physiol 133:29–36.

Xiong LM, Ishitani M, Lee H, Zhu JK. 2001a. The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. Plant Cell 13:2063–2083.

Xiong LM, Gong ZZ, Rock CD, Subramanian S, Guo Y, and others. 2001b. Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in Arabidopsis. Dev Cell 1:771–781.

Xiong LM, Lee HJ, Ishitani M, Zhu JK. 2002. Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. J Biol Chem 277:8588–8596.

Yoshida K, Igarashi E, Mukai M, Hirata K, Miyamoto K. 2003. Induction of tolerance to oxidative stress in the green alga, Chlamydomonas reinhardtii, by abscisic acid. Plant Cell Environ 26:451–457.

Zeevaart JAD. 1999. ''Abscisic acid metabolism and its regulation'' In: Hooykaas P. Hall M. Libbenga K. editors. Biochemistry and Molecular Biology of Plant Hormones Amsterdam, The Netherlands: Elsevier Science. pp 189–207.

Zeevaart JAD, Heath TG, Gage DA. 1989. Evidence for a universal pathway of abscisic acid biosynthesis in higher plants from 18O incorporation patterns. Plant Physiol 91: 1594–1601.