Elucidation of the Indirect Pathway of Abscisic Acid Biosynthesis by Mutants, Genes, and Enzymes[1]

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Abscisic acid (ABA) was discovered independently by several groups in the early 1960s. Originally believed to be involved in the abscission of fruit and dormancy of woody plants, the role of ABA in these processes is still not clear. ABA is, however, necessary for seed development, adaptation to several abiotic stresses, and sugar sensing. The regulation of these processes is in large part mediated by changes in de novo synthesis of ABA.

Two pathways have been proposed for the synthesis of ABA. In the "direct pathway," which operates in some fungi, ABA is derived from farnesyl diphosphate (Hirai et al., 2000). Because of structural similarities, an "indirect pathway" in which ABA is produced from the cleavage of carotenoids also had been proposed (Taylor and Smith, 1967). The first committed step for ABA synthesis in plants is the oxidative cleavage of a 9-cis-epoxycarotenoid (C_{40}) to produce xanthoxin (C_{15}) and a C_{25} by-product (Fig. 1). The 4′-hydroxyl of xanthoxin is oxidized to a ketone by an NAD-requiring enzyme. As a consequence, there is a nonenzymatic desaturation of the 2′-3′ bond and opening of the epoxide ring to form abscisic aldehyde. In the final step of the pathway, abscisic aldehyde is oxidized to ABA.

Evidence for the indirect pathway in plants had initially come from a variety of biochemical studies, $^{18}O_2$-labeling experiments, and the characterization of ABA-deficient mutants. In recent years, the genes encoding enzymes for many steps in the pathway have been identified. Much of the recent work in characterizing these genes has confirmed previous biochemical studies. Advances in the elucidation of the ABA biosynthetic pathway and its regulation also have allowed the manipulation of ABA levels in transgenic plants. Of particular interest is the cloning and characterization of the nine-cis-epoxycarotenoid dioxygenases (*NCED*s) that catalyze the rate-limiting step in ABA synthesis. The identification of the *NCED*s also has had an impact beyond plant biology. Similar proteins are present in a diverse array of organisms. Their enzymatic activities are responsible for the synthesis of a variety of compounds from carotenoids, including vitamin A in animals.

ABA-DEFICIENT MUTANTS

Our understanding of the functions and synthesis of ABA has been greatly enhanced by the identification and characterization of ABA-deficient mutants (Table I). The ABA-deficient mutants have been identified by the following phenotypes: precocious germination, susceptibility to wilting, an increase in stomatal conductance, and an ability to germinate and grow on media containing a high concentration of Suc or salt. In recent years, these mutants have also been very useful in cloning the genes that encode ABA biosynthetic enzymes.

The pathway of ABA synthesis can be traced back to the early steps of isoprenoid synthesis in plastids (Rodríguez-Concepción and Boronat, 2002). Isoprenoids are an extremely diverse class of natural products that serve a variety of functions in plants. Although it is not a committed step in ABA synthesis, the epoxidation of zeaxanthin seems to be a reasonable place to begin a review on ABA synthesis in plants (Fig. 1). Mutants impaired in the epoxidation of zeaxanthin were first identified by an ABA-deficient phenotype. In addition, the ZEP appears to have a role in the regulation of ABA synthesis in non-chlorophyllous organs.

The *aba1* mutant in Arabidopsis (Karssen et al., 1983; Duckham et al., 1991; Rock and Zeevaart, 1991a) and the *aba2* mutant in *N. plumbaginifolia* (Marin et al., 1996) both contain lesions in the enzyme that catalyzes the epoxidation of zeaxanthin to antheraxanthin and violaxanthin (ZEP in Fig. 1). To avoid confusion, the genes corresponding to these mutants will subsequently be referred to as *AtZEP* and *NpZEP*, respectively.

The *atzep* mutant provided definitive evidence that ABA is derived from an epoxycarotenoid precursor (Duckham et al., 1991; Rock and Zeevaart, 1991a). This mutant also has been used extensively to characterize the role of epoxycarotenoids in the xanthophyll cycle and as components of the light-harvesting complexes (Lokstein et al., 2002).

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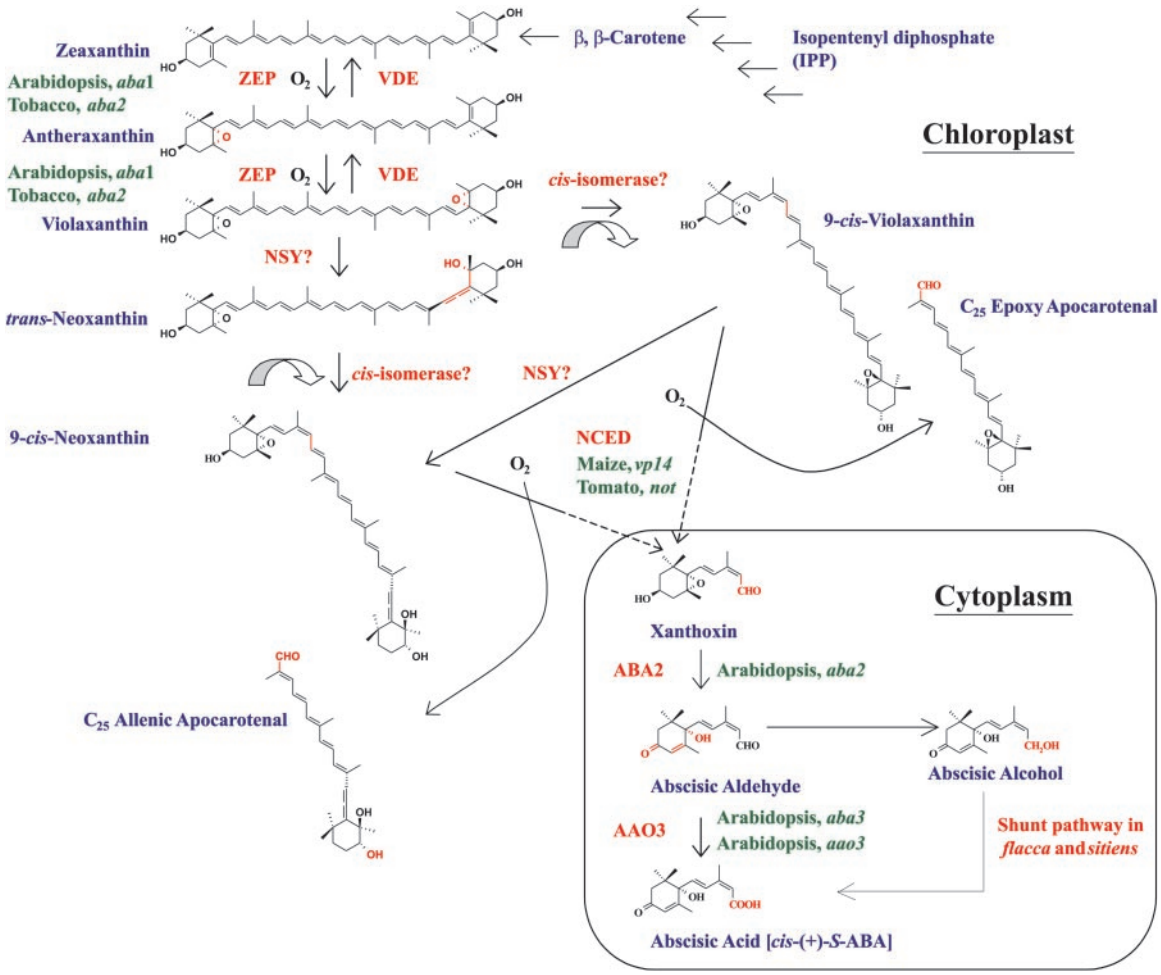

Figure 1. The pathway of ABA synthesis beginning with zeaxanthin. The steps in isoprenoid and carotenoid synthesis before zeaxanthin are reviewed elsewhere (Cunningham and Gantt, 1998; Rohmer, 1999; Hirschberg, 2001; Rodríguez-Concepción and Boronat, 2002). VDE, Violaxanthin de-epoxidase.

An epoxidase mutant in *N. plumbaginifolia*, *npzep*, has been identified and the corresponding gene cloned (Marin et al., 1996). The NpZEP protein, which is similar to some bacterial monooxygenases, was able to catalyze the epoxidation of zeaxanthin to antheraxanthin and violaxanthin. For this activity, it was necessary to add an additional component from chloroplasts. It was later determined that reduced ferredoxin is a necessary cofactor (Bouvier et al., 1996).

Because the level of epoxycarotenoids in green leaves is high relative to the amount of ABA synthesized, it is considered unlikely that ZEP has a regulatory role in these tissues. The expression of *ZEP* transcripts in green tissue does not increase in wild tobacco (*Nicotiana plumbaginifolia*; Audran et al., 1998), tomato (Thompson et al., 2000a), or cowpea (*Vigna unguiculata*; Iuchi et al., 2000) that are subjected to osmotic stress. In etiolated tissues, the concentration of carotenoids is significantly lower and the increased expression of *ZEP* mRNA does correlate with elevated ABA synthesis in roots and seeds (Audran et al., 1998; Borel et al., 2001). The overexpression of *ZEP* in transgenic tobacco resulted in increased seed dormancy (Frey et al., 1999), thus providing further evidence that the level of epoxycarotenoids limits ABA synthesis in some tissues.

The Arabidopsis epoxidase, *AtZEP*, was cloned by sequence similarity to the tobacco *ZEP* (Audran et al., 2001; Xiong et al., 2002). Contrasting reports on the expression of *AtZEP* mRNA have appeared in the literature. In one study, it was found that the level of *AtZEP* mRNA was induced by drought stress in root tissues (Audran et al., 2001). The expression of the *AtZEP* transcript was unaffected in several ABA-deficient and -insensitive mutants (Audran et al., 2001). In another study, it was reported that the expression of *AtZEP* mRNA increased in response to osmotic stress or ABA treatment in both roots and shoots (Xiong et al., 2002). The osmotic induction of *AtZEP* transcript was impaired in ABA-deficient mutants and in the ABA-insensitive mutant, *abi1*. Several additional genes necessary for the later steps in ABA synthesis also were found to be induced by

Table I. *ABA biosynthesis mutants with position of the lesions in the pathway and functions of the wild-type proteins*

Mutant	Species	Biochemical Step	Enzyme	References
aba1/npq2/los6[a]	Arabidopsis	Epoxidation of zeaxanthin	Zeaxanthin epoxidase (ZEP)	Karssen et al. (1983); Rock and Zeevaart (1991a); Niyogi et al. (1998); Xiong et al. (2002)
aba2	*Nicotiana plumbaginifolia*	Epoxidation of zeaxanthin	ZEP	Marin et al. (1996)
osaba1	Rice (*Oryza sativa*)	Epoxidation of zeaxanthin	ZEP	Agrawal et al. (2001)
vp14	Maize (*Zea mays*)	Oxidative cleavage of neoxanthin or violaxanthin	NCED	Schwartz et al. (1997b); Tan et al. (1997)
not (*notabilis*)	Tomato (*Lycopersicon esculentum*)	Oxidative cleavage of neoxanthin or violaxanthin	NCED	Burbidge et al. (1999)
aba2/gin1/isi4/sis4	Arabidopsis	Oxidation of xanthoxin to abscisic aldehyde	Short-chain alcohol dehydrogenase	Léon-Kloosterziel et al. (1996); Schwartz et al. (1997a); Laby et al., 2000; Rook et al. (2001); Cheng et al. (2002)
flc	Tomato	Oxidation of abscisic aldehyde to ABA	Molybdenum cofactor (MoCo) sulfurase	Sagi et al. (2002)
aba3/los5/gin5	Arabidopsis	Oxidation of abscisic aldehyde to ABA	MoCo sulfurase	Léon-Kloosterziel et al., (1996); Schwartz et al. (1997a); Xiong et al. (2001); Cheng et al. (2002)
sit	Tomato	Oxidation of abscisic aldehyde to ABA	Aldehyde oxidase	Okamoto et al. (2002)
aba1	*N. plumbaginifolia*	Oxidation of abscisic aldehyde to ABA	MoCo sulfurase?	Akaba et al. (1998)
nar2A	Barley (*Hordeum vulgare*)	Oxidation of abscisic aldehyde to ABA	MoCo synthesis	Walker-Simmons et al. (1989)
aao3	Arabidopsis	Oxidation of abscisic aldehyde to ABA	Abscisic aldehyde oxidase (AAO3)	Seo et al. (2000b)

[a]In several cases, allelic mutants that were isolated by different laboratories have been assigned different gene symbols.

stress and ABA (Xiong et al., 2001, 2002). The authors suggested that ABA synthesis might be subject to positive feedback regulation.

The significance of *ZEP* up-regulation in green leaves is uncertain. The $^{18}O_2$-labeling experiments, which were instrumental in establishing the indirect pathway of ABA synthesis, also provide some indication of flux through the pathway. The 1′-hydroxyl in ABA is derived from the epoxide in the carotenoid precursor. In $^{18}O_2$-labeling experiments, there is little incorporation of ^{18}O at this position for time points less than 8 h (Zeevaart et al., 1989). Therefore, de novo synthesis of epoxycarotenoids appears to be unnecessary for ABA synthesis in leaves.

THE CLEAVAGE REACTION

The first committed step in ABA synthesis is the oxidative cleavage of a 9-cis-epoxycarotenoid. For many years, the pathway of ABA synthesis had been a point of contention because of difficulties in demonstrating this activity in vitro. This problem was eventually resolved by the identification and characterization of an ABA-deficient mutant in maize, *vp14* (*viviparous 14*). Biochemical characterization of *vp14* indicated that there was no lesion in carotenoid synthesis or in the later steps of ABA synthesis (Tan et al., 1997). By the process of elimination, it appeared that *vp14* was impaired in the cleavage reaction.

The *vp14* mutant resulted from a transposon insertion, which allowed the corresponding gene to be cloned (Tan et al., 1997). At the time the *Vp14* gene was identified, the deduced amino acid sequence was most similar to lignostilbene dioxygenases (LSDs) from *Pseudomonas* (*Sphingomonas*) *paucimobilis*. The LSDs catalyze a double-bond cleavage reaction (Kamoda and Samejima, 1991) that is very similar to the cleavage reaction in ABA synthesis. The recombinant VP14 protein was able to cleave 9-cis-neoxanthin and 9-cis-violaxanthin to form xanthoxin and a C_{25} by-product (Schwartz et al., 1997b). The characteristics of the cleavage reaction in its substrate specificity and the site of cleavage (11–12 position) were consistent with predictions. A 9-cis double bond in the carotenoid precursor was necessary for activity. The product of this cleavage reaction is cis-xanthoxin, which is readily converted to ABA [cis-(+)-*S*-ABA] by plants. Cleavage of an all trans-isomer would result in trans-xanthoxin, which is converted to biologically inactive trans-ABA.

Additional ABA synthetic cleavage enzymes have been identified and characterized in a variety of plant species (Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2000, 2001). The recombinant enzymes from these species display the same substrate specificity as VP14. The nomenclature that has been adopted for these enzymes is NCEDs,

which is consistent with either 9-cis-violaxanthin or 9-cis-neoxanthin as a substrate.

Although the NCEDs display significant substrate plasticity in vitro, circumstantial evidence favors neoxanthin as the primary precursor of ABA. Neoxanthin exists almost entirely as a 9-cis-isomer, whereas only a small proportion of the violaxanthin is present as a 9-cis-isomer (Strand et al., 2000). In addition, the K_m for the recombinant PvNCED1 and VP14 is lower with neoxanthin as substrate relative to 9-cis-violaxanthin (Qin and Zeevaart, 1999; Schwartz et al., 2003). Definitive evidence of the endogenous substrate would require identification of the C_{25} by-product in planta. Previous efforts to identify the C_{25} compounds in vegetative tissue have been unsuccessful. It has been suggested that these compounds are rapidly degraded after the cleavage reaction (Parry and Horgan, 1991). The C_{25}-epoxy-apocarotenal and related compounds have been identified in fruits that produce high levels of ABA during ripening (Molnár and Szabolics, 1980; Gross and Eckhardt, 1981; see also Parry and Horgan, 1991).

Carotenoids in plants are synthesized within plastids and are associated with the thylakoid and envelope membranes. Therefore, it was expected that the cleavage reaction would also occur in chloroplasts. The PvNCED1 from bean (*Phaseolus vulgaris*) was imported into pea (*Pisum sativum*) chloroplasts, where it was found to associate exclusively with the thylakoid membrane (Qin and Zeevaart, 1999). An N-terminal targeting sequence from a cowpea enzyme, VuNCED1, was capable of targeting the green fluorescent protein to chloroplasts (Iuchi et al., 2000). After in vitro import assays, the VP14 protein was found in the stroma and on the thylakoid membrane exposed to the stroma (Tan et al., 2001). In this study, deletion or disruption of a putative amphipathic-helix in the N terminus of VP14 interfered with the association of VP14 with thylakoids. The binding of VP14 to the thylakoid was saturable, suggesting that it associates with specific components in the thylakoid membrane that have not yet been identified.

The *not* mutant in tomato also is impaired in the cleavage step (Burbidge et al., 1999). Both the *vp14* and *not* mutants have weak phenotypes relative to other ABA-deficient mutants. The *vp14* null mutant shows only a 35% reduction of ABA levels in stressed leaves and a 70% reduction in developing embryos (Tan et al., 1997). This indicates that there are multiple NCEDs involved in ABA synthesis. In avocado (*Persea americana*), *PaNCED1* and *PaNCED3* were both shown to encode ABA biosynthetic enzymes (Chernys and Zeevaart, 2000).

In the Arabidopsis genome, there are nine hypothetical proteins that share sequence similarity to NCEDs (Fig. 2). In a phylogenetic analysis of the NCEDs and other similar proteins, five of the Arabidopsis proteins are clustered with previously characterized NCEDs. It has been reported that AtNCED2, 3, 6, and 9 are able to catalyze the cleavage reaction in ABA synthesis (Iuchi et al., 2001). Based upon sequence similarity, it is expected that AtNCED5 is also an ABA synthetic enzyme. However, the biochemical role of AtNCED5 has not been verified experimentally.

Presumably, the different NCEDs in Arabidopsis are expressed in different tissues and at different developmental stages. The *AtNCED3* transcript is induced by water stress and reduced expression results in a wilty phenotype (Iuchi et al., 2001), indicating that this gene is an important regulator of ABA levels during water stress. The expression of *AtNCED9* also is elevated slightly in response to water stress (Iuchi et al., 2001). The expression pattern and physiological role of the other *AtNCED* genes has not been reported yet. One or more of these genes is expected to display elevated expression during seed development when ABA begins to accumulate.

SYNTHESIS OF APOCAROTENOIDS

A variety of natural products like ABA are derived from the oxidative cleavage of carotenoids. Collectively referred to as apocarotenoids, these compounds serve important functions in a range of organisms. Retinal is a chromophore used for phototaxis in green algae (Nagel et al., 2002; Sineshchekov et al., 2002) and for light-driven proton pumping in *Halobacterium* NRC-1. (Kolbe et al., 2000).

In animals, vitamin A is necessary for normal vision and development. Based on sequence similarity to NCEDs, a vitamin A biosynthetic enzyme was identified in fruitfly (von Lintig and Vogt, 2000) and subsequently in other species (Wyss et al., 2000; Redmond et al., 2001; Lindqvist and Andersson, 2002). Hypothetical proteins that are similar to NCEDs are also present in a variety of prokaryotes (Fig. 2). The biochemical and biological roles of the putative cleavage enzymes in prokaryotes have not been reported.

All of the proteins in the alignment (Fig. 3) have been shown to catalyze a double-bond cleavage reaction. With the exception of SpLSD, the substrates are carotenoids. The N terminus of the ZmVP14, which does not align well with the other proteins, contains a chloroplast-targeting sequence of approximately 45 amino acids (Tan et al., 2001). The AtCCD7 protein also contains a predicted chloroplast-targeting sequence. The specific functions for conserved residues have not been yet been defined. There are several highly conserved His and acidic residues that may be necessary for coordinating iron in the active site.

There are four hypothetical proteins in Arabidopsis that share some degree of sequence similarity with the NCEDs, but are not thought to be involved in ABA synthesis (Fig. 2). Recent findings demonstrate

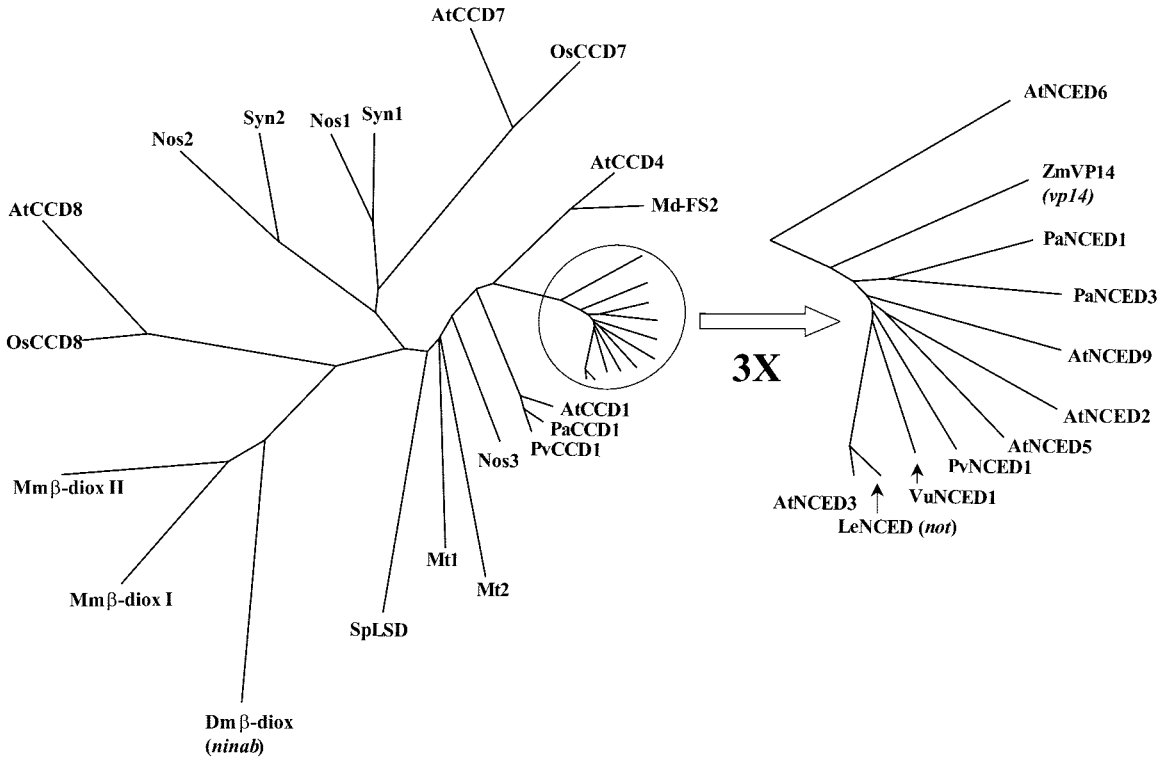

Figure 2. Unrooted phylogenetic tree of NCEDs, carotenoid cleavage dioxygenases (CCDs), and related proteins. The proteins that have been demonstrated to have NCED activity branch together with other likely NCEDs. Hypothetical proteins from plants that are not involved in ABA synthesis are referred to as CCDs. Alignments were performed with ClustalW and displayed with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). AtCCD1, NP_191911.1; AtCCD4, NP_193652.1; AtCCD7, NP_182026; AtCCD8, NP_195007; AtNCED2, NP_193569.1; AtNCED3, NP_188062.1; AtNCED5, NP_174302.1; AtNCED6, NP_189064; AtNCED9, NP_177960; LeNCED, CAB10168; Md-FS2, CAB07784; OsCCD7, nucleotide sequence 61,958 to 59,544; OsCCD8, BAB63485; PaCCD1, AAK00622.1; PaNCED1, AAK00632.1; PaNCED3, AAK00623; PvCCD1, AAK38744; PvNCED1, AAF26356.1; VuNCED1, BAB11932.1; ZmVP14, AAB62181; Dm β-diox, CAB93141; Mm β-diox I, AAG33982; Mm β-diox II, CAC28026; Mt1, CAB09380; Mt2, CAB08511; Nos1, BAB75983; Nos2, BAB76594; Nos3, BAB73063; SpLSD, AAB35856.2; Syn1, BAA18428; Syn2, BAA18465. At, Arabidopsis; Le, tomato; Md-FS2, *Malus* × *domestica*; Os, rice; Pa, avocado; Pv, bean; Vu, cowpea; Zm, maize; Dm, fruitfly (*Drosophila melanogaster*); Mm, *Mus musculus*; Mt, *Mycobacterium tuberculosis* H37Rv; Nos, *Nostoc* sp. PCC 7120; Sp, *S. paucimobilis*; Syn, *Synechocystis* sp. PCC 6803.

that at least two of these proteins are able to catalyze carotenoid cleavage reactions. To distinguish these enzymes from the NCEDs, the nomenclature of CCDs has been adopted.

The AtCCD1 protein and a likely ortholog in bean catalyze a symmetric 9-10 (9′-10′) cleavage with several different carotenoids (Schwartz et al., 2001). A variety of volatile and semivolatile compounds, such as the ionones and β-damascenone, are derived from 9-10 cleavage reactions. These compounds, which are often produced in flowers and fruits, are believed to serve as attractants for pollination and seed dispersal. Several products derived from 9-10, (9′-10′) cleavage reactions also accumulate in the roots of plants inoculated with arbuscular mycorrhizal fungi (Walter et al., 2000). The function of these compounds in mycorrhizae has not been determined yet. Recently, a 7-8 (7′-8′) CCD has been cloned from *Crocus sativus*, which specifically catalyzes the synthesis of crocetin dialdehyde (C_{20}) and hydroxy-β-cyclocitral (C_{10}) from zeaxanthin (Bouvier et al., 2003). Crocetin dialdehyde is a precursor of crocin, the primary pigment in saffron.

The most disparate members of this enzyme family in Arabidopsis are AtCCD7 and AtCCD8. The recombinant AtCCD7 protein is able to cleave β,β-carotene at the 9-10 position (S.H. Schwartz and J.A.D. Zeevaart, unpublished data). The *AtCCD8* gene corresponds to the *max4* mutant in Arabidopsis, which exhibits extensive lateral shoot growth. The *CCD8/MAX4* gene in Arabidopsis is orthologous to the *RMS1* gene in pea (O. Leyser, personal communication). Grafting experiments indicate that the *rms1* mutant is impaired in the synthesis of a signal molecule that originates in the wild-type rootstock and inhibits outgrowth of lateral buds on the mutant scion (Beveridge et al., 2000). There is also evidence for a similar signaling molecule in Arabidopsis (Turnbull et al., 2002). The biochemical function and role of AtCCD8 in this process have not been determined yet.

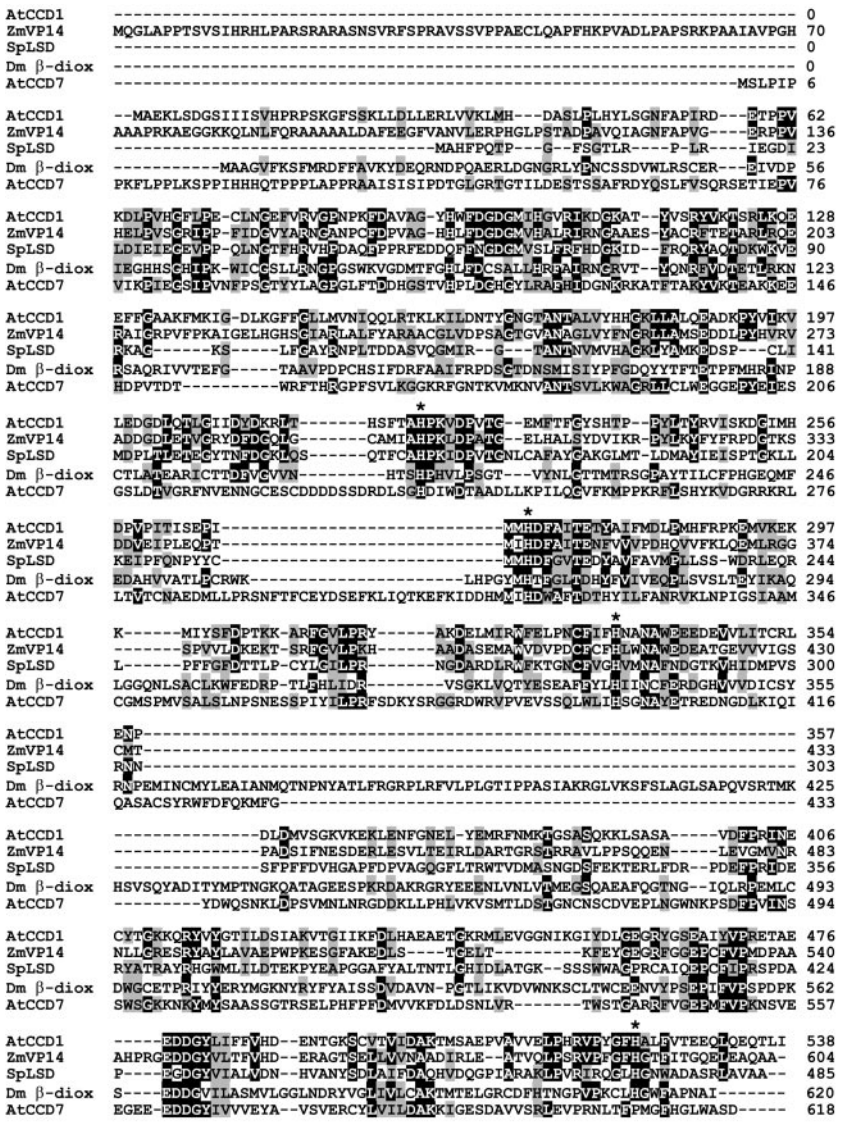

Figure 3. An alignment of several carotenoid cleavage enzymes with ClustalW. Residues that are identical in four or more of the proteins are shaded in black. Similar residues are shaded in gray. GenBank accession numbers and species names can be found in the legend of Figure 2. Conserved His residues are indicated with asterisks.

THE LATER STEPS. CONVERSION OF XANTHOXIN TO ABA

In contrast to the cleavage reaction, the later steps in ABA synthesis have been characterized extensively by feeding potential intermediates to intact plants and cell-free extracts (Sindhu and Walton, 1987). By this approach, the sequence of reactions subsequent to cleavage has been determined. The first steps are the oxidation of the 4′-hydroxyl to a ketone followed by the nonenzymatic desaturation of 2′-3′ bond and opening of the epoxide ring. The final step in the pathway is the oxidation of abscisic aldehyde to ABA.

The *aba2* mutant in Arabidopsis was first identified by screening for the ability to germinate in the presence of the GA biosynthetic inhibitor, paclobutrazol (Léon-Kloosterziel et al., 1996). By feeding potential intermediates to extracts of the *aba2* mutant, it was determined that this mutant was impaired in the conversion of xanthoxin to abscisic aldehyde (Schwartz et al., 1997a). The *aba2* is the only mutant identified to date that is blocked at this step in the pathway. Additional alleles of *aba2* have since been identified in screens for a sugar-insensitive phenotype (Laby et al., 2000; Cheng et al., 2002; see Table I), altered stomatal conductance (Merlot et al., 2002), and germination and growth on a medium containing a high NaCl concentration (González-Guzmán et al., 2002). The gene corresponding to *aba2* has recently been cloned and the gene product was found to be similar to short chain dehydrogenases/reductases (Cheng et al., 2002; González-Guzmán et al., 2002). As expected, the ABA2 protein was able to catalyze the conversion of xanthoxin to abscisic aldehyde utilizing NAD as a cofactor. The *ABA2* transcript level was not affected by stress (González-Guzmán et al., 2002) but was induced by Glc (Cheng et al., 2002). It has not yet been reported whether the

overexpression of *ABA2* would have any effect on ABA levels.

Mutants impaired in the final step of ABA synthesis, the oxidation of abscisic aldehyde to ABA, have been identified in a variety of plants. A loss of this abscisic aldehyde oxidase activity may result from a mutation in the aldehyde oxidase apoprotein or a lesion in the synthesis of a MoCo that the enzyme requires for activity. A lesion in an early step of MoCo synthesis would affect a number of activities. For example, the *nar2a* mutant in barley lacks aldehyde oxidase, xanthine dehydrogenase, and nitrate reductase activities (Walker-Simmons et al., 1989). The *aba3* mutant in Arabidopsis and the *flacca* mutant in tomato lack aldehyde oxidase activity, but the activity of nitrate reductase is unaffected. This phenotype results from a defect in the formation of a desulfo moiety of the MoCo that is specifically required by certain hydroxylases (Schwartz et al., 1997a; Akaba et al., 1998; Sagi et al., 1999). The gene corresponding to *aba3* has been cloned and the N terminus of the deduced protein is similar to the NifS sulfurase (Bittner et al., 2001; Xiong et al., 2001). Using Cys as a sulfur donor, the recombinant protein was able to activate aldehyde oxidase activity (Bittner et al., 2001). The *flacca* mutant also results from a mutation in this sulfurase (Sagi et al., 1999, 2002). It was found that *ABA3* expression increased in response to osmotic stress or ABA (Bittner et al., 2001; Xiong et al., 2001).

Four abscisic aldehyde oxidase (AAO) genes have been identified in Arabidopsis (*AAO1* through *4*). Of the aldehyde oxidases characterized so far, only AAO3 uses abscisic aldehyde efficiently as a substrate (Seo et al., 2000a). A wilty, ABA-deficient mutant with a lesion in *AAO3* has been identified (Seo et al., 2000b), demonstrating that AAO3 is responsible for ABA synthesis in vegetative tissues. In contrast to *aba3*, the *aao3* mutants are not subject to precocious germination. Therefore, another aldehyde oxidase appears to be necessary for ABA synthesis in some tissues. In plants subjected to dehydration, *AAO3* mRNA expression was elevated. However, the level of the corresponding protein was unaffected by water stress (Seo et al., 2000a). In addition, feeding experiments and assays with cell-free preparations indicate that the conversion of xanthoxin to ABA is unaffected by stress (Sindhu and Walton, 1987; Schwartz et al., 1997a).

Several variations in the later steps of the pathway may be responsible for a small portion of ABA synthesis. It has been suggested that oxidation of the aldehyde may occur subsequent to cleavage and before the ring modifications (Cowan, 2000), indicating that xanthoxic acid would be an intermediate in the pathway. However, the conversion of xanthoxic acid is very low in cell-free extracts (Sindhu and Walton, 1987). Also, the ABA2 protein is unable to convert xanthoxic acid to ABA (Cheng et al., 2002). Both the *flacca* and *sitiens* mutants in tomato are blocked in the final step of the pathway, the oxidation of abscisic aldehyde to ABA, and accumulate 2-trans-ABA alcohol (Linforth et al., 1987). These mutants are able to synthesize some ABA by a shunt pathway in which abscisic alcohol is oxidized to ABA (Rock et al., 1991b).

TRANSGENIC PLANTS WITH ELEVATED ABA LEVELS

Inhibitors of transcription and translation block stress-induced ABA accumulation (Quarrie and Lister, 1984; Guerrero and Mullet, 1986), indicating gene expression is up-regulated for one or more steps in ABA synthesis. The genes encoding most of the enzymes in the ABA biosynthetic pathway have now been identified. Based upon elevated expression during stress, a regulatory role has been proposed for several of the genes (Audran et al., 1998, 2001; Seo et al., 2000b; Bittner et al., 2001; Xiong et al., 2001, 2002).

In etiolated tissues, the levels of epoxycarotenoids are low and it appears that elevated expression of ZEP may be important for ABA synthesis (Frey et al., 1999). In green tissue, however, most of the biochemical evidence indicates that the NCED-catalyzed cleavage reaction is the primary regulatory step in ABA synthesis. In all instances studied to date, stress-induced ABA accumulation correlates well with increased expression of *NCED* mRNA (Tan et al., 1997; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2000, 2001; Thompson et al., 2000a) and also with NCED protein levels (Qin and Zeevaart, 1999). The expression of *PaNCED1* and *PaNCED3* also increased before the accumulation of high ABA levels during fruit ripening in avocado (Chernys and Zeevaart, 2000).

The overexpression of *NCEDs* is sufficient for elevated ABA synthesis. Overexpression of the *LeNCED1* in tomato (Thompson et al., 2000b), *PvNCED1* in tobacco (Qin and Zeevaart, 2002), and *AtNCED3* in Arabidopsis (Iuchi et al., 2001) resulted in increased ABA levels. In *N. plumbaginifolia*, induced expression of *PvNCED1* resulted in decreased stomatal conductance and increased stress tolerance (Fig. 4). Similar results were obtained with the other species listed above. For the overexpression of *LeNCED1* (Thompson et al., 2000b) and *PvNCED1* (Qin and Zeevaart, 2002), increased seed dormancy was also reported.

CATABOLISM OF ABA

The level of ABA in plants is controlled not only by its synthesis, but also through its catabolism. One of the primary catabolites of ABA is phaseic acid (PA). The conversion of ABA to PA begins with the hydroxylation of the 8′ position by ABA 8′-hydroxylase. The 8′ hydroxyl appears to be an unstable intermediate that spontaneously rearranges to form

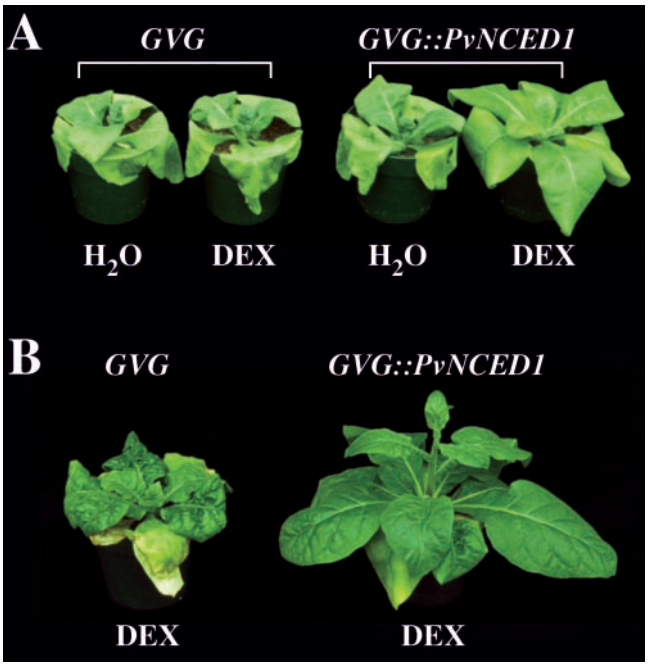

Figure 4. Enhanced drought tolerance and recovery of *N. plumbaginifolia* plants overexpressing *PvNCED1*. A, Homozygous plants transformed with vector only (*GVG*) or with *PvNCED1* gene under control of a dexamethasone-inducible promoter *GVG* 7 d after spraying with water or 30 μM dexamethasone (DEX), and without watering. B, Difference in recovery of control and plant overexpressing *PvNCED1* 9 d after daily watering was resumed.

PA. The ABA 8′-hydroxylase is a cytochrome P450 (Krochko et al., 1998), which may be induced by ABA (Windsor and Zeevaart, 1997). This negative feedback regulation is consistent with time course measurements of ABA and PA accumulation in stressed plants (Zeevaart, 1980) and recent work with *NCED* overexpression in plants (Qin and Zeevaart, 2002). ABA may also be inactivated by the formation of ABA Glc ester in some tissues. An ABA glucosyltransferase gene from adzuki bean (*Vigna angularis*) has been cloned recently (Xu et al., 2002). Interestingly, this gene is also up-regulated by ABA. The physiological significance of ABA Glc ester formation and the potential for engineering ABA levels by decreased glucosylation may now be investigated.

FUTURE DIRECTIONS

There are several steps in ABA biosynthesis preceding the cleavage reaction that are not well characterized. The epoxycarotenoid precursor must have a 9-cis configuration to be cleaved by an NCED and for subsequent conversion to ABA [cis-(+)-*S*-ABA]. The formation of these 9-cis isomers has not yet been established. An enzyme that catalyzes a similar reaction, the cis/trans isomerization of prolycopene to lycopene, has recently been identified (Isaacson et al., 2002; Park et al., 2002). This isomerase appears to be necessary only in non-photosynthetic tissue. In light-grown tissue, photo-isomerization of lycopene is sufficient. It has not been established whether the 9-cis isomerization of neoxanthin and violaxanthin is an enzymatic reaction. Alternatively, the 9-cis conformations of some epoxycarotenoids could be stabilized by carotenoid-binding proteins.

In most plant tissues, neoxanthin is the predominant carotenoid with a 9-cis conformation and is considered the most likely precursor of ABA (Strand et al., 2000). Neoxanthin is derived through the opening of an epoxy ring in violaxanthin followed by an intramolecular rearrangement to form an allenic bond. Allenic carotenoids, such as neoxanthin, are among the most abundant carotenoids in nature. Therefore, an understanding of their synthesis and functions in photosynthetic organisms is of considerable interest. Two genes that encode neoxanthin synthases (NSY) have been identified in potato (*Solanum tuberosum*) and tomato (Al-Babili et al., 2000; Bouvier et al., 2000). The NSY gene products are similar to lycopene cyclases from various plants and a capsanthin-capsorubin synthase from pepper (*Capsicum annuum*). Transient expression in tobacco and in vitro assays both demonstrated that the tomato NSY was capable of converting violaxanthin to neoxanthin (Bouvier et al., 2000). No lycopene cyclase activity was found by co-expression in a lycopene-accumulating strain of *Escherichia coli* (Bouvier et al., 2000). However, the *NSY* gene corresponds to the *old-gold* mutant in tomato, which accumulates higher levels of lycopene due to the loss of a fruit-specific lycopene β-cyclase, CYC-B (Ronen et al., 2000; Hirschberg, 2001). It has been suggested that the NSY is a bifunctional enzyme capable of converting lycopene to β,β-carotene or violaxanthin to neoxanthin (Hirschberg, 2001). Presumably, there is an additional gene responsible for neoxanthin synthesis in plants, because the *old-gold* mutant is able to produce neoxanthin. Moreover, no ortholog of the *NSY* gene is apparent in the Arabidopsis genome.

The oxidative cleavage products of carotenoids serve important roles in both plants and animals. Based upon sequence similarity to NCEDs, putative cleavage enzymes have been identified in a number of plants and prokaryotes. The characterization of CCDs in plants suggests that apocarotenoids have various roles in growth and development. The synthesis of apocarotenoids is well documented in cyanobacteria and a carotenoid cleavage activity has been described in the cyanobacterium *Microcystis* PCC7806 (Jüttner and Höflacher, 1985). However, the biological functions of these compounds in cyanobacteria and other prokaryotes have not yet been determined.

Despite the important roles that apocarotenoids serve in various organisms and the growing number of putative cleavage enzymes appearing in the sequence databases, little is known about the mecha-

nism by which these enzymes catalyze reactions. In an isotopic labeling experiment with β,β-carotene 15, 15′-dioxygenase from chicken (*Gallus gallus*), approximately 50% of the cleavage products contained oxygen derived from O_2 (Leuenberger et al., 2001). In this experiment, the second oxygen was derived from water, and the authors proposed a monooxygenase mechanism. However, no reducing equivalents are required for assays with any of the recombinant enzymes that have been characterized. In addition, $^{18}O_2$-labeling experiments with plants indicate that the initial cleavage product in ABA synthesis, xanthoxin, results entirely from O_2 (Zeevaart et al., 1989). At this point, the mechanism by which these enzymes catalyze reactions is still uncertain.

The biochemical aspects of ABA synthesis, such as the intermediates in the pathway and the sequence of reactions, have become well established. The genes that encode most of the enzymes in the pathway have now been cloned. Although elevated expression in response to osmotic stress has been reported for several of these genes, the significance of this upregulation is still uncertain. Previous biochemical studies and the recent work with transgenic plants clearly demonstrate that transcriptional regulation of the *NCEDs* is the major control point in ABA synthesis. The initial perception of stress and the signal transduction pathway leading to elevated *NCED* expression remain to be elucidated.

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