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Control of abscisic acid synthesis

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Abstract

The abscisic acid (ABA) biosynthetic pathway involves the formation of a 9-*cis*-epoxycarotenoid precursor. Oxidative cleavage then results in the formation of xanthoxin, which is subsequently converted to ABA. A number of steps in the pathway may control ABA synthesis, but particular attention has been given to the enzyme involved in the oxidative cleavage reaction, i.e. 9-*cis*-epoxycarotenoid dioxygenase (NCED). Cloning of a gene encoding this enzyme in maize was first reported in 1997. Mapping and DNA sequencing studies indicated that a wilty tomato mutant was due to a deletion in the gene encoding an enzyme with a very similar amino acid sequence to this maize NCED. The potential use of this gene in altering ABA content will be discussed together with other genes encoding ABA biosynthetic enzymes.

Key words: Abscisic acid biosynthesis, mutants, cloning, plant stress, over-expression.

Introduction

Abscisic acid (ABA) is a phytohormone which performs several specific functions in plant growth and development. There is now good evidence that ABA plays a role in seed development and primary dormancy (Frey *et al.*, 1999). In addition, ABA levels in vegetative tissues can be elevated in response to various environmental stresses; most notably drought. For these reasons ABA is sometimes referred to as a stress hormone (Zeevaart, 1999).

Knowledge of the ABA biosynthetic pathway, combined with the cloning of genes directly involved in biosynthesis (Marin *et al.*, 1996; Tan *et al.*, 1997), have made the prospects of manipulating ABA levels in plants a realistic objective. Using antisense technology, it is now possible to investigate symptoms of ABA deficiency in species which are important from a physiological and/or economic viewpoint, but which do not have any conventional ABA-deficient mutants. It now also appears to be possible to investigate the effects of ABA over-production, using constructs designed to over-express ABA biosynthetic genes.

In this review, important advances made in our understanding of the control of ABA biosynthesis will be discussed in relation to recent attempts to alter ABA concentrations in transgenic plants.

The ABA biosynthetic pathway

The general framework of the ABA biosynthetic pathway in plants is now well established. ABA synthesis represents a minor branch of the carotenoid pathway; as originally indicated by a number of pioneering experiments (Taylor and Burden, 1973). It has only recently become clear that the isopentenyl pyrophosphate (IPP) C_5 ‘building blocks’ from which ABA is formed, are themselves synthesized from glyceraldehyde phosphate and pyruvate inside plastids (Arigoni *et al.*, 1997; Lichtenthaler *et al.*, 1997).

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Abbreviations: ABA, abscisic acid; ABAld, abscisic aldehyde; ABAO, abscisic aldehyde oxidase; AO, aldehyde oxidase; AX, antheraxanthin; CaMV, cauliflower mosaic virus; DAP, days after pollination; EST, expressed sequence tag; IPP, isopentenyl pyrophosphate; LHC, light harvesting complex; LSD, lignostilbene-α,β-dioxygenase; MoCo, molybdenum cofactor; MPT, molybdopterin; Mu, mutator transposon; NCE, 9-*cis*-epoxycarotenoid; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NR, nitrate reductase; NX, neoxanthin; ORF, open reading frame; PCR, polymerase chain reaction; TAO, tomato aldehyde oxidase; VX, violaxanthin; XAN, xanthoxin or xanthoxal; XDH, xanthine dehydrogenase; ZEP, zeaxanthin epoxidase; ZX, zeaxanthin.

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It is therefore important to note that the ultimate precursor of ABA is not mevalonic acid. This represents a major revision of the information given in many earlier reviews (Milborrow, 1974); as has been pointed out more recently by a number of authors (Milborrow and Lee, 1998).

The genes and enzymes involved in the assembly of the C_{40} compound β-carotene, from IPP precursor molecules, have been discussed in a recent comprehensive review (Cunningham and Gantt, 1998). The two rings of β-carotene can be hydroxylated to form a xanthophyll known as zeaxanthin (Fig. 1). The ABA biosynthetic pathway can in some ways be regarded as starting from this xanthophyll (Taylor, 1991) since mutants blocked downstream of zeaxanthin show symptoms typical of ABA, rather than carotenoid, deficiency.

Important evidence relating to the validity of the pathway shown in Fig. 1 has been provided by a number of labelling studies (involving $^{18}O_2$, $^{2}H_2O$, $^{14}CO_2$, ^{14}C-xanthoxin, ^{13}C-xanthoxin, ^{2}H-ABA aldehyde etc), by stoichiometric experiments involving dark-grown tissues and by the use of cell-free biosynthesis systems. Endogenous ABA levels can be reduced using the carotenoid biosynthesis inhibitors, norflurazon and fluridone. However, the virtually white seedlings which can be obtained following norflurazon and fluridone treatments are deficient in a whole range of metabolic processes resulting from the photobleaching of chlorophyll. Against this background, the phenotypic effects of ABA deficiency seem trivial and ABA is not the limiting factor preventing continued growth. Comprehensive information on these important experiments has been provided recently by three excellent reviews (Cutler and Krochko, 1999; Liontenberg *et al.*, 1999; Zeevaart, 1999) and therefore they are not described further in this review.

The characterization of ABA-deficient mutants impaired at various points in the pathway, has provided very strong evidence that the biosynthetic steps shown in Fig. 1 are mainly correct; although some ambiguities persist and some evidence appears to indicate the existence of alternative/substitute routes (see Cutler and Krochko, 1999, for some discussion on this). Unfortunately, no ABA-deficient mutant plants have yet been shown to be impaired in the various xanthophyll isomerization steps taking place at the C_{40} level of the ABA pathway (Fig. 1). For this reason, considerable uncertainty remains concerning the precise route(s) followed prior to the formation of the first C_{15} intermediate, xanthoxin. None of the following *in vivo* alternatives can be formally ruled out:-

(A) Selective oxidative cleavage (defined by the proposal that only *one* of the two 9-*cis*-xanthophylls detected in plant tissues can be oxidatively cleaved to form the first C_{15} ABA biosynthetic intermediate, xanthoxin). This could involve one or more of the following three routes:-

Route A1: selective cleavage of 9-*cis*-violaxanthin synthesized directly by a single isomerization step from all-*trans*-violaxanthin.

Route A2: selective cleavage of 9′-*cis*-neoxanthin synthesized from all-*trans*-violaxanthin in two isomerization steps via 9-*cis*-violaxanthin.

Route A3: selective cleavage of 9′-*cis*-neoxanthin synthesized from all-*trans*-violaxanthin in two isomerization steps via all-*trans*-neoxanthin.

(B) Non-selective oxidative cleavage (defined by the proposal that *either* of the two 9-*cis*-xanthophylls detected in plant tissues can be oxidatively cleaved to form the first C_{15} ABA biosynthetic intermediate, xanthoxin). This could involve one or more of the following two routes:-

Route B1: all-*trans*-violaxanthin is first converted to 9-*cis*-violaxanthin, most of which is further isomerized to form 9′-*cis*-neoxanthin. Either of these two 9-*cis*-xanthophylls could be used indiscriminately as a substrate for oxidative cleavage.

Route B2: all-*trans*-violaxanthin is simultaneously subjected to two alternative isomerizations, leading to the formation of 9-*cis*-violaxanthin and all-*trans*-neoxanthin. Only the latter would be further isomerized to form 9′-*cis*-neoxanthin. Either of these two 9-*cis*-xanthophylls could be used indiscriminately as a substrate for oxidative cleavage.

At present there is no clear experimental evidence available to demonstrate which of these alternative routes occurs *in planta*. It is important for this uncertainty to be resolved. Several of the other steps in ABA biosynthesis have been characterized using ABA-deficient mutants obtained in a number of different plant species.

The location of genetic lesions in the ABA biosynthetic pathway

ABA-deficient mutants have been obtained in both monocotyledonous and dicotyledonous species. Various mutants have been listed in Table 1 together with a *Chlamydomonas* mutant affecting xanthophyll isomerization which blocks the formation of the allene (neoxanthin). To date no mutants have been identified which impair the isomerization of all-*trans*-xanthophylls to form the 9-*cis*-xanthophylls which are important ABA precursors. Characterization of mutants at this isomerization step would be likely to end some of the uncertainty which continues to surround this part of the pathway (see the previous section and also Fig. 1).

The plant genetic lesions included in Table 1 all result in a wilty phenotype, which is more severe in some cases than others. They undergo phenotypic reversion in response to exogenous ABA and can therefore be regarded as ABA-deficient mutants according to earlier definitions (Taylor, 1991). Mutants affecting the C_{40} level

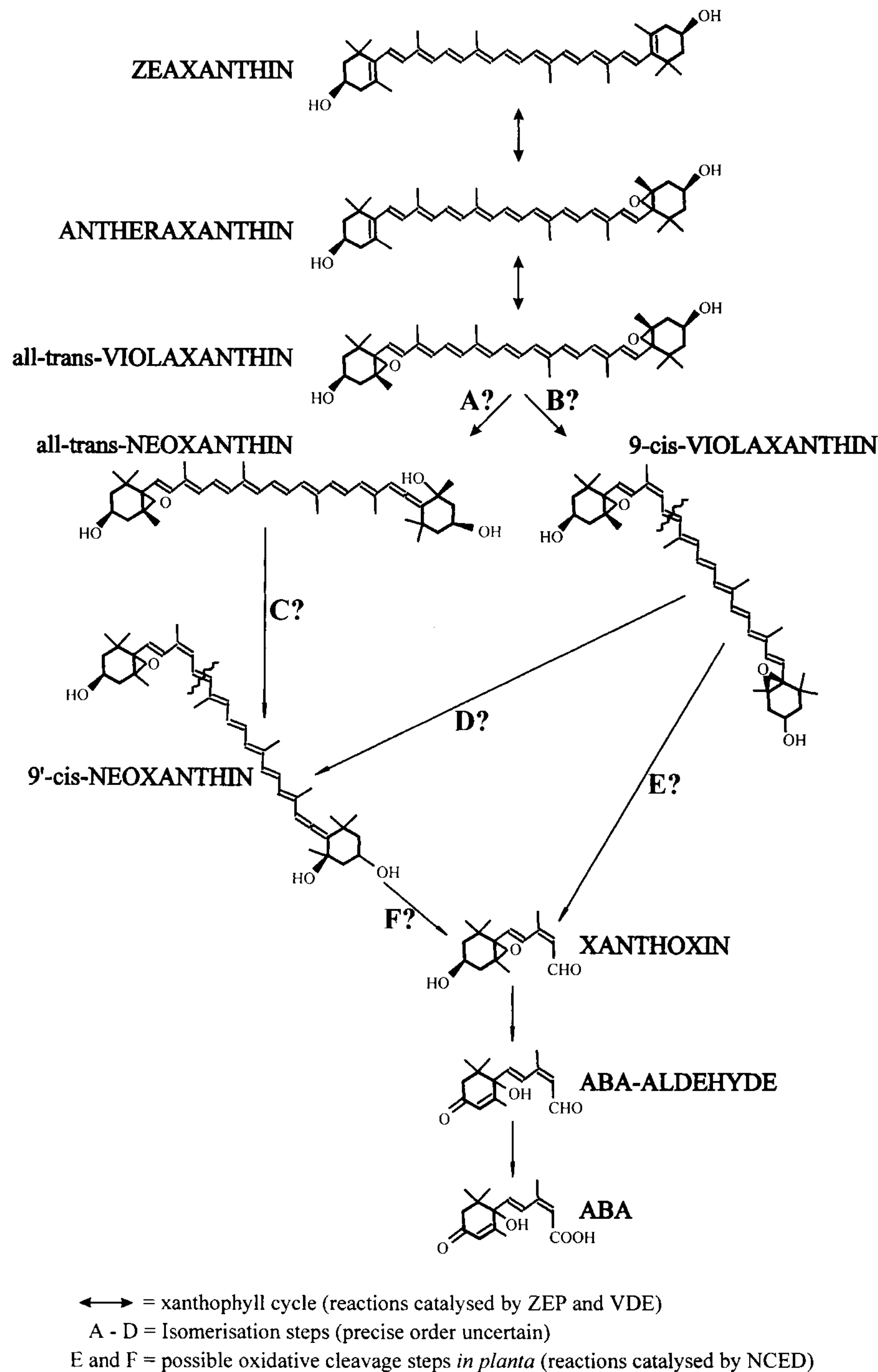

Fig. 1. The ABA biosynthetic pathway.

of the pathway are referred to in Table 1 as forming part of ABA/xanthophyll biosynthesis. Because xanthophylls form important components of the photosynthetic apparatus it is misleading to consider them simply as precursors of ABA. Nevertheless, it is reasonable to point out that the most obvious abnormalities of mutants which are impaired in the epoxidation of zeaxanthin (Duckham *et al.*, 1991; Rock and Zeevaart, 1991; Marin *et al.*, 1996) are associated with their ABA deficiency, rather than their reduced levels of epoxyxanthophylls.

Only three of the categories of mutant shown in Table 1, can be regarded as exclusively affecting ABA biosynthesis. There is strong evidence that the *vp14* mutant of maize (Tan *et al.*, 1997; Schwartz *et al.*, 1997*a*) and the *not* mutant of tomato (Burbidge *et al.*, 1997*b*, 1999) impair the oxidative cleavage reaction which forms the first C_{15}

Table 1. *Biosynthetic steps impaired in ABA-deficient mutants*

Conversion(s)[a]	Pathway	Mutant	Species	Reference
ZX→AX→VX	ABA xanthophyll biosynthesis	*aba1*	*Arabidopsis thaliana*	Duckham *et al.* (1991); Rock and Zeevaart (1991)
		aba2	*Nicotiana plumbaginifolia*	Marin *et al.* (1996)
VX→NX(?)	ABA xanthophyll biosynthesis	M526	*Chlamydomonas reinhardtii*	Katoh *et al.* (1997)
NCE→XAN	ABA biosynthesis	*not*	*Lycopersicon esculentum*	Burbidge *et al.* (1999)
		wilty (?)	*Pisum sativum*	Duckham *et al.* (1989)
		vp14	*Zea mays*	Schwartz *et al.* (1997*a*)
XAN→ABAld	ABA biosynthesis	*aba2*	*Arabidopsis thaliana*	Leon-Kloosterziel *et al.* (1996); Schwartz *et al.* (1997*b*)
ABAld→ABA	ABA biosynthesis	*v11*(*aao3*)	*Arabidopsis thaliana*	Seo *et al.* (2000)
		sit	*Lycopersicon esculentum*	Linforth *et al.* (1987); Taylor *et al.* (1988); Marin and Marion-Poll (1997)
		droopy	*Solanum phureja*	Duckham *et al.* (1989)
ABAld→ABA	MoCo biosynthesis	*nar2a*	*Hordeum vulgare*	Walker-Simmons *et al.*, (1989)
		cnxA	*Nicotiana plumbaginifolia*	Mendel and Schwarz (1999)
ABAld→ABA	MoCo maturation	*aba3*	*Arabidopsis thaliana*	Leon-Kloosterziel *et al.* (1996); Schwartz *et al.* (1997*b*)
		flc	*Lycopersicon esculentum*	Marin and Marion-Poll (1997)
		aba1	*Nicotiana plumbaginifolia*	Leydecker *et al.* (1995); Akaba *et al.* (1998)

[a]*ZX*, zeaxanthin; AX, antheraxanthin; VX, violaxanthin; NX, neoxanthin; NCE, 9-*cis*-epoxycarotenoid; XAN, xanthoxin; ABAld, ABA aldehyde; MoCo, molybdenum cofactor.

intermediate of ABA biosynthesis, i.e. xanthoxin. It is likely that the *wilty* mutant of pea also affects this first committed step in the ABA pathway, although *wilty* pea has not yet been fully characterized. The first step at the C_{15} level of ABA biosynthesis, the conversion of xanthoxin to ABA aldehyde (ABAld), is blocked only by the *aba2* genetic lesion of *Arabidopsis* (Leon-Kloosterziel *et al.*, 1996; Schwartz *et al.*, 1997*b*).

In contrast, a much larger and more diverse collection of mutants are known to be impaired in the oxidation of ABAld to form ABA. In Table 1, only one group of mutants affecting this last step has been assigned directly to the ABA biosynthetic pathway. The *sit* mutant of tomato accumulates the biologically inactive compound *trans* ABA alcohol instead of ABA in response to water stress (Linforth *et al.*, 1987). This mutant converts exogenously supplied (±) ABAld to a mixture of *cis* and *trans* ABA alcohol (Taylor *et al.*, 1988); it is possible that only the natural (+) enantiomer of ABAld forms *trans* ABA alcohol. Virtually identical results were obtained for the *droopy* mutant of *Solanum phureja*, which appears to occupy an equivalent position in the potato genome to the *sit* gene locus on the short arm of chromosome 1 of tomato (Duckham *et al.*, 1989). Marin and Marion-Poll showed that *sit* tomato mutants were only defective for aldehyde oxidase (AO^-) and were not impaired in two other enzymes which also require a molybdenum cofactor (MoCo); nitrate reductase (NR^+) and xanthine dehydrogenase (XDH^+) (Marin and Marion-Poll, 1997). From this it can be predicted that both *sit* and *droopy* are defective in the apoenzyme of ABAld oxidase (ABAO). Recently, strong evidence has been obtained that a mutant of *Arabidopsis*, previously known as *v11* and now renamed *aao3*, is also impaired in the apoenzyme of ABAO (Seo *et al*., 2000).

It should be noted that two additional categories of mutant are defective in the conversion of ABAld to ABA, but are not involved in encoding ABA biosynthetic enzymes. One group of mutants affects the supply of the co-factor either by affecting synthesis of the molybdopterin (MPT) or by in some way restricting the transfer of Mo to MPT to form MoCo. In Table 1 these are classified as MoCo synthesis mutants which are pleiotropically defective in the three main types of molybdoenzymes (AO^-, NR^-, XDH^-). The second category has been referred to as affecting MoCo maturation, following the review by Mendel and Schwarz (Mendel and Schwarz, 1999). These authors regard the cofactor containing the dioxo form of Mo as the end product of MoCo biosynthesis. This is the form of the cofactor which is incorporated into the nitrate reductase apoprotein. After parallel incorporation of MoCo into aldehyde oxidase and xanthine dehydrogenase apoproteins, a sulphurylase adds inorganic sulphur to the Mo centre converting dioxo MoCo to the monooxo form. The tomato mutant *flc* (Marin and Marion-Poll, 1997), *aba3* of *Arabidopsis* (Schwartz *et al.*, 1997*b*) and *Nicotiana plumbaginifolia* mutant alleles at the *aba1* locus (Leydecker *et al.*, 1995; Akaba *et al.*, 1998) all affect genes encoding this sulphurylase enzyme. These mutants have normal nitrate reductase (NR^+), but are simultaneously impaired in aldehyde oxidase activities (AO^-) and xanthine dehydrogenase (XDH^-).

Mutants affecting MoCo synthesis and maturation have been discussed recently (Mendel and Schwarz, 1999). As

they are indirectly, rather than directly, associated with ABA biosynthesis these mutants will not be considered further in this review. Of the remaining ABA-deficient mutants listed in Table 1, two categories have already been characterized at the molecular level in some detail and a third is currently being analysed to identify the precise DNA sequence changes involved. These categories will be discussed in turn.

Molecular characterization of zeaxanthin epoxidase (ZEP)

The first major breakthrough in understanding the molecular genetics of ABA biosynthesis involved the cloning of zeaxanthin epoxidase (Marin *et al*., 1996). A programme of non-targeted transposon mutagenesis was carried out in *Nicotiana plumbaginifolia* using the maize *Activator* (*Ac*) transposon. A plant was identified which exhibited an unstable wilty mutant phenotype. In progeny of this plant, a specific *Ac* insertion was found to co-segregate with the mutant phenotype. Inverse PCR was used to generate a DNA fragment flanking this *Ac* element. This fragment was used to screen a cDNA library from which a hybridizing full-length, single copy cDNA clone was isolated (Marin *et al*., 1996).

A stable mutant (*aba2-s1*), selected from the *Ac*-tagged line, was shown to have arisen from by an imprecise transposon excision. The stable mutant plants accumulated very high levels of zeaxanthin and had abnormally low concentrations of the epoxyxanthophylls, i.e. antheraxanthin, violaxanthin and neoxanthin (Marin *et al*., 1996). A virtually identical pattern of xanthophyll abnormalities was reported previously for the *Arabidopsis* mutant *aba1* (Duckham *et al*., 1991; Rock and Zeevaart, 1991; Table 1). The wild-type phenotype was restored to *N. plumbaginifolia aba2* following transformation with a construct based on the *ABA2* cDNA under the control of the CaMV 35S promoter. In addition, this construct complemented the *Arabidopsis aba1* genetic lesion. These are orthologous mutants, both impaired in the synthesis of the enzyme zeaxanthin epoxidase (ZEP). The complete open reading frames encoding ZEP have been reported for pepper (Bouvier *et al*., 1996) and tomato (Burbidge *et al*., 1997*a*) in addition to DNA sequence data on *N. plumbaginifolia* and *Arabidopsis*.

Zeaxanthin epoxidase gene expression

An internal 1.4kb *Hind*III fragment for the *ABA2* cDNA was used as a probe to investigate steady-state *ABA2* mRNA levels in various tissues of *N. plumbaginifolia*. Changes in transcript level during development at different times of day and also in response to water stress, were determined in detail in this species (Audran *et al*., 1998). Parallel Northern analyses of tomato plants using a probe from the full-length *LeZEP1* cDNA have also been reported recently (Thompson *et al*., 2000*a*). Similar general conclusions can be drawn from both of these studies.

Diurnal variation

Steady-state *NpZEP* mRNA was analysed in leaves from plants at the rosette stage during a single day consisting of 16/8 h light/dark (Audran *et al*., 1998). The *NpZEP* mRNA levels increased dramatically during the light period reaching a maximum after 3–5 h. Steady-state *NpZEP* mRNA then fell continuously during the later part of the light period and reached a minimum in the dark phase. Audran *et al*. also observed that there was a small increase in *NpZEP* mRNA levels 30 min before the next light period was due to start (Audran *et al*., 1998).

Expression patterns in leaves of tomato plants were assayed at 6 h intervals throughout 3 d consisting of 12/12 h light/dark followed by 2 d of continuous darkness (Thompson *et al*., 2000*a*). The highest *LeZEP* mRNA steady-state levels were observed 5 h into the light period (in agreement with Audran *et al*., 1998). After 11 h of light the *LeZEP* mRNA had fallen substantially from the peak of abundance; minimum values were observed after 5 h of darkness. As with *N. plumbaginifolia*, in tomato leaves the *LeZEP1* mRNA level was observed to increase in anticipation of the light period (Audran *et al*., 1998; Thompson *et al*., 2000*a*). The *LeZEP* mRNA was found to oscillate with a phase very similar to *light harvesting complex II (LHCII)* mRNA, although there was a slightly earlier decline in *LeZEP* mRNA in the later phase of the light period. In the tomato experiment, the rises in *LeZEP* mRNA and *LHCII* mRNA continued during a 48 h period of darkness, suggesting that *ZEP* mRNA may also be under the control of a circadian oscillator. This similarity between *ZEP* and *LHCII* mRNAs is consistent with the proposal that phases of mRNA accumulation are co-ordinated for individual complexes (Oelmuller *et al*., 1995). Expression of genes encoding the enzymes of the xanthophyll cycle may be co-ordinated with those of photosystem II, as a result of their involvement in photoprotection.

A Western blot analysis was carried out in *N. plumbaginifolia* (Audran *et al*., 1998). Despite the diurnal rhythm for *NpZEP* mRNA no oscillations in steady-state protein levels were detected. Although superficially surprising, this could have resulted from putative rises in newly synthesized *ZEP* being obscured by high background levels of stable *ZEP* protein. In parallel experiments with tomato *LHCII* protein, oscillations were observed by *in vivo* labelling (Riesselmann and Piechulla, 1992), but no changes in steady-state protein levels had been detected by earlier workers (Piechulla and Gruissem, 1987).

Drought-induced expression

Because of the strong diurnal rhythm, steady-state *ZEP* mRNA levels in leaves can appear to either rise or fall during a period of several hours following the application

of a water-stress stimulus. It was found that *NpZEP* mRNA level decreased 2–3-fold in *N. plumbaginifolia* leaves after 4 h of dehydration and 1.5–5-fold after 8 h of dehydration (Audran *et al.*, 1998). A similar fall was detected in steady-state *NpZEP* mRNA in the leaves of non-dehydrated *N. plumbaginifolia* plants over the same time-course, as a result of the normal diurnal rhythm. This demonstrates the importance of designing experiments with suitable controls to allow for these time of day effects.

In both *N. plumbaginifolia* (Marin *et al.*, 1996; Audran *et al.*, 1998) and tomato (Burbidge *et al.*, 1997*a*; Thompson *et al.*, 2000*a*) leaves, no rise in steady-state *ZEP* mRNA levels have been reported to occur in response to a variety of dehydration treatments. Because the products of *ZEP*, the epoxyxanthophylls (e.g. all-*trans*-violaxanthin) are present at high levels in leaves (Parry and Horgan, 1992), it has been concluded that 'the availability of xanthophyll precursors might not be limiting for ABA synthesis, even under water stress. An increased gene expression would therefore not be needed or would be restricted to certain tissues and might not be detected' (Audran *et al.*, 1998).

The epoxyxanthophyll levels in roots are much lower than in the leaves (Parry and Horgan, 1992) and therefore could be limiting for sustained ABA synthesis. On this basis it might reasonably have been predicted that in roots it would be possible to increase steady-state *ZEP* mRNA levels by water stress treatments. In the systems investigated so far, this appears to be true.

It was found that *NpZEP* mRNA levels in roots were increased by between 2.5–5-fold after 4 h of dehydration, rising to between 3–7-fold after an 8 h period of water stress (Audran *et al.*, 1998). The elevated levels of steady-state *NpZEP* mRNA were still maintained after 24 h of dehydration. Low and constant levels of steady-state *NpZEP* mRNA were observed in unstressed roots over a 24 h period (Audran *et al.*, 1998). A more gradually imposed drought treatment in tomato resulted in a 4-fold increase in steady-state *LeZEP* mRNA levels in the roots (Thompson *et al.*, 2000*a*) confirming the earlier results obtained for *N. plumbaginifolia*. The full extent of the drought-induced increase in *LeZEP* mRNA was masked by the presence of an additional hybridization signal when Northern blots were washed at 60 °C rather than at 68 °C. There appears to be an additional '*ZEP*-like' mRNA which is present in tomato roots and is not responsive to drought stress (Thompson *et al.*, 2000*a*).

Developmental changes in expression

The abundance of *NpZEP* mRNA was found to change substantially during seed development (Audran *et al.*, 1998). Steady-state *NpZEP* mRNA levels increased to a maximum in seeds 7–10 d after pollination (DAP) and then fell substantially as the seed matured prior to capsule opening at 22 DAP. This followed the expected time-course for changes in ABA content during seed development. In *N. plumbaginifolia* flowers the levels of *ZEP* mRNA were constant during development, but higher steady-state *NpZEP* mRNA levels were observed during the later stages of flower maturation (Audran *et al.*, 1998).

Artificially-induced modifications in expression

The report of the cloning of *NpZEP* (Marin *et al.*, 1996) offered the first prospect of manipulating ABA levels by genetic engineering. Antisense technology could be used to down-regulate a specific step in ABA biosynthesis and to create the first ABA-deficient transformants to add to the collection of ABA-deficient mutants shown in Table 1.

Antisense constructs were produced based on a 1.36 kb *Hind*III internal fragment and a 3′ *Xba*I fragment of 0.81 kb obtained from the *NpZEP* cDNA (Frey *et al.*, 1999). Northern analysis of leaves from homozygous transgenic *N. plumbaginifolia* plants revealed a substantially reduced steady-state *NpZEP* mRNA level in an antisense line involving the 1.36 kb fragment and a much less obvious down-regulation with the 0.81 kb construct. Despite the antisense suppression of mRNA in leaves, it was reported that none of the 19 antisense plants studied exhibited a wilty phenotype and that water loss from detached leaves was similar to that of wild-type plants. Mutant *aba2-s1* plants, in contrast, were extremely wilty and were described as unable to grow under greenhouse conditions unless they were grafted onto wild-type tobacco stocks (Frey *et al.*, 1999). The lack of a wilty phenotype in the *NpZEP* antisense transformants of *N. plumbaginifolia* was therefore unexpected. It is conceivable that the regions of the *NpZEP* gene used to build the antisense constructs were for some reason sub-optimal in providing a leaf phenotype. Nevertheless, it was observed that two out of five transgenic lines obtained with the 0.81 kb antisense construct and six out of 14 carrying the 1.36 kb antisense sequence, exhibited reduced seed dormancy (Frey *et al.*, 1999). Data were reported for two of the lines which showed the greatest effect on seed dormancy. Half of the seed in these antisense lines had germinated after 6–7 d; this contrasted with wild-type seed which took 13–14 d to attain this level of germination. This lack of seed dormancy was correlated with reduced steady-state *NpZEP* mRNA during seed development and lower ABA levels in mature seeds.

Homozygous tomato transgenics carrying an *LeZEP1* antisense construct have been produced (Parker *et al.*, 1999). These tomato transformants did show a wilty 'mutant' phenotype, but were less severely affected than *notabilis* mutants. However, in combination with *notabilis* the antisense transgene produced an extremely wilty phenotype. A similar effect was described previously for *notabilis/sitiens* double mutants, which had a much more severe phenotype than either mutant alone (Taylor and

Tarr, 1984). Because tomato seeds have almost no dormancy, in contrast to those of *N. plumbaginifolia*, it is difficult to detect an effect of the *LeZEP1* antisense construct on germination rate. However, the mature flowers of tomato which are normally bright yellow have a much paler colour in the *LeZEP1* antisense transformants. This was associated with the accumulation of zeaxanthin in the corolla (Fig. 2) at the expense of the epoxyxanthophylls, e.g. all-*trans*-violaxanthin and 9-*cis*-neoxanthin (Parker *et al*., 2000). The increased expression of *NpZEP* mRNA as the flowers mature in *N. plumbaginifolia* (Audran *et al*., 1998) may be part of a developmental programme to produce yellow flowers in the Solanaceae, which is no longer fully functional in this white-flowered species.

Constructs were also designed in which the complete ORF of the *NpZEP* cDNA was placed under the control of a constitutive 35S promoter, in the sense rather than antisense orientation (Frey *et al*., 1999). Transgenic *N. plumbaginifolia* plants over-expressing *NpZEP* mRNA were obtained. In homozygous over-expressing lines a maximum delay in germination of about 3 d was reported in this species (Frey *et al*., 1999). This effect was correlated with increased steady-state *NpZEP* mRNA levels during seed development and increased ABA levels in mature seed (Frey *et al*., 1999). This is consistent with the idea that epoxyxanthophyll levels in non-photosynthetic tissues, such as developing seeds and roots, could be limiting for sustained ABA synthesis. Expression of *ZEP* genes may be involved in the regulation of ABA biosynthesis in these non-photosynthetic tissues.

Molecular characterization of 9-*cis*-epoxycarotenoid dioxygenase (NCED)

The second major breakthrough in understanding the molecular genetics of ABA biosynthesis was the cloning of 9-*cis*-epoxycarotenoid dioxygenase (Tan *et al*., 1997). This involved characterization of the *viviparous 14* (*vp14*) gene locus in maize (*Zea mays*). As shown in Table 1, mutants impaired at this gene locus are unable to convert the final C_{40} precursor(s) (9-*cis*-violaxanthin and/or 9′-*cis*-neoxanthin) into the first C_{15} intermediate of ABA biosynthesis (xanthoxin) in an oxidative cleavage reaction (Fig. 1). The argument has often been made (most recently and persuasively by Qin and Zeevaart, 1999) that 'the limiting step of ABA biosynthesis in plants is presumably the cleavage of 9-*cis*-epoxycarotenoids, the first committed step of ABA biosynthesis'. It is therefore difficult to overstate the importance of cloning this gene for studies on the control of ABA biosynthesis.

A programme of non-targeted transposon mutagenesis was carried out in maize using the highly active transposon (*MuI*) in the Robertson's *Mutator* strain. Complete or partial precocious germination of seeds on the cob was used as a screen for new viviparous mutants; a phenotype which can be caused by genetic lesions in ABA biosynthesis. Two new *viviparous* mutants showed some signs of precocious germination but did not emerge fully on the cob. Both had weak penetrance and neither of the mutants appeared to be prone to water-stress under field conditions. Complementation testing showed that the two mutant lines were allelic with each other, but not with any other known *viviparous* mutants. The new mutant alleles were termed *vp14–2274* and *vp14–3250*.

Genomic DNA flanking one copy of the *Mu1* element that co-segregated with *vp14–2274* allele was cloned into λgt10 and used as a probe to isolate cDNA and genomic clones of the wild-type *Vp14* gene. Further molecular analyses showed that in *vp14–2274* the *Mu1* element had integrated into the coding sequence, completely disrupting the ORF, resulting in a null allele. In the other allele, *vp14–3250*, the *Mu1* transposon had inserted in the promoter region at a position 1 kb upstream from the start of the coding sequence (Tan *et al*., 1997).

Fig. 2. Mature tomato flowers of, from left to right; wild-type, *notabilis*, antisense ZEP, *notabilis*/antisense ZEP double mutant. Note that flowers containing the transgenic antisense ZEP construct exhibit a reduced corolla pigmentation. (This figure was kindly provided by Rachel Parker, Nottingham, UK.)

The ORF of the *Vp14* gene contains no introns and has a deduced sequence of 604 amino acids (Tan *et al.*, 1997). The motif FDGDGM in VP14 is very similar to the FNGDGM motif of the two subunits of lignostilbene-α,β-dioxygenase (LSD) from the bacterium *Pseudomonas paucimobilis* (Kamoda and Saburi, 1995). A second VP14 motif, MIHDFAI, is also similar to that of the LSD α subunit MMHDFGV (Kamoda and Saburi, 1995). These and many other amino acid sequence homologies are consistent with VP14 encoding a dioxygenase (Schwartz *et al.*, 1997*a*). More direct proof that VP14 was specifically involved in the oxidative cleavage reaction in ABA biosynthesis was obtained by producing a recombinant VP14 protein in *E. coli*. This was shown to cleave specifically xanthophyll substrates provided that they had the 9-*cis* configuration. The putative ABA precursors, 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin were both cleaved by recombinant VP14 to form xanthoxin *in vitro* (Schwartz *et al.*, 1997*a*).

The ABA-deficient mutant of tomato, *notabilis* (*not*), had been shown to metabolise xanthoxin to ABA readily (Parry *et al.*, 1988). Subsequent analysis of leaf xanthophyll levels revealed no abnormalities in *not* mutant homozygotes (Parry *et al.*, 1990). From this it could be inferred that *not* mutants were probably impaired in the same step of the pathway as the *vp14* mutant of maize. Degenerate primers were designed (D McCarty, personal communication) based on the two conserved motifs between VP14 and LSD which were discussed in the previous paragraph. These were used to generate a 540 bp PCR product from tomato genomic DNA, which was used as a probe to screen a cDNA library generated from wilted tomato leaf tissue. A full-length wild-type cDNA clone was isolated (Burbidge *et al.*, 1997*b*) which shared 63% identity with VP14 and encoded a protein of 605 amino acids (Burbidge *et al.*, 1999). Primers designed to the extreme 5′ and 3′ ends of the coding region were used to PCR-amplify the equivalent sequence from genomic DNA extracted from *not* mutant plants. DNA sequence analysis revealed only one consistent difference, a single base-pair deletion in the *not* allele which corrupted a restriction enzyme cleavage site (*Tsp*RI). Southern analysis confirmed that this deletion was real and thus demonstrated that this wilty mutant had a frameshift mutation disrupting the ORF encoding a 9-*cis*-epoxycarotenoid dioxygenase of tomato (Burbidge *et al.*, 1999). This is now referred to as an *LeNCED* gene and *Vp14* is referred to as a *ZmNCED* gene (Liontenberg *et al.*, 1999). The deduced amino acid sequences encoded by the wild-type allele of the *not* gene locus (now known as *LeNCED1*), VP14 from maize and similar sequences from *Arabidopsis* and bean (*Phaseolus vulgaris*) have recently been compared (Qin and Zeevaart, 1999).

9-*cis*-epoxycarotenoid dioxygenase gene expression

Information on the expression of an *NCED* gene was first published quite recently (Tan *et al.*, 1997). Since then reports have appeared describing *NCED* gene expression in tomato, *Arabidopsis* and in bean (*Phaseolus vulgaris*). A number of general conclusions can be drawn from these studies.

Diurnal variation

Northern analyses were carried out using an *LeNCED1* probe in parallel to those discussed earlier in relation to steady-state levels of *LeZEP* mRNA(Thompson *et al.*, 2000*a*). It was a surprise to find that *LeNCED1* mRNA levels also showed a strong diurnal rhythm in leaves, but with a clearly different phase from that of *LeZEP* mRNA. It appears that in tomato leaves *NCED1* mRNA abundance reaches a peak at the end of the light period, rather than in the middle. Unlike *ZEP* mRNA, there was apparently no anticipation of the fact that the lights were about to go out. The *LeNCED1* mRNA levels then fell by between 3- and 5-fold with a minimum recorded value after 5 h of darkness. The steady-state mRNA level was still at this low level when measured 1 h before the lights were due to come on, showing no sign that this event was being anticipated. This again was in contrast to *LeZEP* mRNA. During 48 h of continuous darkness, both *LeZEP* and *LHCII* mRNAs displayed one additional complete cycle and began to rise for a second cycle. This was not the case with *LeNCED1* mRNA which remained at a low level throughout the extended dark period, showing no sign that it was being controlled by a circadian oscillator. It appears that this gene is subject to control by a mechanism that is sensitive to light and dark, but is otherwise insensitive to the time of day (Thompson *et al.*, 2000*a*). Care must be taken to ensure that experiments monitoring the response of *NCED* mRNAs to other environmental stimuli do not span a light/dark transition. Further experiments should also be carried out to test for possible diurnal fluctuations in *NCED* mRNAs at high relative humidity, when fluctuations in water potential have been prevented. If such studies confirm that there is a genuine light effect, then the phenomenon requires an explanation. It is conceivable that the plant prepares for a possible emergency requirement for ABA biosynthesis by turning on this gene at dawn. The plant may anticipate that water stress would most likely occur during the day. Additional control mechanisms may determine whether this diurnal rise in *NCED* mRNA eventually results in elevated ABA levels.

Drought-induced expression

In maize, dehydrated detached leaves accumulated higher levels of *ZmNCED* mRNA after a 5 h incubation period

than corresponding controls which were not water-stressed (Tan *et al*., 1997). In tomato, a 3-fold increase in *LeNCED1* mRNA was observed in leaves taken from whole plants which were not watered. The levels were relatively low at 12.00 h on day 2 of the drought and showed the full 3-fold rise by the next time point at 16.00 h on the same day (Burbidge *et al*., 1997*b*). More recently, steady-state *LeNCED1* mRNA levels were determined at earlier time points after dehydration in detached tomato leaflets (Thompson *et al*., 2000*a*). At the first time point, only 15 min after detachment, a small increase in *LeNCED1* mRNA abundance was detected. The increase was complete 45 min after detachment. In bean leaves a similarly rapid response was detected with *PvNCED1* mRNA being induced strongly within 30 min of the start of the stress treatment (Qin and Zeevaart, 1999). These authors also reported that the mature form of the PvNCED1 protein could be detected after 1 h of stress. In both the tomato and the bean, these changes preceded a rise in ABA levels in the leaves. This is consistent with a cause and effect relationship; although it is necessary to upregulate steady-state *NCED* mRNA levels in the absence of water stress in order to demonstrate this directly. The rapidity of the responses in both tomato and bean leaves, suggests that an initial reduction in turgor almost instantly triggers expression of this gene; which therefore must be extremely pressure sensitive.

In roots, the steady-state *LeNCED1* mRNA levels were almost 5-fold lower than in the leaves on non-stressed tomato plants (Thompson *et al*., 2000*a*). After 54 h without water being supplied, an 8-fold rise in *LeNCED1* mRNA was detected in the leaves and an increase of over 60-fold was observed in the roots (Thompson *et al*., 2000*a*). Bean roots responded similarly to those of tomato, when they were detached and subjected to osmotic stress (Qin and Zeevaart, 1999). The bean roots accumulated *PvNCED1* mRNA rapidly, within 1 h of the start of the stress treatment. As with leaves, rises in *PvNCED1* mRNA and protein levels preceded the subsequent increase in ABA (Qin and Zeevaart, 1999). In both the leaves and the roots *PvNCED1* mRNA was less abundant following re-hydration (Qin and Zeevaart, 1999). This is consistent with an important role for *NCED* mRNA in the control of ABA biosynthesis in both leaves and roots. In the roots, at least, it seems probable that the increase in steady-state *ZEP* mRNA is also required to maintain the supply of epoxycarotenoids for continued ABA biosynthesis (see earlier sections).

Developmental changes in expression

The abundance of *NCED* mRNA can clearly vary in a tissue-specific manner, in that unstressed tomato roots have almost 5-fold lower levels of *LeNCED1* mRNA than leaves of the same plants (Thompson *et al*., 2000*a*). Although *NpZEP* mRNA increased as flowers matured and peaked one-third to one-half of the way through the process of seed development in *N. plumbaginifolia* (Audran *et al*., 1998), corresponding Northern data for *NpNCED* are not currently available. This aspect of *NCED* expression has not yet been investigated as thoroughly as the dramatic response to water stress.

Artificially-induced modifications in expression

Work on transgenic plants with altered *NCED* gene expression is about a year behind that described in the corresponding section on *ZEP*. A number of studies on this topic are likely to be published shortly. Antisense and sense constructs involving the tomato *LeNCED1* gene have been produced as a result of a collaboration between the groups at Nottingham (UK) and at HRI (Wellesbourne, UK). Early work with these constructs has been mainly focused on those with the *NCED1* ORF in the sense orientation. A total of 61 independent primary transformants have been obtained (Thompson *et al*., 2000*b*). The majority of these transgenic tomato plants (39 in total) had a strong tendency to wilt and are therefore likely to be showing co-suppression effects. Another group of 19 plants appeared to be normal and have not been investigated further.

All of the primary transformants were transferred to a controlled environment set to 90% relative humidity. Under these conditions three of the transformants grew slowly and were observed to guttate, i.e. to exude droplets of liquid at the leaf margins. Because all of the other 58 plants in the same environment were not showing this symptom, these three primary transformants were referred to as ‘over-guttating’. ABA has been shown to result in 3-fold increases in apparent hydraulic conductivity of sunflower roots resulting in substantial increases in the volume of xylem sap exuding from the cut surface of de-topped plants (Glinka, 1980; Glinka and Abir, 1983). Increased volumes of xylem sap flowing from the roots to the shoots, combined with stomatal closure, probably results in exudation from the hydathodes at the leaf margins. Since this could be interpreted as a symptom of excessive ABA levels, these ‘over-guttating’ transformants were selected for further analysis.

One of the three over-guttating transgenics was found to contain multiple inserts and this presents difficulties in establishing homozygous lines. Most of the recent experiments have involved homozygous progeny derived from the other two primary transformants. Progeny from one of these primary transformants was shown to have 17-fold higher *LeNCED1* mRNA abundance in their leaves than corresponding wild-type plants and a substantial and significant increase in bulk leaf ABA content. Reduced stomatal conductance and increased seed dormancy were also noted (Thompson *et al*., 2000*b*). Progeny of the

second primary transformant had almost 4-fold higher *LeNCED1* mRNA abundance in their leaves than corresponding wild-type plants. This did not result in a detectable increase in bulk leaf ABA content, but in spite of this a reduction in stomatal conductance and an increase in seed dormancy was observed. The seed ABA levels were significantly higher in both of these transgenic lines (Thompson *et al.*, 2000*b*). It is possible that in the second line, any potential increase in ABA, resulting from its 4-fold higher *LeNCED1* mRNA level was balanced by increased catabolism in the leaves (as suggested by Zeevaart, 1999). However, the resulting increased flux of ABA, a possible change in compartmentation, or a possible increase in root-sourced ABA, could explain the reduced stomatal conductance. In the other line, the 17-fold higher steady-state *LeNCED1* mRNA level appears to have been sufficient to overwhelm the capacity for increased catabolism to prevent ABA from accumulating to excessive levels in the leaves. This study provides direct evidence that increasing steady-state *NCED* mRNA alone is sufficient to result in enhanced ABA accumulation. This confirms that NCED is a key rate-limiting enzyme in the control of ABA biosynthesis.

Molecular characterization of ABA aldehyde oxidase (ABAO)

The most recent breakthrough in understanding the molecular genetics of ABA biosynthesis involves the identification of an *Arabidopsis* gene encoding an ABA aldehyde (ABAld) oxidase (ABAO) which is specific to ABA biosynthesis (Seo *et al.*, 2000). At the time of writing, it is only possible to refer to this in the form of abstracts. This represents the culmination of a groundbreaking series of papers on plant AOs by Koshiba and co-workers. Koshiba and Matsuyama investigated indole-3-acetaldehyde oxidase activity in maize seedlings (Koshiba and Matsuyama, 1993). The first plant AO was subsequently purified from maize seedlings and found to be a 300 kDa homodimer which was clearly a molybdoenzyme (Koshiba *et al.*, 1996). Alternative evidence on the amino acid sequence of a plant AO was obtained by database searching with a genomic DNA sequence obtained from tomato chromosome 11, which revealed homology with human (Wright *et al.*, 1993) and bovine (Calzi *et al.*, 1995) AOs. This suggested a function for the tomato ORF in encoding an aldehyde oxidase which was termed TAO1 (Ori *et al.*, 1997). The predicted amino acid sequence of TAO1 and the purified maize AO revealed many conserved regions in plant AOs (Sekimoto *et al.*, 1998). Plant AOs are members of complex multigene families; four AO cDNAs from *Arabidopsis* were obtained and positioned on four of the five chromosomes in the genome, i.e. *atAO-1* on chromsome 5; *atAO-2* on chromosome 3; *atAO-3* on chromsome 2, and *atAO-4* on chromosome 1 (Sekimoto *et al.*, 1998). Two of these *Arabidopsis* cDNAs were obtained following PCR using degenerate primers based on conserved regions between maize and bovine AOs (*atAO-1* and *atAO-2*). The other two were obtained by searching the *Arabidopsis* EST (*atAO-3*) and genomic (*atAO-4*) databases (Sekimoto *et al.*, 1998). It now appears certain that the *Arabidopsis* EST E10BIT7 (Newman *et al.*, 1994) was derived from an mRNA encoding an AO enzyme specifically involved in ABA biosynthesis (ABAO). The evidence for this conclusion is as follows:-

(i) Expressing genes encoding enzymes with a MoCo binding domain has been difficult in *E. coli*. Expression in various yeasts has been more successful, e.g. *Arabidopsis* nitrate reductase (NR-holoenzyme) was expressed in an active form in the yeast *Pichia* (Su *et al.*, 1996, 1997). Consequently, a *Pichia* heterologous expression system was used to demonstrate that an enzyme encoded by *Arabidopsis atAO-3* had substrate preference/specificity for ABAld (Seo *et al.*, 1999—poster 454 at the IBC at St Louis). Experiments of this type provide a means of testing the suggestion that an AO converts xanthoxin to xanthoxic acid, rather than ABAld to ABA (Lee and Milborrow, 1997).

(ii) An *Arabidopsis* ABA-deficient wilty mutant (originally termed *v11*) co-mapped with *atAO-3* on chromosome 2 and subsequent DNA sequencing of the *atAO-3* region in the mutant revealed a point mutation. The wilty mutant was therefore renamed *aao3* and it was concluded that the gene product of the wild-type allele was the ABAO enzyme involved in ABA biosynthesis in leaves (Seo *et al.*, 2000).

There has been insufficient time, compared with ZEP and NCED, to obtain a full picture of the role of ABAO in the control of ABA biosynthesis; consequently, it is not yet possible to review ABAO on the basis of the sections/subheadings previously used for ZEP and NCED. However, it is interesting to note that Seo *et al.* have found that in *Arabidopsis* the *AAO3* mRNA is mainly expressed in leaves and that steady-state mRNA levels are rapidly increased in dehydrated leaf tissue (Seo *et al.*, 2000).

Concluding remarks

From this review it can be seen that three of the steps in the ABA biosynthetic pathway can now be investigated at the level of gene expression. This is providing a fascinating insight into the way that plants respond to environmental stimuli by turning on ABA biosynthesis. Steady-state levels of all three mRNAs appear to increase in response to water stress in at least some plant tissues.

It is important to know how expression of the three genes is co-ordinated to ensure that ABA synthesis can be sustained efficiently. It is also necessary to remember that the balance between ABA synthesis and metabolism is vital in determining whether ABA accumulates in whole plants (see Cutler and Krochko, 1999, for a review of both aspects). ABA is metabolized by ABA 8′-hydroxylase activity which is induced by ABA itself (Cutler *et al*., 1997). This effect is clearly antagonistic to attempts to increase ABA levels by over-expressing ABA biosynthesis genes. If ABA were also to inhibit its own synthesis by some form of feedback, then a combination of these two effects would tend to frustrate attempts to produce transgenic plants with elevated ABA levels. No suppression of drought-induced increases in steady-state *LeNCED1* (Thompson *et al*., 2000*a*) and *PvNCED1* (Qin and Zeevaart, 1999) mRNA by ABA has been detected. In practice, it has proved possible to produce transgenic plants with an increased ABA content by over-expressing ABA biosynthetic genes (Frey *et al*., 1999; Thompson *et al*., 2000*b*). ABA biosynthesis is now subject to human as well as environmental control.

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