Regulation of Abscisic Acid Biosynthesis[1]

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Plant growth and development are regulated by internal signals and by external environmental conditions. One important regulator that coordinates growth and development with responses to the environment is the sesquiterpenoid hormone abscisic acid (ABA). ABA plays important roles in many cellular processes including seed development, dormancy, germination, vegetative growth, and environmental stress responses. These diverse functions of ABA involve complex regulatory mechanisms that control its production, degradation, signal perception, and transduction. Because of the key role of ABA in plant stress responses, understanding these regulatory mechanisms will help devise rational strategies to breed or genetically engineer crop plants with increased tolerance to adverse environmental conditions.

Since the discovery of ABA in the early 1960s, much effort has been devoted to understanding how ABA is synthesized. Through genetic and biochemical studies, the pathway for ABA biosynthesis in higher plants is now understood in great detail. Recently, all the major genes for the enzymes in the biosynthesis pathway have been identified (Schwartz et al., 2003). The new challenge is to understand how these biosynthesis genes, and the biosynthetic pathway as a whole, are regulated. Although much remains to be learned about the regulatory mechanism, evidence thus far indicates that ABA biosynthesis is subject to complex regulation during plant development and in response to environmental stresses. In this Update, we first present a brief overview of the functions of ABA and the biosynthesis pathway. We then focus on the regulation of ABA production and attempt to provide some future directions in ABA biosynthesis studies.

BIOLOGICAL FUNCTIONS OF ABA

Under non-stressful conditions, ABA in plant cells is maintained at low levels. Some low levels of ABA may be required for normal plant growth, as evidenced by reduced vigor observed in ABA-deficient mutant plants that can be restored to the wild-type level of growth by exogenous ABA (Finkelstein and Rock, 2002). Because all ABA-deficient mutants still have certain basal levels of ABA that are not dramatically lower than those in the wild type under normal growth conditions, it is difficult to uncover the cellular processes that require a very small amount of ABA. As a consequence, our knowledge of ABA functions has been gained mainly from observations with ABA at elevated levels, either from endogenous or exogenous sources. ABA levels can increase dramatically during seed maturation and in response to environmental stresses. Thus, ABA functions have been most extensively studied in these two processes.

During seed development, ABA is known to initiate the following programs: embryo maturation, synthesis of storage reserves and late embryogenesis-abundant (LEA) proteins, and initiation of seed dormancy, although ABA is not the sole regulator of these processes. In particular, the induction of LEA protein synthesis to preserve the viability of embryos in the extremely dry condition of seeds is related to the role of ABA in promoting synthesis of LEA-like proteins in vegetative tissues to tolerate dehydration stress. Embryos from ABA antibody-expressing plants lose their viability as a result of desiccation intolerance (Phillips et al., 1997).

In vegetative tissues, ABA levels increase when plants encounter adverse environmental conditions such as drought, salt, and to a lesser extent, low temperatures. Although a higher level of exogenous ABA inhibits plant growth under non-stressful conditions, an increased ABA content is beneficial for plants under environmental stress as a result of ABA-induced changes at the cellular and whole-plant levels. ABA promotes the closure of stomata to minimize transpirational water loss. It also mitigates stress damage through the activation of many stress-responsive genes that encode enzymes for the biosynthesis of compatible osmolytes and LEA-like proteins, which collectively increase plant stress tolerance (Hasegawa et al., 2000; Bray, 2002; Finkelstein et al., 2002). In addition, ABA has been shown to offset the inhibitory effect of stress-induced ethylene on plant growth (Sharp, 2002). Plant mutants defective in ABA biosynthesis are more susceptible to the environmental stresses and have been isolated in stress sensitivity screens (Xiong et al., 2002b). Importantly, manipulating ABA levels by changing the

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expression of key ABA biosynthetic genes provides an effective means to increase plant stress resistance.

THE ABA BIOSYNTHESIS PATHWAY

ABA-deficient mutants have been instrumental for revealing the pathway of ABA biosynthesis. By virtue of their precocious germination of seeds and the wilty appearance of the plants, mutants defective in ABA biosynthesis were isolated from a number of plant species including maize (*Zea mays*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), and Arabidopsis. Before the molecular identities of the affected genes were known, a major route for ABA biosynthesis was revealed by profiling ABA biosynthetic intermediates in combination with feeding assays using these mutants. These studies suggested that ABA in higher plants is synthesized from an "indirect" pathway through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde (Fig. 1; Taylor et al., 2000; Finkelstein and Rock, 2002; Seo and Koshiba, 2002; Schwartz et al., 2003). By now, major ABA-deficient mutants, genes, and enzymes have been characterized in Arabidopsis (Schwartz et al., 2003). The information from Arabidopsis is applicable to other plant species because the pathway and the respective genes are highly conserved in angiosperms. To avoid confusion, in this Update, genes are named after their products instead of the respective genetic loci.

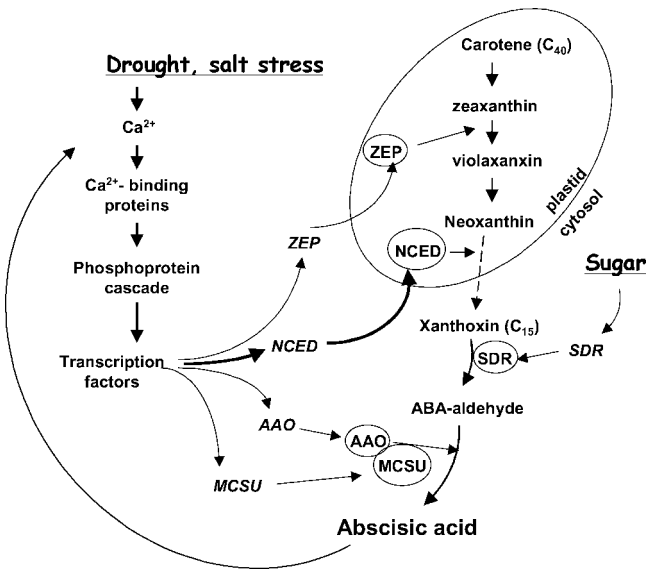

Figure 1. Regulation of ABA biosynthesis. ABA is derived from C_{40} epoxycarotenoid precursors through an oxidative cleavage reaction in plastids. The C_{15} intermediate xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde in cytosol. Abiotic stresses such as drought and salt activate the biosynthetic genes (italicized), probably through a Ca^{2+}-dependent phosphorelay cascade as shown on the left. ABA feedback stimulates the expression of the biosynthetic genes, which is also likely through a Ca^{2+}-dependent phosphoprotein cascade. Among the biosynthetic genes, *NCED* is strongly upregulated by stress (indicated with a thick arrow), whereas *SDR* is regulated by sugar. ABA biosynthetic enzymes are shown in small ovals. The NCED step probably limits ABA biosynthesis in leaves (indicated with a dashed arrow). ZEP, zeaxanthin epoxidase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; AAO, ABA-aldehyde oxidase; MCSU, MoCo sulfurase.

The first step that is more specific to the ABA biosynthesis pathway is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which occurs in plastids. This step is catalyzed by a zeaxanthin epoxidase (ZEP), whose molecular identity was first revealed in tobacco (Marin et al., 1996). After a series of structural modifications, violaxanthin is converted to 9-*cis*-epoxycarotenoid. Oxidative cleavage of the major epoxycarotenoid 9-*cis*-neoxanthin by the 9-*cis*-epoxycarotenoid dioxygenase (NCED) yields a C_{15} intermediate, xanthoxin (Schwartz et al., 1997). This step was considered the first committed step in the ABA biosynthesis pathway. The *ZmNCED* gene was isolated using the maize *vp14* mutant (Tan et al., 1997). The product xanthoxin is then exported to the cytosol, where it is converted to ABA through a two-step reaction via ABA-aldehyde. A short-chain alcohol dehydrogenase/reductase (SDR), encoded by the *AtABA2* gene (Rook et al., 2001; Cheng et al., 2002; Gonzalez-Guzman et al., 2002), catalyzes the first step of this reaction and generates ABA aldehyde. ABA aldehyde oxidase (AAO) then catalyzes the last step in the biosynthesis pathway. Mutations in either the aldehyde oxidase apoprotein (e.g. Seo et al., 2000b) or molybdenum cofactor (MoCo) synthase (e.g. *Ataba3* mutant) would impair ABA biosynthesis. The *AtABA3* gene encodes a MoCo sulfurase that catalyzes the sulfurylation of a dioxo form of MoCo to a sulfurylated mono-oxo form (Bittner et al., 2001; Xiong et al., 2001b). This form of MoCo is required by aldehyde oxidase and xanthine dehydrogenase for their activities.

DEVELOPMENTAL REGULATION OF ABA BIOSYNTHESIS

Seed maturation and germination expose the young embryo to dramatic osmotic stresses. ABA is the major factor that is required to escort the embryo upon entering and exiting its quiescent state. ABA in developing seeds can either be derived from maternal tissues or be synthesized de novo in the embryo. Studies in Arabidopsis suggest that during seed development, there appear to be two peaks of ABA accumulation (for review, see Bentsink and Koornneef, 2002; Finkelstein et al., 2002). The first one occurs about halfway during seed development (approximately 10 d after pollination). This ABA is likely to be derived from maternal tissues because in reciprocal crosses, the peak only occurred when the wild-type but not ABA-deficient mutants were used as the female. ABA at this stage promotes the synthesis of

storage proteins. Embryos from ABA antibody-expressing plants either did not accumulate or accumulated a much lower level of storage proteins relative to the wild-type embryos (Phillips et al., 1997). The second peak with less significant ABA accumulation (about one-third of the first peak) is from biosynthesis in the embryo and may activate the synthesis of LEA proteins that prepare the embryo for desiccation. This peak of ABA also initiates seed dormancy. ABA levels fall rapidly in the later stage of seed maturation and are very low in dry seeds. During seed imbibition, de novo ABA biosynthesis in the embryo is a determinant of seed dormancy (Bentsink and Koornneef, 2002; Finkelstein et al., 2002). ABA at this stage also maintains, within a narrow time window, the imbibed embryo in a reversible state between dormancy and germination by regulating the basic Leu Zip transcription factor ABI5 (Lopez-Molina et al., 2001). These important roles of ABA and its dynamics of accumulation in the embryo suggest that ABA biosynthesis is under tight developmental regulation in the embryo.

Transcripts for all the ABA biosynthetic genes are detected in embryos/developing seeds, although more detailed analysis of the expression of individual genes during seed development has not been reported except for *AtZEP1*. In situ hybridization detected *AtZEP* expression in the embryo from globular to desiccation stages (Audran et al., 2001), which indicates that the *ZEP* gene was expressed during embryogenesis before the first peak of ABA accumulation in developing seeds. In addition to suggesting a potential role of low-level ABA in embryogenesis, it also raises an interesting question regarding which signal(s) activates *ZEP* (and other ABA biosynthetic genes) in developing embryos. Although the possibility of a unique developmental signal that induces one or more of the ABA biosynthetic genes cannot be ruled out, current experimental evidence implies that soluble sugars, osmotic stress, and ABA itself are likely to be the signals that activate ABA biosynthesis in developing seeds.

Maternal ABA has been shown to be required for the first peak accumulation of ABA in developing seeds, yet it is not clear whether the ABA was directly derived from maternal tissues or rather that maternal ABA serves only as a signal for de novo synthesis of ABA in developing embryos. This question is relevant because ABA can positively regulate its own biosynthesis by activating ABA biosynthetic genes (see below). Because *ZEP*, *SDR1*, *AAO3*, and *MCSU* genes are all induced by sugars to various extents (Cheng et al., 2002), sugar levels may regulate ABA biosynthesis in the embryo. This mechanism is perhaps more important for *SDR1* because *SDR1* is not induced by either osmotic stress or ABA. At the onset of seed maturation, osmotic stress may become more important in activating de novo ABA biosynthesis, which is responsible for embryo desiccation tolerance and dormancy.

The activation of individual genes in seeds may eventually be responsible for ABA biosynthesis and accumulation in seeds. For instance, the expression of *NtZEP* reaches its maximum between one-third and one-half of seed development, which correlates with increased ABA accumulation during this period (Audran et al., 1998). Likewise, seeds from plants overexpressing *NtZEP* had a higher ABA level and showed enhanced dormancy (Frey et al., 1999), suggesting that NtZEP may regulate ABA biosynthesis in seeds and during seed germination. Similarly, in tomato, NCED may also regulate ABA levels in the seeds. Overexpression of the *LeNCED1* gene increased ABA levels in imbibed seeds and extended seed dormancy (Thompson et al., 2000b). These experimental data suggest that ABA biosynthesis in developing and imbibing seeds may be regulated at multiple steps.

ABIOTIC STRESS REGULATION OF ABA BIOSYNTHESIS

Certain environmental signals such as light have been suggested to regulate ABA biosynthesis directly or indirectly. The environmental conditions that most dramatically activate ABA biosynthesis, however, are drought and salt stress. Increased ABA levels under these abiotic stresses result mainly from increased de novo biosynthesis. The degradation of ABA appears to be suppressed by stress and activated by ABA and stress relief.

Drought and salt stresses induce ABA biosynthesis largely through transcriptional regulation of ABA biosynthetic genes because blocking transcription by using transcription inhibitors impairs stress-induced ABA biosynthesis. Therefore, transcriptional regulation of ABA biosynthetic genes holds the key to understanding how ABA biosynthesis is regulated, although regulation of the specific activities of ABA biosynthesis enzymes also exists.

ZEP was the first gene in the ABA biosynthesis pathway to be cloned, and its expression and regulation have been scrutinized in a number of plant species. ZEP genes were expressed ubiquitously in every plant part with a higher basal expression in leaves (Audran et al., 1998; Xiong et al., 2002a). It was thought that ZEP does not limit ABA biosynthesis in photosynthetic tissues because on a molar basis, the amount of 9-*cis*-epoxycarotenoid (precursor downstream of the ZEP-catalyzed reaction) in photosynthetic tissues such as leaves is several times higher than the amount of ABA produced during stress. In tobacco and tomato plants, the transcript levels of *ZEP* genes in leaves were also not regulated by drought stress but were found to be regulated diurnally with high transcript levels in the day, which may reflect regulation by the circadian rhythm (Au-

dran et al., 1998; Thompson et al., 2000a) rather than the periods of diurnal water potential changes in plants. Despite the diurnal variation in transcript levels, no change in the level of ZEP protein was found. The potential circadian regulation could be related to the involvement of ZEP products in the light-harvesting complex but not to the role of ZEP proteins in ABA biosynthesis. In contrast to leaves, the amount of epoxycarotenoid precursors is lower in roots, where ZEP, therefore, may limit ABA biosynthesis. Consistent with this speculation, ZEP genes in roots were clearly regulated by drought stress. Its transcript levels increased severalfold after drought stress both in tobacco and tomato plants (Audran et al., 1998; Thompson et al., 2000a).

The regulation of ABA biosynthetic genes may vary not only between different plant parts and developmental stages but also between different plant species. Like the ZEP genes in tobacco and tomato, the Arabidopsis *ZEP* gene also had a basal transcript level under non-stressful conditions. However, drought, salt, and polyethylene glycol clearly increased its expression level both in the shoot and in the root (Xiong et al., 2002a), demonstrating that the *AtZEP* gene is under stress regulation. Variation in the regulation of the ZEP genes observed in different experiments may be partly related to the relative basal transcript levels. A high basal transcript level may mask stress inducibility of the genes.

Expression studies with other ABA biosynthetic genes (*NCED*, *AtAAO3*, *MCSU*, and *AtSDR1*) are less controversial. With the notion that the cleavage step is rate limiting in ABA biosynthesis, the expression of *NCED* gene(s) has received particular attention. Drought stress treatments were shown to induce *NCED* expression in maize (Tan et al., 1997), tomato (Burbidge et al., 1999), bean (*Phaseolus vulgaris*; Qin and Zeevaart, 1999), Arabidopsis (Iuchi et al., 2001), cowpea (*Vigna unguiculata*; Iuchi et al., 2000), and avocado (*Persea americana*; Chernys and Zeevaart, 2000). Significant increases in *NCED* transcript levels can be detected within 15 to 30 min after leaf detachment or dehydration treatment (Qin and Zeevaart, 1999; Thompson et al., 2000a), indicating that the activation of *NCED* genes can be fairly quick.

In fact, with the exception of *AtSDR1*, whose expression appears not to be regulated by stress (Cheng et al., 2002; Gonzalez-Guzman et al., 2002), all the other ABA biosynthetic genes are up-regulated by drought and salt stress (Seo et al., 2000b; Iuchi et al., 2001; Xiong et al., 2001b; Xiong et al., 2002a), although their protein levels have not been examined in most cases. Because ABA biosynthesis does increase dramatically upon stress treatment, it is expected that the protein levels of these genes increase after the transcript levels, as was seen with the *NCED* gene (Qin and Zeevaart, 1999). Nonetheless, a study with *AtAAO3* suggested that its protein levels did not change as the transcript levels (Seo et al., 2000b). At present, it is not clear whether this is only an exception reflecting the particular experimental conditions used. In contrast to the clear regulation of these genes by drought and salt stress, the expression of *AtZEP* (Xiong et al., 2002a), *NCED* (Qin and Zeevaart, 1999), and *AtMCSU* (Xiong et al., 2001b) was not obviously up-regulated by cold. This is consistent with the observation that the magnitude of increase in ABA contents in plants subjected to cold treatment was much less than that in drought-stressed plants (Thomashow, 1999).

The unique regulation of the *SDR* gene is intriguing. *AtSDR1* (*ABA2/GIN1*) promoter activity was detected mainly in vascular tissues. Thus, its predominant expression tissues appear to be separated from those for other ABA biosynthetic genes that are expressed more ubiquitously (Cheng et al., 2002). The gene is expressed constitutively at a relatively low level and is not induced by drought stress. Rather, its expression is enhanced by sugar. During seed development, changes in sugar levels in the maturing seeds may have an impact on ABA biosynthesis, as discussed above. Sugar levels also vary diurnally and are influenced by abiotic stress. However, short-term stress treatments under laboratory conditions had no obvious effect on *AtSDR1* promoter activity (Cheng et al., 2002).

SELF-REGULATION OF ABA BIOSYNTHETIC GENES

Many biosynthetic pathways are regulated by their end products. ABA has long been thought to negatively regulate ABA accumulation by activating its catabolic enzymes (Cutler and Krochko, 1999). The activity of a cytochrome P450 enzyme, ABA 8′-hydroxylase, which catalyzes the first step of ABA degradation, was stimulated by exogenous ABA (e.g. Uknes and Ho, 1984). Studies with transgenic tobacco overexpressing *NCED* gene (or under an inducible promoter) showed that ABA overproduction correlated with the overaccumulation of the catabolite, phaseic acid (Qin and Zeevaart, 2002). These studies support the notion that ABA might restrict its own accumulation by activating its degradation, at least under non-stressful conditions. On the other hand, whether ABA can stimulate or inhibit its own biosynthesis was unclear.

Because the *NCED* gene product has been suggested to catalyze the rate-limiting step in the ABA biosynthesis pathway, whether or not this gene is regulated by ABA is very relevant to the question of whether ABA can auto-regulate its own biosynthesis. In tomato plants, it was found that the *NCED* gene was not induced by exogenous ABA (Thompson et al., 2000a). Similarly, in cowpea, ABA was unable to activate *NCED* genes (Iuchi et al., 2000). These observations would support the idea that ABA may stimulate its own degradation but not its production.

However, when the expression of ABA biosynthetic genes was examined across wide genetic backgrounds, a different picture emerged. To our surprise, we have found that *ZEP, AAO3*, and *MCSU* in Arabidopsis are all up-regulated by ABA, in addition to being regulated by stress. Exogenous ABA significantly enhanced the expression of these genes (Xiong et al., 2001a, 2001b, 2002a). Moreover, these genes appear also to be regulated by endogenous ABA. It was observed that in any of the ABA-deficient mutants *los5*, *aba1*, *aba2*, or *aba3*, the transcript levels for all the inducible ABA biosynthetic genes under stress conditions were significantly lower than those in the wild-type plants (Xiong et al., 2002a), although their basal transcript levels were unaffected in these mutants under non-stressful conditions (Audran et al., 2001; Xiong et al., 2002a). Thus, ABA deficiency in the Arabidopsis *aba* mutants may not be simply a consequence of the lesions in the biosynthetic enzymes but also may be because of the significantly reduced expression of other ABA biosynthetic genes as a result of the primary lesions.

Furthermore, it was found that even the *NCED* gene was induced by ABA in certain genetic backgrounds (e.g. ABA-deficient mutants and certain ecotypes such as Landsberg; Xiong et al., 2002a). Recently, Cheng et al. (2002) also reported that the *AtNCED3* gene (and *AtZEP* and *AtAAO3*) could be induced by ABA in the Landsberg background. In addition, *AtNCED3* transcript levels under drought and salt stress treatments were significantly reduced in the ABA-deficient mutants *los5* and *los6* as compared with those in wild-type seedlings, demonstrating that ABA is required for full activation of *AtNCED3* by osmotic stress (Xiong et al., 2002a). Taken together, these observations strongly suggest a positive feedback regulation of ABA biosynthesis by ABA. This may underscore a stress adaptation mechanism where an initial induction of ABA biosynthesis rapidly stimulates further biosynthesis of ABA through this positive feedback loop (Fig. 1).

To better understand this feedback regulatory circuit, it is important to determine which signaling components may mediate this self-regulation of ABA biosynthetic genes. One such component is *SAD1* (supersensitive to ABA and drought 1). A recessive mutation in *SAD1* confers hypersensitivity to exogenous ABA during seed germination, vegetative growth, and in gene expression (Xiong et al., 2001a). Interestingly, *sad1* mutant plants are also impaired in drought-induced ABA biosynthesis. Further characterization of *sad1* found that the mutant was defective in the self-regulatory loop because the *sad1* mutation impairs ABA regulation of the *AAO3* and *MCSU* genes (Xiong et al., 2001a). Both gene products are required for the last step of ABA biosynthesis, i.e. the conversion of ABA aldehyde to ABA (Fig. 1). Significantly, a feeding assay showed that the conversion of ABA-aldehyde to ABA was impaired in the *sad1* mutant (Xiong et al., 2001a). This defect may well be responsible for the impaired drought-induced ABA biosynthesis in the mutant. It is likely that SAD1, an Sm-like small ribonucleoprotein that is predicted to be involved in mRNA splicing, export, and degradation, may regulate the turnover rates for the transcripts of an early ABA signaling component(s). The components(s) may in turn regulate the feedback circuit. One such component is likely to be an ABI1-like protein phosphatase 2C because stress induction of this gene was impaired in *sad1* (Xiong et al., 2001a). Although a mutation in the mRNA cap-binding protein also reduced the expression of another PP2C gene, this ABA-hypersensitive mutant, *abh1*, was not ABA deficient (Hugouvieux et al., 2001). Thus, at least some of the PP2Cs, including ABI1 itself, may be involved in this self-regulation circuit as discussed below.

Arabidopsis ABI1 and ABI2 are homologous 2C-type protein phosphatases that may negatively regulate ABA signaling. The *abi1-1* and *abi2-1* mutations confer ABA insensitivity in seed germination, vegetative growth, and the expression of certain ABA-regulated genes (Finkelstein et al., 2002). In *abi1*, the self-regulation loop of ABA biosynthesis is partially impaired because in this mutant, ABA fails to activate the expression of the *NCED* gene (in Landsberg background), and this mutation significantly reduced the transcript levels of *ZEP* and *AAO3* under ABA treatment. Strikingly, all these genes were unaffected in *abi2* (Xiong et al., 2002a). Despite their sequence homology and overlapping functions, ABI1 and ABI2 are often found to have different roles in ABA signaling. For example, it was shown that ABA-induced reactive oxygen species (ROS) production was impaired in *abi1* but not in *abi2*. In contrast, hydrogen peroxide activates plasma membrane Ca^{2+} channels and induces stomatal closure in *abi1* but not in *abi2* (Murata et al., 2001). Because ROS is involved in ABA signaling and probably also in regulating ABA biosynthesis (Zhao et al., 2001), it is possible that this self-regulation of ABA biosynthetic genes may be partly mediated by ROS through a protein phosphorylation cascade (Fig. 1). In any case, the involvement of ABI1 in the feedback loop further suggests that signaling for ABA biosynthesis is ABA dependent and that there is cross talk between the signaling pathway for ABA biosynthesis and the pathway for ABA responsiveness. Future studies are needed to identify the interplay between these two pathways and the significance of self-regulation of ABA biosynthesis in overall ABA accumulation under abiotic stress conditions.

DIFFERENTIAL REGULATION OF ABA BIOSYNTHETIC GENES AND THE RATE-LIMITING STEP IN THE BIOSYNTHESIS PATHWAY

Genes involved in ABA biosynthesis exist either as a single copy or a gene family, and the family mem-

bers may be subjected to differential regulation as discussed above. For Arabidopsis, whose genome has been completely sequenced, the copy numbers of ABA biosynthetic genes are known (Table I). It seems that *ZEP*, *MCSU*, and *SDR* are single-copy genes, whereas *NCED* and *AAO* belong to gene families. Current data suggest that for those belonging to gene families, each member is regulated differently by stresses. In addition, they may be expressed in a tissue- and developmental stage-specific manner. For example, *AtAAO3* was expressed in leaves but not in the root, and *AAO4* was mainly expressed in siliques (Seo et al., 2000a), whereas *AtAAO3* may be less expressed in seeds. Thus, *aao3* mutant seeds were not obviously changed in dormancy (Seo et al., 2000b), which is clearly different from other known ABA-deficient mutants whose seeds are much less dormant. This may explain why *aao3* mutants were not isolated in germination screens. It is also likely that family members may be regulated differentially by different stresses or may have different threshold of stress induction.

Because the ABA biosynthesis pathway involves multiple gene products, there could be a rate-limiting step in the pathway. Finding this limiting step is important for genetic manipulation of the pathway. With the complex regulation mechanisms for individual ABA biosynthetic genes that may vary among plant species, together with the mobile nature of ABA or its immediate precursors, it would be difficult to draw a consensus regarding the regulatory patterns of these genes or the rate-limiting step for ABA biosynthesis in the whole plant. Nonetheless, it was recognized generally that the step catalyzed by NCED, i.e. the oxidative cleavage of neoxanthin, is rate limiting (Tan et al., 1997; Qin and Zeevaart, 1999; Taylor et al., 2000; Thompson et al., 2000b). This may be valid in leaves, where most studies on ABA biosynthesis are concerned. Consistent with this prediction, constitutive or inducible overexpression of the *NCED* genes resulted in increased ABA biosynthesis and reduced transpiration water loss (Iuchi et al., 2001; Qin and Zeevaart, 2002; Thompson et al., 2000b). However, if the rate-limiting step is solely at the NCED, then one would expect that overexpression of other ABA biosynthetic genes would not significantly increase ABA biosynthesis, at least not in the leaves. Nonetheless, experimental evidence indicated that even overexpressing the *AtZEP* gene, whose product catalyzes the least rate-limiting step considered in the pathway, could result in an increased stress gene induction in Arabidopsis seedlings (Xiong et al., 2002a). Furthermore, overexpression of *NtZEP* led to increased seed dormancy and delayed seed germination in tobacco (Frey et al., 1999), presumably as a result of increased ABA biosynthesis in imbibed embryos. These observations would suggest that ZEP might limit ABA biosynthesis also, particularly in non-photosynthetic tissues such as seeds and roots. It would be interesting to see whether up-regulating the expression level of other ABA biosynthetic genes can cause enhanced ABA biosynthesis.

The possibility that overexpressing any of the ABA biosynthetic genes probably have an impact on ABA biosynthesis may in fact have to do with the self-regulation mechanism discussed in the previous section. This is because a limited initial increase in ABA biosynthesis from overexpressing a single ABA biosynthetic gene may result in a coordinately increased induction of other ABA biosynthetic genes through the self-regulatory loop (Fig. 1). This possibility can be confirmed by examining the expression of other ABA biosynthetic genes in the overexpressors. In

Table I. *ABA biosynthetic genes and their regulation in Arabidopsis*

Not all the genes listed are involved in ABA biosynthesis; those known to be involved are indicated in bold. Gene symbols are given in Figure 1.

Genes	Arabidopsis Genome Initative Identification	Mutants	Expression Pattern	Stress Inducibility
ZEP	At5g67030	*aba1, los6,npq2*	Ubiquitous	Inducible in roots, also in leaves
SDR1	At1g52340	*aba2,gin1, sis4, isi4, sre1, san3*	Ubiquitous? Vascular tissues	Not induced by stress but by sugar
NCED1/CCD1	At3g63520			Not inducible?
NCED2	At4g18350			Not inducible
NCED3	At3g14440	*nced3*	Ubiquitous?	Strongly inducible
NCED4	At4g19170			Not inducible
NCED5	At1g78390			Not inducible
NCED6	At3g24220			Not inducible
NCED9	At1g30100			Weakly inducible
AAO1	At5g20960		Roots and seeds	
AAO2	At3g43600		Roots	
AAO3	At2g27150	*aao3*	Leaves but not siliques/ seeds	Inducible in leaves but not in roots
AAO4	At1g04580		Mainly in siliques	
MCSU	At1g16540	*aba3, los5, frs1*	Ubiquitous	Inducible

addition, the fact that *NCED*s are either not up-regulated or weakly up-regulated by ABA may cause the NCED step to become rate limiting late in ABA biosynthesis in leaves. As a consequence, regulating NCED genes may have a more significant impact on overall ABA biosynthesis.

UNRAVELING THE REGULATORY CIRCUIT FOR ABA BIOSYNTHESIS

Obviously, the regulation of ABA biosynthesis is of great importance in controlling ABA level and fine-tuning plant stress responses and developmental programs. Because stress-inducible ABA biosynthetic genes contain both the *DRE*- and *ABRE*-like cis-elements in their promoters (Xiong et al., 2001b; Bray, 2002), it is possible, therefore, that these genes may be similarly regulated as the DRE/CRT class of stress-responsive genes (Xiong et al., 2002b). Circumstantial evidence has suggested that the signal transduction pathways for stress-induced ABA biosynthesis may involve redox signals, Ca^{2+} signaling, and protein phosphorylation and dephosphorylation events. Yet, biochemical or genetic studies of each of these signaling processes in relation to ABA biosynthesis have been lacking. In the near future, molecular studies should identify the cis-elements in ABA biosynthetic genes and the respective transcription factors that are responsible for the activation of these genes. Extensive genetic analysis will also be needed to dissect such a complex signal transduction process.

Exhaustive screens of seed germination, either in the presence of GA synthesis inhibitors or high concentrations of salt, have identified lesions in ABA biosynthesis (and in ABA responsiveness). These mutants also were repeatedly recovered in screens for sensitivity to sugars (Finkelstein et al., 2002; Rolland et al., 2002). In fact, some loci were identified in more than six independent screening schemes! Nonetheless, no component that directly regulates ABA biosynthesis has emerged in these screens. Because sugar, ethylene, and ABA biosynthesis have a complicated interplay in seed germination and in some other physiological processes (Ghassemian et al., 2000; Hansen and Grossmann, 2000; Gonzalez-Guzman et al., 2002), there is a possibility that some of the existing signaling components in other hormone response pathways may be involved in ABA biosynthesis regulation but have escaped our attention. One such example may be *era3* (*enhanced response to ABA 3*)/*ein2* (*ethylene insensitive 2*). *era3*/*ein2* plants have a basal ABA level twice of that in the wild type, which is probably due to increased biosynthesis because the ZEP transcript level was also higher in *ein2* (Ghassemian et al., 2000). Yet, the effect of the *ein2* mutation on ABA biosynthesis could be a consequence of the complex interaction between different hormones rather than a specific regulation of ABA biosynthesis. Aside from possible genetic redundancy of the regulatory pathways, the difficulty in identifying regulatory loci in seed-based screens may also have to do with the possibility that these screens are not suitable for the identification of the components. To uncover the regulatory pathways for ABA biosynthesis, one may need additional approaches.

Given the roles of ABA in stress responses at the vegetative stage, screens based on stress responses in vegetative tissues would likely identify new loci that are important in regulating ABA biosynthesis or signaling. Due to the difficulty in manipulating drought stress in a quantitative and reproducible way, screens for altered drought tolerance have not been widely used. Alternative screens such as those for guard cell regulation (e.g. Mustilli et al., 2002) and gas exchange (e.g. sensitivity to CO_2) may discover novel loci that regulate ABA accumulation or signaling. Because gene expression is more sensitive to stress regulation than are some of the visible phenotypes, molecular genetic approaches such as the one used in the screen for stress signal transduction mutants (Ishitani et al., 1997) may be more productive in identifying signal transduction components. In this approach, the promoters of stress-inducible ABA biosynthetic genes (in particular, *AtNCED3*, *AtMCSU*, and *AtAAO3*) can be transcriptionally fused to a reporter gene; then, mutants with altered reporter gene expressions in response to abiotic stress can be isolated. These screens may uncover novel regulatory mechanisms in ABA biosynthesis. With the availability of the complete genomic information of Arabidopsis and large bodies of expression data, reverse genetics approaches should also facilitate the identification of new regulatory components in the signaling pathway leading to ABA biosynthesis. A complete understanding of the regulation of ABA biosynthesis will require a combination of genetics, genomics, molecular biology, and biochemical approaches.

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