

Abscisic Acid Biosynthesis and Catabolism

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Abstract

The level of abscisic acid (ABA) in any particular tissue in a plant is determined by the rate of biosynthesis and catabolism of the hormone. Therefore, identifying all the genes involved in the metabolism is essential for a complete understanding of how this hormone directs plant growth and development. To date, almost all the biosynthetic genes have been identified through the isolation of auxotrophic mutants. On the other hand, among several ABA catabolic pathways, current genomic approaches revealed that *Arabidopsis* CYP707A genes encode ABA 8'-hydroxylases, which catalyze the first committed step in the predominant ABA catabolic pathway. Identification of ABA metabolic genes has revealed that multiple metabolic steps are differentially regulated to fine-tune the ABA level at both transcriptional and post-transcriptional levels. Furthermore, recent ongoing studies have given new insights into the regulation and site of ABA metabolism in relation to its physiological roles.

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INTRODUCTION

ABA belongs to a class of metabolites known as isoprenoids, also called terpenoids. They derive from a common five-carbon (C_5) precursor, isopentenyl (IDP). Until recently, it was thought that all isoprenoids were synthesized from MVA. However, recently, an alternative pathway to synthesize IDP was discovered, first in certain eubacteria and then in higher plants (65a, 89a). Plastidic isoprenoids, including carotenoids, originate from IDP synthesized from this MVA-independent pathway, called the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (24, 89).

Although ABA contains 15 carbon atoms, in plants it is not derived directly from the C_{15} sesquiterpene precursor, farnesyl diphosphate (FDP), but is rather formed by cleavage of C_{40} carotenoids originating from the MEP pathway (47, 56, 72). Evidence for ABA synthesis from carotenoids has been obtained by ^{18}O labeling experiments, molecular genetic analysis of auxotrophs, and biochemical studies. The mile-

stones of the discovery of this "indirect pathway" are described in detail in the previous review on ABA metabolism in this series (128).

ABA BIOSYNTHETIC AND CATABOLIC PATHWAYS

The molecular basis of ABA metabolism was established by genetic approaches. Most of *viviparous* mutants in maize are defective in carotenoid biosynthesis (68). These mutants showed an albino phenotype with a reduced ABA level. In contrast, in a variety of plant species, phenotypes of mutants defective in downstream of xanthophyll cycle are most likely due to ABA deficiency, which is characterized by a wilted plant and production of nondormant seeds. Several recent reviews described the upstream of ABA biosynthesis, particularly MEP and carotenoid pathways (24, 28, 89). Therefore, we focus on the current advances in ABA biosynthetic steps following xanthophyll formation and on the catabolic pathway. Several other reviews describing the ABA metabolic pathway were recently published (20, 98, 101, 120).

ABA Biosynthesis

Epoxy-carotenoid synthesis. Zeaxanthin is produced as a *trans*-isomer after cyclization and hydroxylation of all-*trans*-lycopene via β -carotene. The following steps consist of the synthesis of *cis*-isomers of violaxanthin and neoxanthin that will be cleaved to form a C_{15} precursor of ABA (**Figure 1**).

Conversion of zeaxanthin to violaxanthin is catalysed by zeaxanthin epoxidase (ZEP) via the intermediate antheraxanthin. The ZEP gene, which was first cloned in *Nicotiana plumbaginifolia* by insertional mutagenesis, encodes a protein with sequence similarities to FAD-binding monooxygenases that requires ferredoxin (14, 67). Mutants impaired in ZEP have been isolated in several species, including *Arabidopsis* (60, 69, 77, 119), *N. plumbaginifolia* (67), and rice (1). They accumulate zeaxanthin and show a severe reduction in ABA content, which

ABA: abscisic acid
MVA: mevalonic acid
MEP pathway: Isopentenyl diphosphate derives in plastids from pyruvate and glyceraldehyde 3-phosphate via the formation of 2-C-methyl-D-erythritol-4-phosphate (MEP). In higher plants, both MVA and MEP pathways coexist, in contrast to many eubacteria and green algae, in which only the MEP pathway is present.

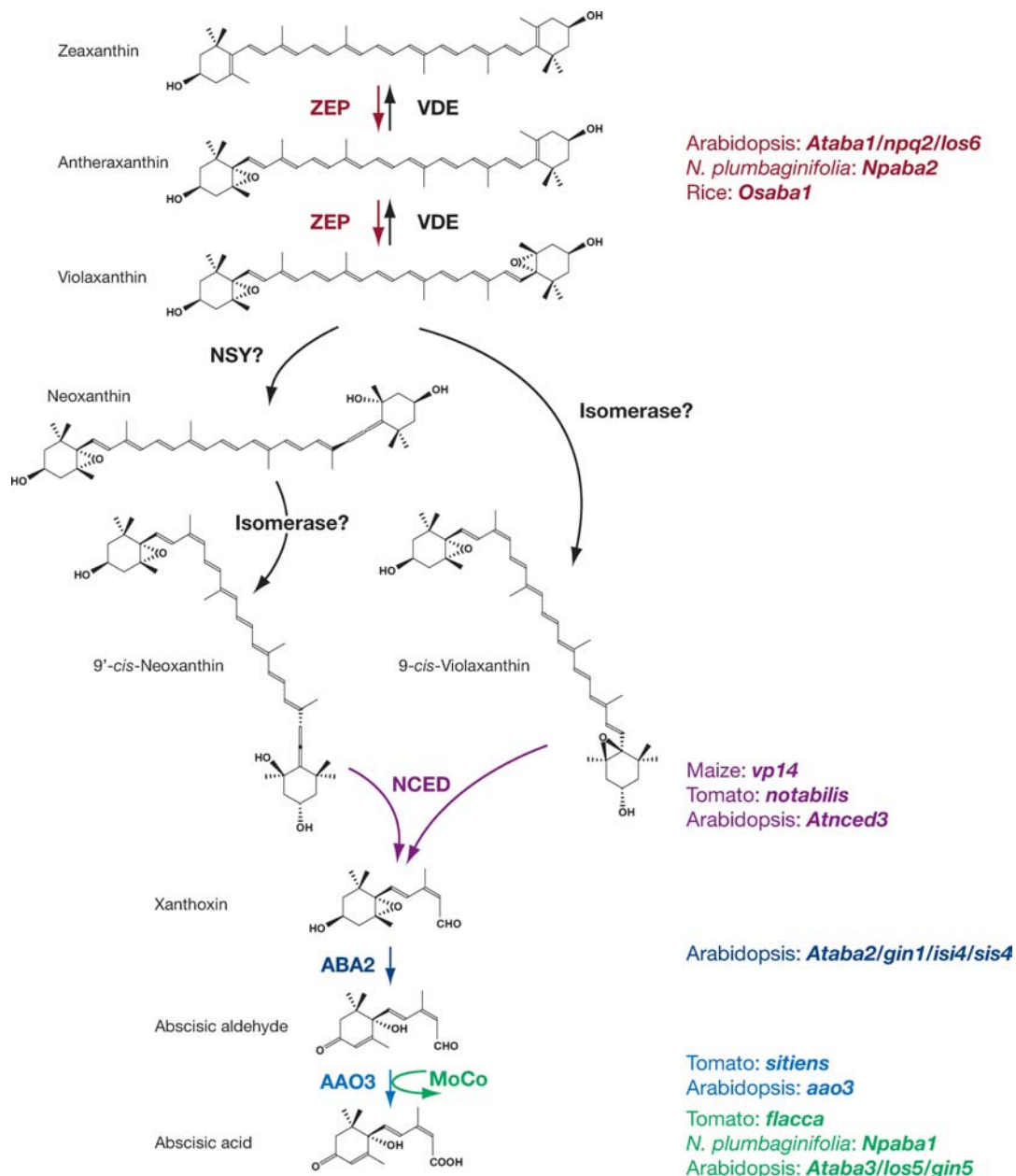


Figure 1

ABA biosynthetic pathway. Synthesis of violaxanthin is catalyzed by zeaxanthin epoxidase (ZEP). A reverse reaction occurs in chloroplasts in high light conditions catalyzed by violaxanthin de-epoxidase (VDE). The formation of *cis*-isomers of violaxanthin and neoxanthin may require two enzymes, a neoxanthin synthase (NSY) and an isomerase. Cleavage of *cis*-xanthophylls is catalyzed by a family of 9'-*cis*-epoxycarotenoid dioxygenases (NCED). Xanthoxin is then converted by a short-chain alcohol dehydrogenase (ABA2) into absciscic aldehyde, which is oxidized into ABA by an absciscic aldehyde oxidase (AAO3). AAO3 protein contains a molybdenum cofactor activated by a MoCo sulfurase. A list of defective mutants, which have been named separately depending on species or selective screens, is given on the right side of each enzymatic step.

SDR: short-chain dehydrogenase/reductase

leads to a wilted phenotype and production of nondormant seeds. In *Arabidopsis*, mutations causing amino acid substitutions in the monooxygenase domain impair enzyme function, indicating that this domain might be important for activity (69, 119).

Synthesis of neoxanthin from violaxanthin is not fully elucidated. By homology to lycopene β -cyclase (*LCYB*) and capsanthin capsorubin synthase from pepper, putative neoxanthin synthase (*NSY*) genes of tomato and potato have been isolated (2, 13). However, no *NSY* homologous gene could be found in the *Arabidopsis* genome that contains a unique *LCYB* gene (28). Furthermore, mutations in the putative tomato *NSY* gene were later found to affect β -carotene synthesis from lycopene, therefore proving that this gene encoded a *LCYB* isoform (90). Recently, mutants lacking neoxanthin isomers were identified in *Arabidopsis* (H. North & A. Marion-Poll, unpublished results) and tomato (J. Hirschberg, personal communication). The *Arabidopsis* gene has been cloned (H. North & A. Marion-Poll, unpublished) and further biochemical analysis will indicate whether the encoded protein exhibits *NSY* activity and produces only all-*trans* neoxanthin or both neoxanthin isomers. The gene encoding a *trans-cis* isomerase has not yet been found.

Xanthophyll cleavage. Nine-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes cleave the *cis*-isomers of violaxanthin and neoxanthin to a C₁₅ product, xanthoxin, and a C₂₅ metabolite (98). The first *NCED* gene (*VP14*) was cloned in maize by insertional mutagenesis (99, 109). Maize *VP14* recombinant protein was able to cleave 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin but not *trans*-xanthophyll isomers (99). Enzyme activity requires iron and oxygen to form a *cis*-isomer of xanthoxin (99). In all plant species analyzed, *NCED* genes belong to a multigene family. In accordance, *nced* mutants, such as *vp14* of maize and *notabilis* of tomato, exhibit mild ABA-deficient phenotypes due to gene redundancy (17, 109). In *Arabidopsis*, nine *NCED*-related sequences have

been identified, and the sequence and functional analyses indicate that five of them (*AtNCED2*, 3, 5, 6, and 9) are most probably involved in ABA biosynthesis (50, 98). Recently, a new leading compound for ABA biosynthesis inhibitors targeting the *NCED* was developed (39). This inhibitor might facilitate the study of ABA-mediated physiology in many plant species for which genetic approaches are not available.

As is the case for other carotenoid biosynthesis enzymes, *NCED* proteins from various species are chloroplast-targeted (51, 83, 107, 108). Because the following enzymatic reaction takes place in the cytosol (18), xanthoxin is presumed to migrate from plastid to cytosol by an unknown mechanism.

C₁₅ cytosolic pathway. ABA, the biologically active form, is produced from *cis*-xanthoxin by two enzymatic steps via the intermediate abscisic aldehyde (**Figure 1**). To date, genes encoding these enzymes have been identified only from *Arabidopsis*. The conversion of xanthoxin to abscisic aldehyde is catalyzed by *AtABA2*, belonging to the *SDR* family. This gene was identified by map-based cloning (18, 35) after the isolation of numerous *Arabidopsis* mutant alleles from various genetic screens (35, 65, 69, 74, 85, 91). *AtABA2* protein is encoded by a single gene in the *Arabidopsis* genome; therefore, loss-of-function of this gene leads to a severe ABA deficiency. Mutations have been identified in putative functional domains (NAD binding domain, catalytic center, subunit interacting helix, and substrate binding site) that affect ABA production, indicating the importance of these domains for enzyme activity (35). Furthermore, intragenic complementation between mutant alleles suggests that *AtABA2* might have a multimeric structure in accordance with the dimeric or tetrameric structure for most *SDR* proteins from various organisms (54, 69, 91).

The oxidation of the abscisic aldehyde to the carboxylic acid is the final step in ABA biosynthesis, catalyzed by an abscisic aldehyde oxidase. Among four *Arabidopsis* aldehyde

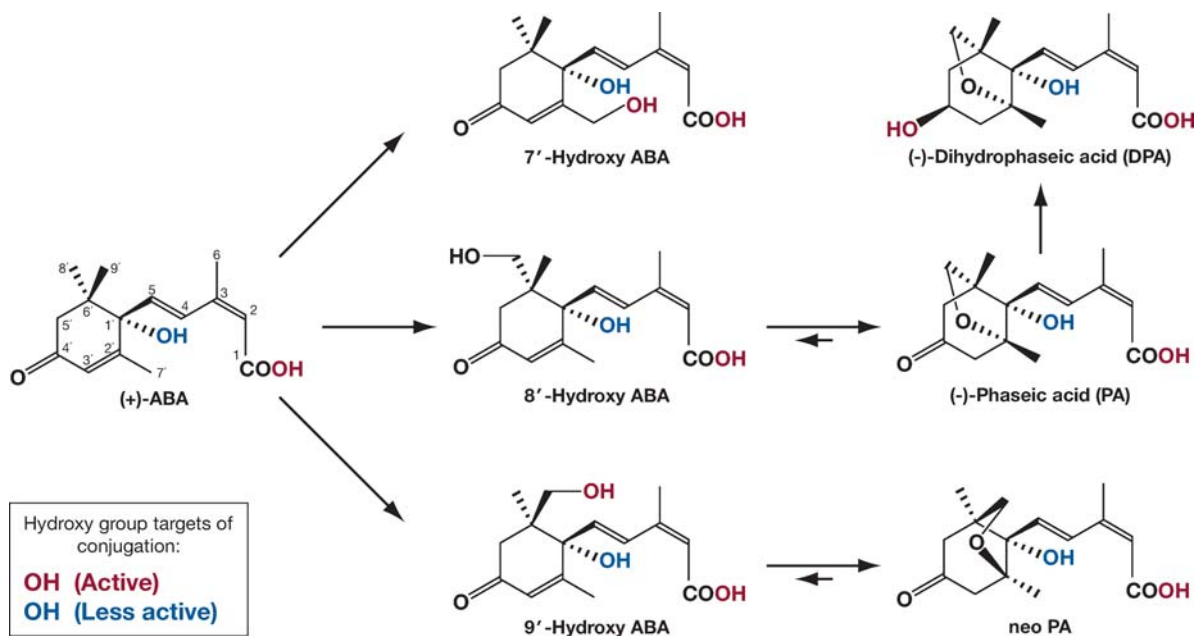


Figure 2

ABA catabolic pathways. Three different hydroxylation pathways are shown. The 8'-hydroxylation is thought to be the predominant pathway for ABA catabolism. Asterisks indicate targets for the conjugation. Red and blue asterisks indicate active and less-active hydroxy groups for conjugation, respectively.

oxidases (AAOs), *AAO3* encodes an enzyme active on abscisic aldehyde (102). The *aao3-1* mutant, containing a mutation in an intron-splicing site, showed a wilted phenotype but only a minor reduction in seed dormancy, compared to other *Arabidopsis aba* mutants affected on unique genes. This mild seed phenotype was thought to be attributed to gene redundancy and it had been postulated that other AAO genes might also be involved in ABA biosynthesis (100). However, identification of null *aao3* alleles exhibiting significant ABA-deficient phenotypes in seeds indicated that *AAO3* is most likely the only AAO gene involved in ABA synthesis (34, 99a).

Aldehyde oxidase requires a molybdenum cofactor (MoCo) for its catalytic activity. Therefore, mutations in the genes for MoCo biosynthesis lead to ABA deficiency. Consistent with this, mutations in the *FLACCA* in tomato (92) and *AtABA3* in *Arabidopsis* (10, 122) en-

coding a MoCo sulfurylase confer the expected ABA-deficient phenotypes.

ABA Catabolism

ABA catabolism is largely categorized into two types of reactions, hydroxylation and conjugation (Figure 2). There are three different ABA hydroxylation pathways that oxidize one of the methyl groups of the ring structure (C-7', C-8', and C-9'). Three forms of hydroxylated ABA contain substantial biological activities (130, 131), but hydroxylation triggers further inactivation steps. The hydroxylation at C-8' position is commonly thought to be the predominant ABA catabolic pathway (20, 128). In addition to hydroxylation pathways, ABA and its hydroxylated catabolites [8'-hydroxy ABA, PA, DPA, and *epi*-DPA] are conjugated to glucose (15, 45). A minor inactive form, 2-*trans*-ABA, was also identified. The *cis-trans* isomerization is a photo-permissive equilibrium

PA: phaseic acid
DPA: dihydrophaseic acid

reaction and is not an enzymatic conversion in tomato (70).

P450: Cytochrome P450 monooxygenases are heme-containing enzymes that catalyze the oxidative reaction of diverse organic compounds by utilizing atmospheric O₂.

EST: expressed sequence tag

ABA hydroxylation. Among the ABA catabolic pathways, the 8'-hydroxylation is reportedly the major regulatory step in many physiological events controlled by ABA. In accordance, PA and DPA are the most widespread and abundant ABA catabolites (20, 128). In addition, ABA analogs modified at the C-8' methyl group that are resistant to the hydroxylation exhibit stronger ABA-like activities compared to other substitutions (21, 113, 115). ABA 8'-hydroxylation is catalyzed by a cytochrome P450 monooxygenase (P450) and 8'-hydroxy ABA is then isomerized spontaneously to PA (32, 61). Ninety-eight percent of 8'-hydroxy ABA exist as PA at the equilibrium under normal laboratory conditions (114). Although this isomerization occurs quickly in vitro, this reaction is thought to be catalyzed enzymatically in vivo (71). PA is further catabolized to DPA by a soluble reductase (32).

ABA is biologically inactivated in a stepwise manner during the course of catabolism. The 8'-hydroxy ABA contains substantial biological activity (4, 131). Spontaneous cyclization to form PA causes a significant reduction in biological activity, although the degree of reduction varies among bioassays (8, 9, 38, 44, 88, 131). Recent reports showed that the ABA-binding proteins from apple fruit and barley aleurone layers are unable to bind to PA (87, 129), suggesting that PA is an inactive catabolite at least for some physiological processes. DPA is inactive in various bioassays; therefore ABA inactivation is complete by this stage (116).

The 7'-hydroxy ABA is found in a variety of plant species as the minor catabolite (116, 128), and 9'-hydroxy ABA and its isomer neoPA were recently identified as abundant ABA catabolites in *Brassica napus* immature seed (130). In addition, this 9'-hydroxylated product appears to exist also in other plant species such as pea, orange, barley, and *Arabidopsis* (130). Further investigation of this catabolic route should elucidate new aspects of ABA catabolism.

Identification of CYP707A genes encoding ABA 8'-hydroxylase. Recently, *Arabidopsis* P450 CYP707A genes were identified by the reverse genetic approach to encode ABA 8'-hydroxylases (62, 93). Biochemical analysis showed that the recombinant CYP707A protein converts ABA to PA in vitro, but none of the other hydroxylated catabolites (such as 7'-hydroxy or 9'-hydroxy ABA) were produced. CYP707A does not appear to be involved in cyclization of 8'-hydroxy ABA to PA because ABA is primarily converted to 8'-hydroxy ABA in a short incubation period and then 8'-hydroxy ABA is autoisomerized to PA (93). The activity of CYP707A was inhibited by a P450 inhibitor tetcyclasis, which was originally developed as an inhibitor of GA biosynthesis, but not by another P450 inhibitor metyrapone (62). This indicates that CYP707A discriminates between two different known P450 inhibitors. Therefore, it might be possible to develop a specific inhibitor of this enzyme in the future.

CYP707A appears to be widespread in many plant species. CYP707A-related sequences are found in rice genome and among ESTs from tomato, soybean, and maize (<http://drnelson.utmem.edu/CytochromeP450.html>). CYP707A sequences are also identified in lettuce and wheat (T. Toyomasu, N. Kawakami & E. Nambara, unpublished results).

ABA conjugation. The carboxyl (at the C-1) and hydroxyl groups of ABA and its oxidative catabolites are the potential targets for conjugation with glucose (**Figure 2**). ABA glucosyl ester (ABA-GE) is the most widespread conjugate (15). In addition to the glucosyl esters, other conjugates with the hydroxyl groups of ABA and its hydroxylated catabolites are also reported. ABA conjugates had been thought to be physiologically inactive and accumulate in vacuoles during aging (16, 63). However, recently ABA-GE was proposed to be involved in long-distance transport of ABA (42, 118). ABA-GE was identified as an allelopathic substance of *Citrus junis* (57), and soil in agricultural fields contains higher concentrations of

ABA-GE (up to 30 nM) than ABA. It has been hypothesized that ABA-GE is taken up by the root (96). Furthermore, ABA-GE is the most abundant catabolite in the sunflower xylem sap (40). β -D-glucosidase releases ABA from ABA-GE in wheat, barley, and sunflower (23, 64, 95). The β -D-glucosidase activity is enhanced by salinity and is inhibited competitively by ABA-GE or zeatin riboside. However, because ABA-GE cannot migrate passively through the plasma membrane, the molecular mechanism underlying the transport of ABA or its conjugates remains unclear.

Identification of the AOG gene encoding ABA glucosyltransferase. The *AOG* gene encoding ABA glucosyltransferase was identified from adzuki bean as the first reported gene for ABA catabolism (123). The AOG recombinant protein can conjugate ABA with UDP-D-glucose. AOG exhibits a broad substrate specificity compared to other ABA catabolic enzyme CYP707As. AOG catalyzes the conjugation of 2-*trans*-ABA to glucose more efficiently than natural 2-*cis*-ABA, consistent with previous feeding experiments in tomato (70). AOG can also use an ABA analog (-)-*R*-ABA or cinnamic acid as substrates, but not the immediate ABA catabolite PA. Therefore, PA and DPA glucosylation might be catalyzed by different enzymes (123).

REGULATION OF ABA METABOLISM IN RELATION TO ITS PHYSIOLOGICAL ROLES

Regulatory Steps, Factors, and Levels

The endogenous ABA level is modulated by the precise balance between biosynthesis and catabolism of this hormone. With regard to ABA biosynthesis, NCED has been proposed to be the regulatory enzyme because its expression is well correlated to endogenous ABA content (98) and its overexpression confers a significant ABA accumulation (50, 84, 111). On the other hand, ABA 8'-hydroxylase is most likely

the major regulatory enzyme in many physiological processes, as described below (20, 62, 93, 128).

Aside from these two main regulatory steps in the ABA metabolic pathway, metabolic steps upstream of ABA metabolism also contribute to determining the ABA level. Overexpression of genes encoding regulatory enzymes for the MEP pathway (1-deoxy-D-xylulose 5-phosphate synthase), carotenoid biosynthesis (phytoene synthase), and xanthophyll cycle (*ZEP*) causes an enhanced accumulation of ABA in seeds or seedlings (25, 29, 66). Taken together, this indicates that the regulation of ABA metabolism is not merely restricted to specific steps in ABA metabolism (i.e., NCED and CYP707A), but is also coordinated with the upstream metabolism.

To date, the regulation of ABA metabolism has been studied mostly at the transcription level. This process is differentially regulated by external and endogenous signals. In particular, the expression of *AtNCED3*, *AAO3*, *AtABA3*, and *AtZEP*, but not *AtABA2*, genes are induced by dehydration in *Arabidopsis*, as detailed below, whereas the expression of *AtABA2*, *AtZEP*, and *AAO3*, but not *AtNCED3*, are induced by application of glucose that induces ABA accumulation (18). Moreover, all four *Arabidopsis* *CYP707A* genes are induced by osmotic stresses (62). In addition to external signals, the expression of the *CYP707A3* is positively regulated by gibberellin (GA) and brassinolide (93), indicating that CYP707A genes function as the node of hormone interactions. Aside from these interactions, several reports indicate that many biosynthetic and catabolic genes are also up-regulated by the application of ABA, suggesting that ABA might regulate its own accumulation (18, 62, 93, 119, 121, 123).

Genetic analysis of the *sad1* (supersensitive to ABA and drought) mutant of *Arabidopsis* indicated that ABA biosynthesis is also regulated at the level of mRNA stability. The *SAD1* locus encodes a peptide similar to multifunctional Sm-like snRNP proteins required for mRNA processing (121). The *sad1* mutant shows reduced levels of ABA and PA, and expression

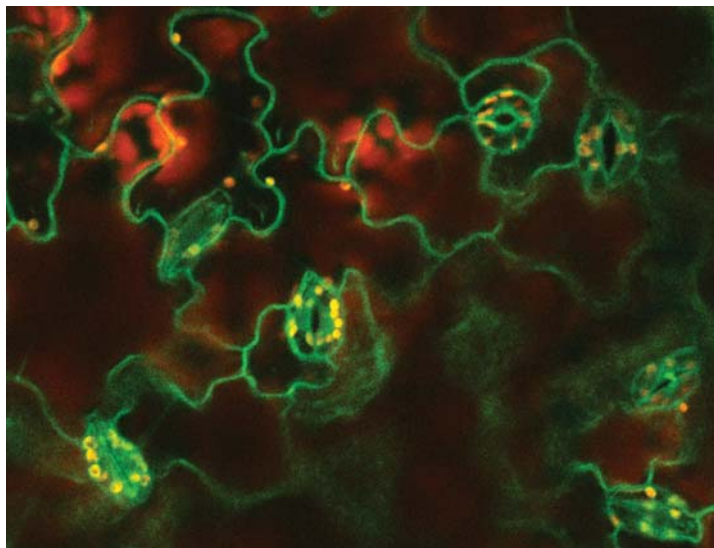


Figure 3

Localization of the pAAO3::AAO3-GFP protein in guard cells. A representative view of turgid transgenic *Arabidopsis* carrying pAAO3::AAO3-GFP is shown.

and feeding analyses demonstrated that SAD1 is a positive regulator of *AAO3* and *AtABA3*. Although it is unclear how SAD1 functions in regulating feedback on ABA metabolism and signaling, the identification of several RNA processing genes through genetic screens suggests that RNA processing is closely tied to the regulation of these processes.

The Sites of ABA Biosynthesis

Study on the site of ABA biosynthesis is essential to link the understanding of the regulation of ABA metabolism to physiology and development. Especially, in contrast to stress-induced ABA accumulation, little is known about the role and function of ABA in plant growth and development under unstressed conditions. Identification of ABA metabolic genes enables the study of where and when these genes are expressed in the plant life cycle.

In turgid tissues the expression of *AtNCEDs*, *AtABA2*, and *AAO3* genes is observed in vascular bundles (18, 59, 108). Koiwai et al. (2004) reported that the AAO3 protein is abundantly localized in phloem companion cells and xylem parenchyma cells of turgid plants (59). Therefore, vascular tissues are probably the main site of ABA biosynthesis in unstressed plants and

ABA and its precursors might be synthesized in vascular tissues and transported to target cells such as stomata.

The localization and regulation of the expression of biosynthetic genes in guard cells is particularly interesting with regard to the role of ABA in stomatal closure. Although many studies have given evidence for the transport of ABA to target cells (94), recent data indicate that ABA synthesis is also active in guard cells. Through reporter gene analysis *AtNCED2* and *AtNCED3* transcripts were expressed in guard cells of senescent leaves and cotyledons, respectively (108). In addition, the *AAO3* encoding the enzyme for the final step in ABA biosynthesis is induced in guard cells upon stress. In the same study, by immuno-fluorescence and expression studies using a reporter-fused protein, the AAO3 protein was also present in guard cells (59) (**Figure 3**). It is, therefore, probable that ABA synthesized inside guard cells, in concert with transported ABA, triggers the downstream signaling cascade leading to stomatal closure.

Nevertheless, it remains to be elucidated whether ABA synthesis is still restricted to the same sites or becomes activated in other plant tissues under stress conditions.

Regulation of ABA metabolism depends on internal and external signals, as well as developmental stages, organs, or tissues. This is illustrated in the two sections below, which describe in more detail the regulation of ABA metabolism genes in two physiological processes highly controlled by the hormone, i.e., seed physiology and stress tolerance.

ABA Metabolism in Seeds

Embryogenesis and seed maturation. ABA has a dual role in embryo growth during seed development, as deduced from the physiological analysis of ABA-deficient mutants. In early embryogenesis, ABA prevents seed abortion and promotes embryo growth (18, 30). In contrast, during late embryogenesis when the hormone level increases, ABA blocks the embryo growth by counteracting the action of GA to

promote germination (86, 117). Recent studies show that the transcription factor FUS3 prevents vivipary in *Arabidopsis* seeds by positively regulating ABA levels and downregulating GA synthesis (31, 73). In addition, maternal ABA can inhibit viviparous germination in *fus3* mutants defective in embryo growth arrest (86). It will be interesting to see if other developmental mutants that affect embryogenesis impinge on ABA metabolism.

Despite the low levels of ABA generally detected during early embryogenesis, the ABA biosynthetic pathway is apparently active at this stage. *AtZEP*, *AtNCED5*, and *AtNCED6* transcripts have been detected in *Arabidopsis* young embryos (7, 108). In maternal tissues, the *AtZEP* gene was expressed in testa, *AtNCED3* and *AtABA2* in funicles, and *AtNCED5* and *AtNCED6* in maternal nucellar tissues of newly fertilized ovules (18, 108). These tissues might provide ABA or its precursors to the embryo, and in agreement, high ABA levels have been found in the pedicel/placento-chalazal complex of maize kernels (53).

A major increase in ABA levels occurs during the maturation phase in relation to the positive regulation of a number of genes for seed reserves (27). Carotenoid precursors accumulate in seeds of most plant species and their synthesis is expected to precede their cleavage into xanthoxin. Consistent with this, maximal *ZEP* gene expression in *N. plumbaginifolia* appears to peak earlier (6) than that found in *Arabidopsis* *NCED* genes. Indeed, *AtNCED5* and *AtNCED6* show the strongest expression in *Arabidopsis* embryos at mid- to late-developmental stages (108). A decrease in the ABA level during the desiccation phase is expected to result from decreased ABA synthesis, as evidenced by very low *ZEP* transcript levels at this stage (6).

Although ABA catabolites have been detected in developing seeds and reproductive organs (19, 103), expression studies of catabolic genes are still limited. *CYP707A1* and *CYP707A3* are expressed abundantly during the mid-stage of *Arabidopsis* seed development and

are downregulated during late embryo development (62).

Seed dormancy and germination. Aside from its role in embryogenesis and seed maturation, ABA is absolutely required to induce seed dormancy during late embryogenesis. Genetic studies show that ABA produced by the embryo itself, and not maternal ABA, is necessary to impose dormancy (37, 55). Besides induction of dormancy in developing seeds, ABA is involved in maintaining dormancy during imbibition (3, 22, 36). Germination is preceded by a decrease in ABA levels resulting from both the suppression of de novo synthesis and the activation of catabolism (26, 62). In contrast, dormant seeds generally maintain endogenous ABA at the high levels, and dormancy is effectively released by the application of fluridone, which blocks the synthesis of carotenoid precursors of ABA. When ABA levels decrease during seed imbibition, concomitant increases in PA/DPA levels were observed in barley (52), lettuce (33), yellow-cedar (97), white pine (26), and *Arabidopsis* (62). In high-temperature-induced dormant lettuce seeds, ABA catabolism is positively regulated by GA because PA/DPA accumulation is accelerated by GA application (33).

In *Arabidopsis*, the catabolic enzyme CYP707A2 plays a major role in the rapid decrease in ABA levels during early seed imbibition (62). *CYP707A2* transcripts accumulate to a high level in dry seed, whereas other CYP707A transcripts are scarce. *CYP707A2* transcript levels increase within six hours of imbibition and decrease thereafter. The *cyp707a2* seeds exhibit hyperdormancy when sown without stratification. Furthermore, *cyp707a2* dry seeds accumulate sixfold more ABA than wild type and this high ABA level is maintained during seed imbibition (62). In addition, gene expression analysis suggests that *CYP707A1* and *CYP707A3* are possibly involved in seed germination and early seedling development because their transcripts are gradually accumulated after 12 hours of imbibition.

ABA Metabolism in Abiotic Stress Adaptation

Transcript levels of several ABA biosynthetic genes are upregulated by osmotic stresses. The expression of the *ZEP* gene is induced by both rapid or progressive drought stress in roots of *N. plumbaginifolia*, tomato, and *Arabidopsis* (6, 7, 110). In leaves, the high carotenoid levels are not likely to contribute positively to ABA synthesis even under stress conditions. In agreement, no upregulation of the *ZEP* gene has been reported in *Arabidopsis* or in *N. plumbaginifolia* and tomato (6, 7, 110). Moreover, *N. plumbaginifolia* transgenic plants overexpressing *ZEP* transcripts do not exhibit higher ABA levels and water stress tolerance compared to wild type (12). However, opposite results were also reported in *Arabidopsis* leaves, in which overexpression of the *ZEP* gene upregulates the expression of genes induced by drought, salt, and osmotic stress (119).

The induction of *NCED* gene expression has been observed in several species, both in roots and in leaves (51, 83, 108, 109, 111). Detailed studies with the *PvNCED1* gene from *Phaseolus vulgaris* provide evidence that the oxidative cleavage of xanthophylls is a major regulatory step of ABA accumulation under drought stress. Water stress-induced ABA accumulation is preceded by large increases in both *PvNCED1* transcript and protein levels in leaves and roots (83). In *Arabidopsis*, among the five *NCED* genes involved in ABA biosynthesis, only *AtNCED3* is highly induced by dehydration, although a positive but minor regulation of the other *NCED* genes was also observed (50, 108). In addition, *AtNCED3* overexpression in transgenic *Arabidopsis* plants increases both ABA levels and desiccation tolerance. This result is also seen in tomato and *N. plumbaginifolia* after transformation with the *LeNCED1* and *PvNCED1* genes, respectively (84, 111). Interestingly, the induction of *AtNCED3* under stress conditions is reduced in carotenoid-deficient mutants, suggesting that the expression level of this gene is correlated

with the levels of its substrates and/or ABA (112). Furthermore, the induction of this gene in response to exogenous ABA is highly enhanced in ABA-deficient mutant backgrounds (119).

Biochemical studies indicate that the activity of the last two biosynthetic enzymes is constitutive (105) and *AtABA2* transcripts are not induced upon osmotic stress in *Arabidopsis* (18). However, the expression of the two other genes, *AAO3* and *AtABA3*, involved in the conversion of abscisic aldehyde into ABA, is upregulated under osmotic stresses (122).

Although it is clear that ABA biosynthesis is responsive to stress conditions, it is becoming evident that the catabolism is also required to determine the ABA level in response to environmental conditions. PA levels and occasionally DPA levels increase following the increase in ABA content. Furthermore, the PA level continues to increase even after ABA levels reach the plateau. However, when dehydrated plants are subsequently rehydrated, the ABA level decreases and a concomitant increase in the PA level is observed in the *P. vulgaris*, *Xanthium strumarium* and *Arabidopsis* (41, 62, 126). In *Arabidopsis*, multiple *CYP707A* genes are expressed in most organs and the ratio of *CYP707A* transcripts varies among tissues. The expression of all *CYP707A* genes is induced by dehydration, although the induction is slower than that of *AtNCED3*, which encodes the key biosynthetic enzyme under dehydration conditions in *Arabidopsis* (62). A significant increase in all *CYP707A* transcripts is observed upon rehydration, in accordance with the increase in PA levels. *CYP707A* are also upregulated by salinity and osmotic stresses (93). In contrast to PA and DPA, ABA conjugate levels do not always vary in parallel to the change in ABA levels, suggesting that the conjugation is regulated in particular tissues and conditions (127). In agreement with this, *AOG* gene expression is significantly induced by dehydration and wounding in adzuki bean hypocotyls, but not in leaves (123).

EVOLUTION OF ABA METABOLISM

ABA Metabolism in Fungi

Some phytopathogens are known to synthesize ABA (81). The ABA level is thought to determine a plant's susceptibility to these fungi by negatively regulating the salicylic acid-dependent defense pathway (5). Studies on fungal ABA biosynthesis have been mostly conducted in *Cercospora rosicola*, *Cercospora cruenta*, *Cercospora pini-densiflorae*, and *Botrytis cinerea*. Feeding experiments using [1-¹³C]-D-glucose clearly demonstrate that fungal ABA is derived from the MVA pathway (47, 124), in contrast to plant ABA, which originates from the MEP pathway.

ABA biosynthesis in fungi can be divided largely into two parts (**Figure 4**). The isomers of ionylideneethanol and/or ionylideneacetate have been identified from several fungi (81). Therefore, the early steps seem to convert FDP derivatives to ionylideneacetate, and the latter steps in their oxidation at C-1' and C-4' to produce ABA. Feeding experiments show that similar but distinct intermediates are identified among fungal genera (81, 128). This suggests that ABA biosynthetic pathway and/or its regulation might be different among these fungi.

In early steps, MVA is converted into ionylideneacetaldehyde via cyclization and isomerization of FDP derivatives. Direct cyclization of the sesquiterpene was proposed in fungal ABA biosynthesis, although experimental evidence is still missing. ¹⁸O₂ labeling experiments demonstrate that the oxygen atom at C-1 of ABA is derived from molecular oxygen in *C. cruenta* and *B. cinerea* (49, 124). This opens two possibilities. One is that dephosphorylation and reduction of FDP occurs to produce alfoarnesene prior to the cyclization, which is then oxidized to ionylideneacetaldehyde. Alternatively, ionylideneacetaldehyde is produced via the cleavage of C₄₀ carotenoids. Some fungi, such as *C. rosicola* and *C. cruenta*, produce β-carotene and other carotenoids (78, 124), but carotenoids (except for phytoene) are not found

in other ABA-synthesizing fungus *B. cinerea* (49, 49a). Recently in *B. cinerea* and *C. cruenta*, alfoarnesene and ionylideneethane were shown to be endogenous compounds that were able to convert to ABA (49, 49a). This indicates that ABA is synthesized by the direct pathway via the cyclization of alfoarnesene and oxidation of ionylideneethane in this fungus.

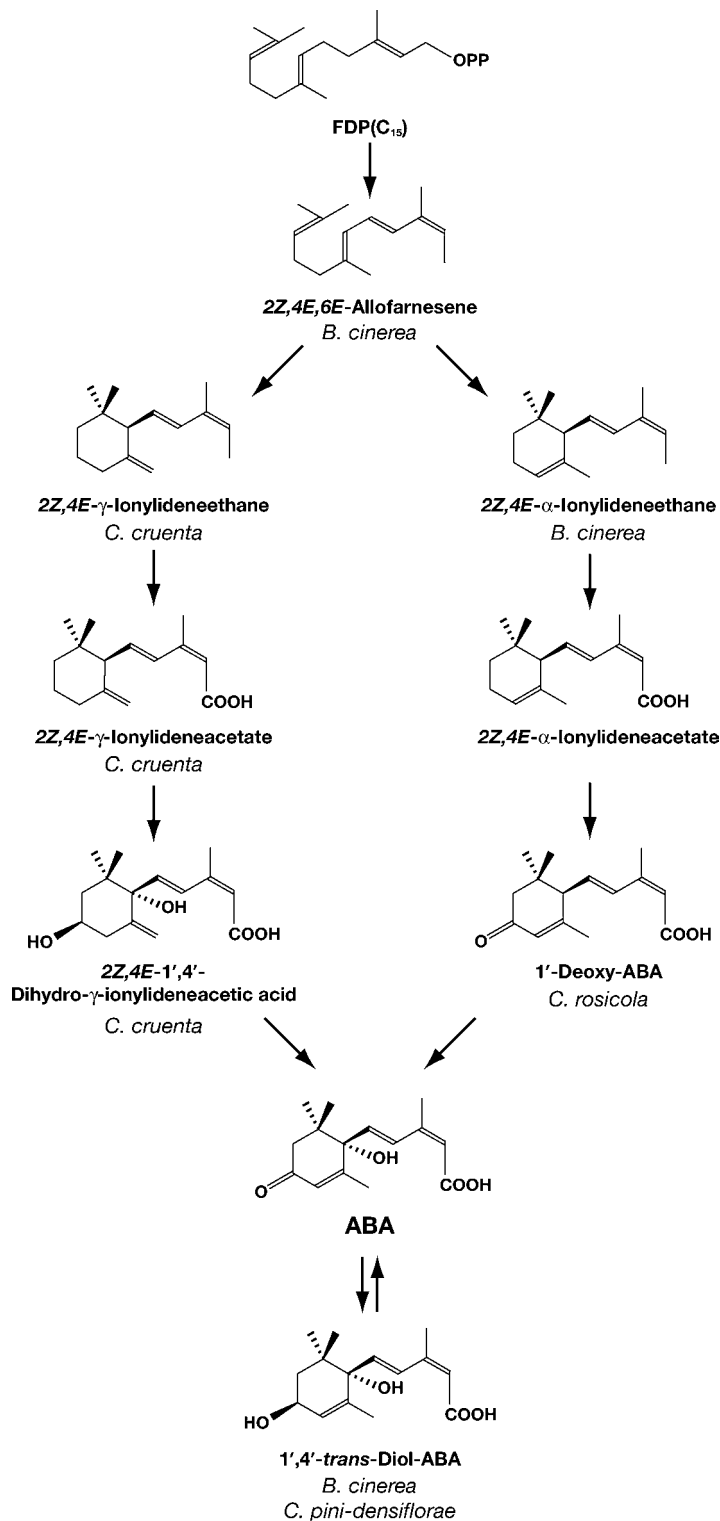
The latter steps involve the oxidation of C-1' and C-4' of ionylideneacetate. α-Ionylideneethanol/ionylideneacetate were converted to ABA and 1'-deoxy ABA in *C. rosicola* (75). 1'-Deoxy ABA is thought to be the precursor of ABA in this fungus, because it is oxidized stereoselectively to ABA (75). On one hand, in *B. cinerea* and *C. pini-densiflorae*, 1',4'-trans-diol ABA is likely the predominant precursor whose endogenous levels are correlated with ABA synthesis (46, 80). On the other hand, 1',4'-trans-dihydro-γ-ionylideneacetaldehyde is thought to be the intermediate of ABA biosynthesis in *C. cruenta* (82). Three oxygen atoms at C-1, C-1', and C-4' derive from atmospheric oxygen in *C. cruenta* and *B. cinerea* (49, 124). In addition, it has been reported that several P450 inhibitors block the ABA synthesis in *C. rosicola* (79), indicating that P450 is most likely involved in these oxidations. This was recently proven by the genomic approach, which showed that targeted inactivation of P450 oxidoreductase reduced ABA production in *B. cinerea* (104). Furthermore, loss-of-function of a P450 gene, *BcABA1*, whose expression is associated with the ABA production, abolished the accumulation of ABA in this fungus (104). The *BcABA1* gene will likely be the first ABA biosynthetic gene identified from fungi. Because functionally related genes are often clustered in fungal genomes, the molecular basis of fungal ABA biosynthesis will be elucidated in the near future.

ABA Metabolism in Lower Plants

In addition to higher plants and fungi, ABA is synthesized in moss, fern, and algae. ABA is found in all divisions and classes of algae, including colorless species (48). In green algae

MVA pathway:

Isopentenyl diphosphate derives in the cytosol from acetyl-coenzymeA via the formation of MVA. It is the only pathway for isoprenoid synthesis in archaeobacteria, fungi, and animals.



Chlamydomonas reinhardtii, application of ABA enhances the resistance to the oxidative stress (125). A study using carotenoid biosynthesis inhibitors suggests the direct pathway is proposed to be active in green algae *Dunaliella* (11). However, this issue is still in debate about whether these organisms synthesize ABA through a direct pathway or a carotenoid-derived indirect pathway.

In *Riccia fluitans*, ABA and its catabolite content fluctuates in response to water status (43). Accumulation of ABA in *Riccia* thalli is inhibited by the application of fluridone, suggesting that ABA is synthesized via carotenoids. Feeding experiments using radio-labeled ABA show PA and DPA as major catabolites, and minor catabolites include ABA-GE. PA/DPA production is inhibited by tetcyclacis, a P450 inhibitor. Therefore, it is likely that *Riccia* synthesizes and catabolizes ABA through the same metabolic pathway as that in higher plants. Interestingly, the same ABA responsive element as in higher plants acts on ABA-mediated transcription in the moss *Physcomitrella patens* (58).

Current genome sequencing and EST projects in many organisms facilitate the understanding of the evolutionary aspects of the ABA metabolic pathway. The EST project of the moss *Physcomitrella patens* reveals genes highly similar to *NCED* and *CYP707A* (76). This indicates that this moss synthesizes and catabolizes ABA through pathways similar to those in higher plants. *Chlamydomonas* contains a gene highly homologous to *ZEP*, consistent with the identification of endogenous xanthophylls (such as 9'-*cis*-neoxanthin) (106). In addition, *Chlamydomonas* contains several genes belonging to the RPE65 family, although protein sequences are more related to the carotenoid-cleaving dioxygenases acting at the 15' position rather than *NCED*, which cleaves at the

Figure 4

Proposed ABA biosynthetic pathways in fungi. Direct (cyclization of C₁₅ terpenoid) is proposed in fungal ABA biosynthesis. Identified potential intermediates with fungal species are shown.

9' or 9 positions of xanthophylls. Interestingly, *Chlamydomonas* also contains a P450 gene that is related to CYP707 (85-clan). Functional anal-

yses of these genes will elucidate the nature of evolution of ABA and carotenoid metabolic pathways in lower plants.

SUMMARY POINTS

1. Both forward and reverse genetic analyses have been instrumental in identifying ABA biosynthetic genes, and most of the genes have been identified in higher plants.
2. *Arabidopsis* CYP707A genes were recently shown to encode ABA 8'-hydroxylases, which catalyze the committed step in the predominant ABA catabolic pathway.
3. Localization analyses of biosynthetic enzymes and their transcripts indicate that the vascular bundles are the active site of ABA synthesis in turgid plants.
4. Expression of ABA metabolism genes is temporarily and spatially regulated during seed development and germination.
5. Transcriptional and post-transcriptional gene regulation plays a crucial role in the ABA accumulation in response to osmotic stresses.
6. ABA biosynthesis in fungi is thought to occur through the direct cyclization of FDP derivatives, and allofarnesene and ionylideneethane are proposed to be intermediates in *Botrytis cinerea*. Furthermore, the first fungal ABA biosynthetic gene, *BcABA1*, was recently identified in *B. cinerea*.

FUTURE ISSUES TO BE RESOLVED

1. In higher plants, the enzymes catalysing the isomerization of xanthophylls are the only biosynthetic enzymes remaining to be identified. Furthermore, recent isolation of genes encoding two major catabolic enzymes encourages the identification of other genes involved in the diverse catabolic pathways.
2. A large research field needs to be explored to unravel the signaling pathways regulating the metabolic genes and their interactions with endogenous and environmental signals.
3. In other organisms, such as fungi, algae, or mosses, the ABA biosynthetic and catabolic pathways require further investigation to identify all the genes, enzymes, precursors, and catabolites. The regulation of ABA produced by phytopathogenic fungi might be of particular interest to study plant-pathogen interaction.

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ERRATA

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