Biochemical Characterization of the aba2 and aba3 Mutants in Arabidopsis thaliana

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Abstract

Abscisic acid (ABA)-deficient mutants in a variety of species have been identified by screening for precocious germination and a wilty phenotype. Mutants at two new loci, aba2 and aba3, have recently been isolated in Arabidopsis thaliana (L.) Heynh. (K.M. Léon-Kloosterziel, M. Alvarez-Gil, G.J. Ruips, S.E. Jacobsen, N.E. Olszewski, S.H. Schwartz, J.A.D. Zeevaart, M. Koornneef, and K.M.L.-K., personal communication). These mutants are impaired in the conversion of xanthoxin to ABA relative to the wild type. The next putative intermediate in ABA synthesis, ABA-aldehyde, was efficiently converted to ABA by extracts from aba2 but not by extracts from aba3 plants. This indicates that the aba2 mutant is blocked in the conversion of xanthoxin to ABA-aldehyde and that aba3 is impaired in the conversion of ABA-aldehyde to ABA. Extracts from the aba3 mutant also lacked additional activities that require a molybdenum cofactor (Moco). Nitrate reductase utilizes a Moco but its activity was unaffected in extracts from aba3 plants. Moco hydroxylases in animals require a desulfo moiety of the cofactor. A sulfido ligand can be added to the Moco by treatment with Na2S. Treatment of aba3 extracts with Na2S restored ABA-aldehyde oxidase activity. Therefore, the genetic lesion in aba3 appears to be in the introduction of S into the Moco.

ABA is a sesquiterpenoid plant growth regulator involved in the induction of seed dormancy and adaptation to a variety of stresses (Zeevaart and Creelman, 1988). The regulation of these physiological processes is in part due to de novo synthesis of ABA. Thus, an understanding of the ABA biosynthetic pathway and its regulation is essential for an appreciation of the factors mediating plant stress responses. The characterization of ABA-deficient mutants has been helpful in elucidating the biosynthetic pathway. For example, the aba1 mutant, which is impaired in the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Duckham et al., 1991; Rock and Zeevaart, 1991), provided definitive evidence that ABA is derived from an epoxy-carotenoid precursor in higher plants.

The first committed step in ABA biosynthesis appears to be the oxidative cleavage of an epoxy-carotenoid precursor to form xanthoxin (Parry et al., 1989). Following the cleavage reaction, xanthoxin is rapidly converted to ABA by a series of ring modifications and the oxidation of the aldehyde to a carboxylic acid. Sindhu and Walton (1987) proposed a pathway based on feeding potential intermediates to a cell-free system and monitoring their conversion to ABA. If a substrate was not efficiently converted to ABA in the cell-free system, it was considered an unlikely intermediate in vivo. The results from these experiments suggest that the ring transformations occur first to produce ABA-aldehyde and that the oxidation of the aldehyde is the final step (Fig. 1). During the conversion of xanthoxin to ABA-aldehyde, three modifications of the ring occur: oxidation of the 4'-hydroxyl to a ketone, desaturation of a 2'-3' bond, and opening of the epoxide ring.

Mutants that are impaired in the ring transformation have not yet been reported. A number of mutants have been identified in the final step of ABA biosynthesis, the oxidation of the aldehyde to the carboxylic acid (Taylor, 1991); fic and sit in tomato, dr in potato, aba1 in Nicotiana plumaginifolia, and nar2a in barley. The nar2a mutant in barley was shown to lack ABA-aldehyde oxidase, xanthine dehydrogenase, and nitrate reductase activities (Walker-Simmons et al., 1989). This pleiotropic phenotype is the result of a lesion in the synthesis of the Moco, which all three enzyme activities require. The aba1 mutant of N. plumaginifolia (Leydecker et al., 1995) and the fic mutant of tomato (Marin and Marion-Poll, 1997) lack aldehyde oxidase and xanthine dehydrogenase but not nitrate reductase. The phenotype of these two mutants may be explained by the existence of two forms of the Moco in eukaryotes (Wahl and Rajagopalan, 1982). Moco hydroxylases such as xanthine dehydrogenase and aldehyde oxidase use a desulfo form of the cofactor, whereas reductive hydroxylases such as nitrate reductase utilize a dioxygen form (Rajagopalan and Johnson, 1992). The lesion in the barley nar2a mutant must occur at an early step in the pathway, since it affects both forms of the cofactor. The lesion in the tobacco mutant appears to be in the modifi-

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Abbreviations: Moco, molybdenum cofactor; WT, wild-type Columbia.
zeaxanthin
abal
abal
Antheraxanthin
all-tram-Violaxanthin
9-cisF-Violaxanthin

HOM
aba24
xanthOxin
aba3
i;
WCOOH

AbsciSic
acid

Zeaxanthin
ab1
Antheraxanthin
ab1
all-trans-Violaxanthin
all-trans-Neoxanthin

9-cis-Violaxanthin


9-cis-Neoxanthin


Figure 1. Proposed pathway of ABA biosynthesis. The biochemical lesions in the aba1, aba2, and aba3 mutants of Arabidopsis thaliana are indicated.

Sequestration of the cofactor to form the desulfo moiety. In a Drosophila melanogaster (ma-1) mutant impaired in the “sulfuration” of the Moco, has been identified (Wahl et al., 1982).

The characterization of ABA-deficient mutants has been valuable in elucidating the function of ABA and the pathway of ABA biosynthesis. The biochemical characterization of mutants at two new loci in A. thaliana, aba2 and aba3 (Léon-Kloosterziel et al., 1996), is described below.

MATERIALS AND METHODS

WT and the mutants aba1–5 (isolation J11), aba2–1 (isolation J14), and aba3–1 (isolation J25) (Léon-Kloosterziel et al., 1996) were grown in a 9-h photoperiod as previously described (Rock and Zeevaart, 1991). Rosettes (approximately 30 d after planting) were frozen in liquid N2 and stored at −80°C until extraction.

Preparation of Enzyme Extracts

Leaves were ground with a mortar and pestle in 0.2 M KPi, pH 7.5, containing 10 mM DTT (3 mL g−1 leaf material). The extracts were filtered through four layers of cheesecloth and centrifuged at 12,000g for 10 min at 4°C. Proteins were precipitated from the supernatant with ammonium sulfate (80% saturation). The ammonium sulfate precipitate was resuspended in 50 mM KPi, pH 7.5, and desalted on an exclusion column (PD-10, Pharmacia) according to the manufacturer’s instructions.

Activity Gels

Extracts were subjected to native gel electrophoresis, and aldehyde oxidase activities were detected with the substrate heptaldehyde (Walker-Simmons et al., 1989). As previously reported, xanthine dehydrogenase activity is difficult to detect in crude extracts from Arabidopsis thaliana (LaBrie et al., 1992). However, the activity was easily detected on activity gels when a 16 to 32% ammonium sulfate fraction was analyzed with hypoxanthine (Aldrich) as a substrate.

Preparation of Substrates

Violaxanthin and neoxanthin isolated from spinach leaves were oxidized with ZnMnO2 (Taylor and Burden, 1972). Xanthoxin was then purified by normal-phase HPLC on a semipreparative column (0.78 × 30 cm; μPorasil, Waters). Xanthoxin was eluted with a linear gradient of 10 to 100% (v/v) ethyl acetate in hexane for 72 min at a flow rate of 2.5 mL min−1. A xanthoxin fraction was collected from 39 to 42 min and dried under a stream of N2. Xanthoxin was further purified by reverse-phase HPLC on a semipreparative column (μBondapak C18, Waters) with a linear gradient of 10 to 60% (v/v) ethanol in water for 40 min at 2.5 mL min−1. A fraction containing cis-xanthoxin was collected from 23 to 25 min. The identity and purity of xanthoxin was confirmed by GC-MS of the trimethylsilyl derivative (Gaskin and MacMillan, 1992). (+)-ABA-aldehyde was provided by Hoffmann-LaRoche (Basel, Switzerland).

Conversion of Xanthoxin and ABA-Aldehyde to ABA

Enzyme assays contained 50 mM KPi, 0.25 mM EDTA, 1 mM PMSF, 1 mM NADP, and the appropriate substrate (xanthoxin or (+)-ABA aldehyde) in a total volume of 200 μL. After the sample was incubated for 1 h at 28°C, 400 μL of acetonitrile was added to stop the reactions. Proteins were pelleted by centrifugation at 10,000g and [3H]ABA was added to the supernatant as an internal standard. The acetonitrile was evaporated in a centrifugal vacuum concentrator (Jouan, Winchester, VA), and 1 mL of 2% (v/v) acetic acid was then added to each sample and ABA was partitioned three times into ethyl acetate. The ethyl acetate fractions were pooled, dried under a stream of N2, and ABA was methylated with diazomethane. Methyl ABA was purified by normal-phase HPLC (Creelman et al., 1987) and analyzed on a gas chromatograph (model 6890, Hewlett-Packard) equipped with an electron capture detector. Samples were injected on a capillary column (HP-5, Hewlett-Packard) and chromatographed isothermally at 188°C. Quantitation of methyl ABA was performed as described previously (Cornish and Zeevaart, 1985).
(+)- and (−)-ABA were separated as their methyl esters on a column packed with Chiralcel OD (Daicel, Los Angeles, CA) with 10% 2-propanol in hexane as the solvent (Railton, 1987).

Inactivation and Reconstitution of ABA-Aldehyde Oxidase Activity

In extracts of WT, aldehyde oxidase was inactivated by treatment with 20 mM KCN for 1 h at room temperature. After the sample was incubated CN− was removed on a spin column, which was prepared by filling a 1-mL syringe with G-25 Sephadex equilibrated in 50 mM KPi, pH 7.5. For reactivation, extracts were made anaerobic by degassing under vacuum and purging with anaerobic hydrogen several times. Anaerobic solutions of dithionite and Na,S were added through a septum, and the extracts were incubated at 37°C for 30 min. Following the incubation, dithionite and Na,S were removed on a G-25 Sephadex spin column and ABA-aldehyde oxidase activity was assayed as described above.

Analysis of Polar Metabolites in the aba3 Mutant

Rosettes were frozen in liquid N₂ and lyophilized. The tissue was extracted with 80% methanol containing butylated hydroxytoluene (0.1 g/L). The methanol was evaporated under vacuum and the pH of the remaining aqueous portion was adjusted to 3.5 with acetic acid. The extract was then partitioned three times with an equal volume of diethyl ether. The aqueous phase was concentrated under a vacuum, filtered through a 0.45-μm HA filter (Millipore), and injected on a semipreparative (0.78 X 30 cm) HPLC column (μBondapak C₁₈, Waters). The column was eluted with a linear gradient of 0 to 50% (v/v) ethanol in water for 35 min at a flow rate of 2.5 mL min⁻¹.

RESULTS

Previous work has shown that the aba2−1 and aba3−1 mutants of Arabidopsis are ABA-deficient (Léon-Kloosterziel et al., 1996). Analysis of total carotenoids in aba2 and aba3 plants indicated no variation compared with WT (data not shown), which suggests that both mutants are impaired in the later steps of ABA biosynthesis. Cell-free extracts of WT A. thaliana were prepared to monitor the conversion of xanthoxin to ABA. The requirements for activity were similar to those reported for cell-free preparations from other species (Sindhu and Walton, 1987). NADP was the only cofactor necessary for activity. DTT, glutathione, or Cys enhanced the conversion of xanthoxin to ABA but were omitted from the assays because they caused nonenzymatic cis- to trans-isomerization of xanthoxin (data not shown). The conversion of xanthoxin to ABA by aba2, aba3, and WT extracts was measured as a function of protein concentration (Fig. 2). Cell-free extracts from the two mutants, aba2 and aba3, showed a substantially reduced ability to convert xanthoxin to ABA.

(+)-ABA-aldehyde was also fed to cell-free extracts to monitor its conversion to ABA. Extracts of aba2 converted (±)-ABA-aldehyde to (±)-ABA as efficiently as WT extracts (Fig. 3). It was established by chiral HPLC that the ABA derived from (±)-ABA-aldehyde consisted of approximately equal amounts of (+) and (−)-ABA (data not shown). Nonstereoselective conversion of (±)-ABA-aldehyde to (±)-ABA by ABA-aldehyde oxidase has also been observed in tomato (Yamamoto and Oritani, 1996). No ABA was detected in assays with extracts from aba3 plants at the protein concentrations tested (Fig. 3). The flic mutant of tomato and the aba1 mutant of N. plumbaginifolia, which are unable to oxidize ABA-aldehyde to ABA, accumulate trans-ABA-alcohol and a glucoside of trans-ABA-alcohol (Linforth et al., 1987; Kraepiel et al., 1994). The flic and sit mutants of tomato (Taylor et al., 1988) and the dr mutant in potato (Duckham et al., 1989) are capable of...
The lesion in aba3 may result from a mutation in the ABA-aldehyde oxidase apoprotein or in an enzyme involved in the synthesis of the Moco (Walker-Simmons et al., 1989; Leydecker et al., 1995). The activities of aldehyde oxidase, xanthine dehydrogenase, and nitrate reductase, which all require a Moco, were measured in extracts from WT and aba3 plants. Aldehyde oxidase activity was tested on an enzyme-activity gel using heptaldehyde as a substrate (Fig. 4). Both WT and aba2 extracts contained three activities capable of oxidizing heptaldehyde. All three aldehyde oxidase activities were absent in aba3 extracts. Likewise, xanthine dehydrogenase was not detectable on an activity gel in extracts from the aba3 mutant (Fig. 5). The activity of nitrate reductase, measured according to the method of Wray and Filner (1970), was not affected in aba3 extracts (data not shown).

During the process of purifying trans-ABA-alcohol, a large UV-absorbing peak unique to aba3 was observed in HPLC chromatograms (Fig. 6). The accumulation of this compound, which has not been identified, is another example of the pleiotropic phenotype of the aba3 mutant.
vated by treating extracts with dithionite and Na₂S. Thus, it is concluded that the pleiotropic phenotype of the aba³ mutant results from a defect in the introduction of sulfur into the Moco.

**DISCUSSION**

The biochemical lesions in the aba² and aba³ mutants of *A. thaliana* were identified by feeding potential ABA precursors to cell-free extracts and monitoring their conversion to ABA. The results indicate that both the aba² and aba³ mutants are impaired in the later steps of ABA biosynthesis. The aba² mutant was unable to convert xanthoxin to ABA but did convert ABA-aldehyde to ABA. Thus, aba² represents a mutant at a new locus impaired in the ring modifications of xanthoxin to produce ABA-aldehyde. Chemical oxidation of the ring hydroxyl with pyridinium chlorochromate is sufficient for the quantitative conversion of xanthoxin to ABA-aldehyde (S.H. Schwartz and J.A.D. Zeevaart, unpublished results), indicating that the ring modifications may result from a single enzymatic step and subsequent rearrangements. The previously characterized aba¹ mutant in *A. thaliana* (Rock et al., 1992) and the newly isolated aba³ mutant have pleiotropic phenotypes that are independent of their ABA deficiency. Because the aba² mutant appears to be specific for ABA biosynthesis, this mutant should be useful for studying the physiological roles of ABA. However, no major differences have been observed in the physiological behavior, independent of the ABA-deficient phenotype, of the aba¹, aba², and aba³ mutants. The aba¹ mutant has been used for studying the role of carotenoids in photoinhibition (Rock et al., 1992; Hurry et al., 1997) and the aba³ mutant may be useful for studying purine catabolism.

Lesions in the conversion of ABA-aldehyde to ABA may result from a defect in the aldehyde-oxidase apoprotein or the Moco, which the aldehyde oxidase requires (Walker-Simmons et al., 1989; Leydecker et al., 1995; Marin and Marion-Poll, 1997). Previously characterized eukaryotic Moco hydroxylases such as xanthine dehydrogenase and aldehyde oxidase require a desulfo moiety of the cofactor (Wahl and Rajagopalan, 1982), whereas nitrate reductase utilizes a variant form of the Moco that does not contain a sulfido ligand (Rajagopalan and Johnson, 1992). Three aldehyde oxidase activities were absent in extracts of the aba³ mutant (Fig. 4), but nitrate reductase was not affected. The inactivation of WT extracts with CN⁻ and the subsequent reactivation with Na₂S suggests that ABA-aldehyde oxidase activity requires a desulfo moiety of the Moco. Treatment of extracts of aba³ with dithionite and Na₂S also resulted in the activation of ABA-aldehyde oxidase activity. Therefore, the genetic lesion in aba³ presumably affects the introduction of sulfur into the Moco (Fig. 8).

The characterization of ABA-deficient mutants has been useful in elucidating the pathway of ABA biosynthesis. Multiple alleles of aba¹ have been identified in Arabidopsis (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996), and there are now mutants at two new loci impaired in the later steps of ABA biosynthesis. No mutants have been reported for the cleavage reaction, which may be the key regulatory step in the pathway. Mutants impaired in the cleavage reaction may not be viable or the existence of multiple isoforms may make them difficult to identify. However, the apparent lability and low abundance of the cleavage enzyme make it unlikely that the activity can be purified by classical biochemical methods. Therefore, the search for additional mutants is of interest.

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