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Biochemical Characterization of the *aba2* and *aba3* Mutants in *Arabidopsis thaliana*[1]

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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. Ruijs, S.E. Jacobsen, N.E. Olszewski, S.H. Schwartz, J.A.D. Zeevaart, M. Koornneef [1996] Plant J 10: 655–661), and the biochemical characterization of these mutants is presented here. Protein extracts from *aba2* and *aba3* plants displayed a greatly reduced ability to convert 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 Na_2S and dithionite. Treatment of *aba3* extracts with Na_2S 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., 1988). 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): *flc* and *sit* in tomato, *dr* in potato, *aba1* in *Nicotiana plumbaginifolia*, 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. plumbaginifolia* (Leydecker et al., 1995) and the *flc* 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 dehydroxylases such as nitrate reductase utilize a dioxo 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.

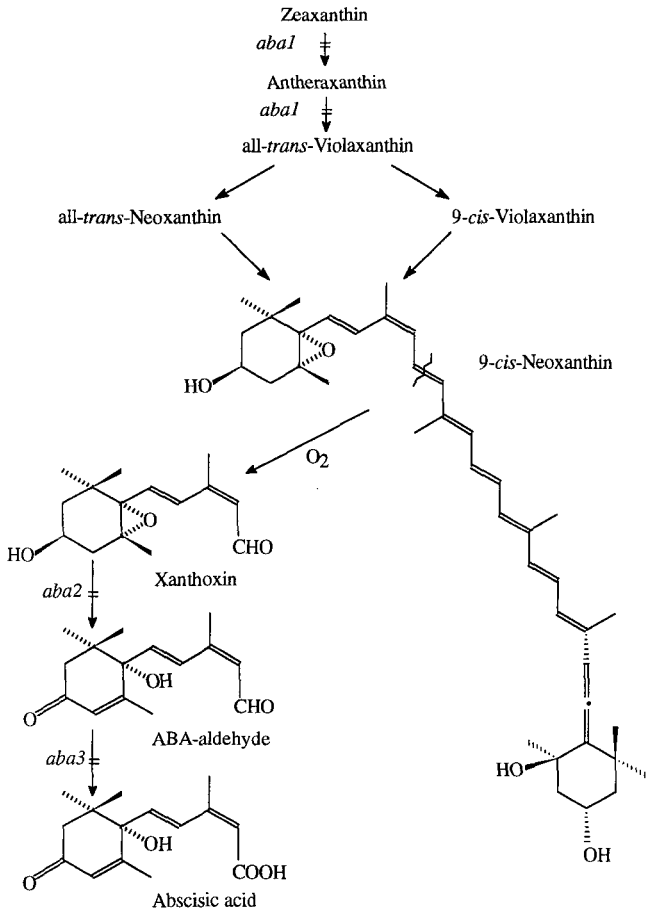

Figure 1. Proposed pathway of ABA biosynthesis. The biochemical lesions in the *aba1*, *aba2*, and *aba3* mutants of *Arabidopsis thaliana* are indicated.

cation 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 N_2 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 12,000*g* 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 $ZnMnO_4$ (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 N_2. Xanthoxin was further purified by reverse-phase HPLC on a semipreparative column (μBondapak C_{18}, 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 acetone was added to stop the reactions. Proteins were pelleted by centrifugation at 10,000*g* and [^{3}H]ABA was added to the supernatant as an internal standard. The acetone 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 N_2, 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_2S were added through a septum, and the extracts were incubated at 37°C for 30 min. Following the incubation, dithionite and Na_2S 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 N_2 and lyophilized. The tissue was extracted with 80% methanol containing butylated hydroxytoluene (0.1 g/L). The methanol was evaporated under a 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 × 30 cm) HPLC column (μBondapak C_{18}, 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}.

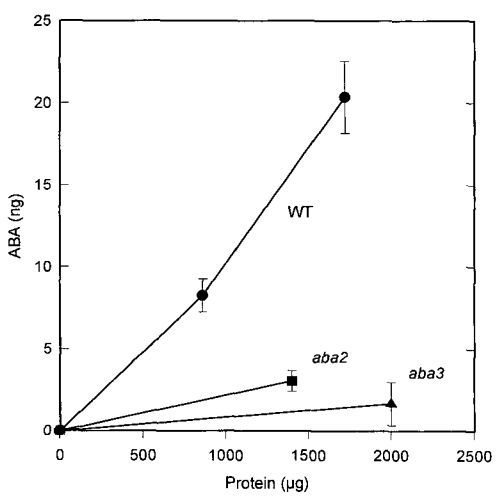

Figure 2. Conversion of xanthoxin to ABA by protein extracts from WT, *aba2*, and *aba3* plants (n = 2). The substrate was 100 ng of xanthoxin per assay.

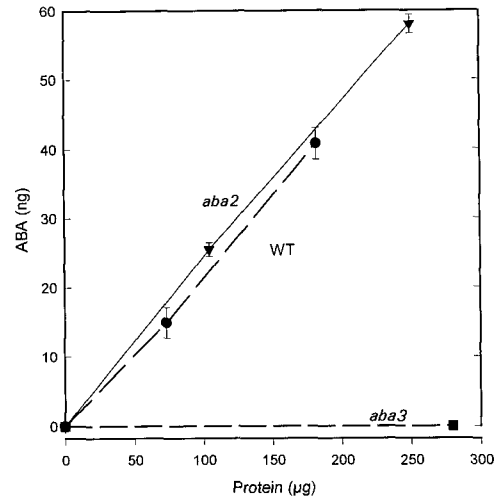

Figure 3. Conversion of ABA-aldehyde to ABA by protein extracts from WT, *aba2*, and *aba3* plants (n = 2). The substrate was 50 ng of ABA-aldehyde per assay.

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 (Yamomoto and Oritani, 1996). No ABA was detected in assays with extracts from *aba3* plants at the protein concentrations tested (Fig. 3). The *flc* 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 *flc* and *sit* mutants of tomato (Taylor et al., 1988) and the *dr* mutant in potato (Duckham et al., 1989) are capable of

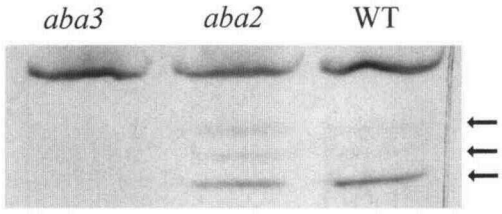

Figure 4. Aldehyde-oxidase activity gel with protein extracts of WT, *aba2*, and *aba3* (80 μg of protein was loaded per lane). The aldehyde oxidase activities are indicated with arrows. The most intense band (at the top) is a staining artifact, which also occurs in the absence of the heptaldehyde substrate.

converting exogenous *cis*-ABA-aldehyde to these compounds. The *aba3* mutant also accumulates *trans*-ABA-alcohol and the glucoside of *trans*-ABA-alcohol (data not shown).

Previous work with several species has shown that the enzymes encoded by *ABA2* and *ABA3* are constitutively expressed (Sindhu and Walton, 1987). This was confirmed for Arabidopsis: Extracts prepared from turgid and water-stressed plants showed no variation in the conversion of xanthoxin to ABA. In addition, extracts from the *aba1–5* mutant, which have a very low ABA content (Léon-Kloosterziel et al., 1996), were able to convert xanthoxin to ABA as well as WT extracts (data not shown).

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.

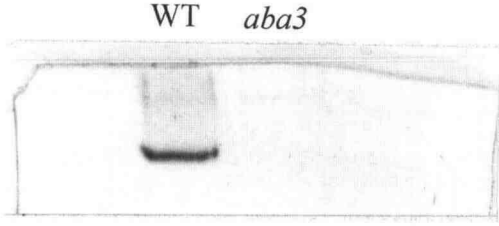

Figure 5. Xanthine-dehydrogenase activity gel with protein extracts of WT and *aba3* (50 μg of a 16 to 32% ammonium sulfate fraction was loaded per lane). The substrate was hypoxanthine.

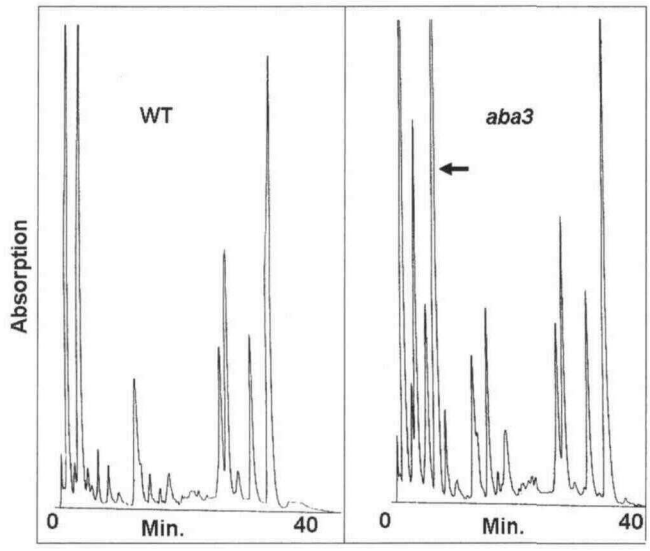

Figure 6. Reverse-phase HPLC chromatogram of aqeous extracts (see "Materials and Methods") from WT and *aba3*. The arrow indicates a compound that accumulates only in the *aba3* mutant.

In eukaryotes a dioxo and a desulfo moiety of the Moco have been identified (Wahl and Rajagopalan, 1982). Nitrate reductase activity is unaffected in the *aba3* mutant, indicating that only one form of the cofactor is absent. The two forms of the Moco can be chemically converted from one to the other (Wahl and Rajagopalan, 1982). Treatment with CN^- will hydrolyze the sulfido ligand. Under strictly anaerobic conditions an S ligand may be added to the Moco by treatment with dithionite and Na_2S. Extracts of WT incubated with CN^- resulted in the inactivation of ABA-aldehyde-oxidase activity, which could subsequently be restored by treatment with Na_2S (Fig. 7). Likewise, the activity of ABA-aldehyde oxidase in *aba3* could be acti-

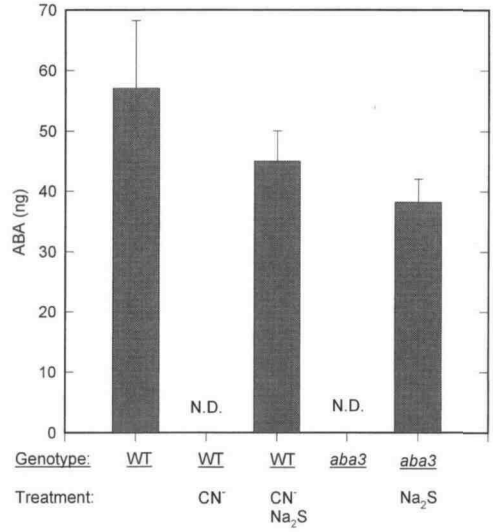

Figure 7. Inactivation of ABA-aldehyde-oxidase activity in WT with KCN and reconstitution of activity in *aba3* by treatment with Na_2S and dithionite. The substrate was 50 ng of ABA-aldehyde per assay. N.D., Not detected.

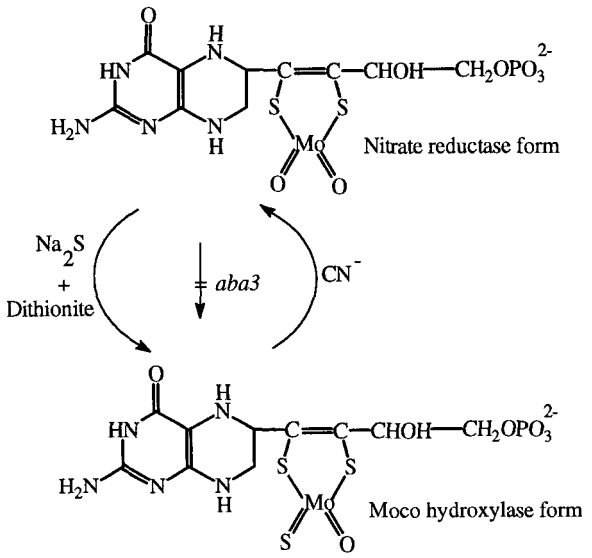

Figure 8. The chemical modification of the Moco and the proposed genetic lesion in the *aba3* mutant.

vated by treating extracts with dithionite and Na_2S. 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 Na_2S suggests that ABA-aldehyde oxidase activity requires a desulfo moiety of the Moco. Treatment of extracts of *aba3* with dithionite and Na_2S 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 isozymes 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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