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Reduced Accumulation of ABA during Water Stress in a Molybdenum Cofactor Mutant of Barley[1]

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ABSTRACT

A barley (*Hordeum vulgare* L.) mutant (Az34) has been identified with low basal levels of abscisic acid (ABA) and with reduced capacity for producing ABA in response to water stress. The mutation is in a gene controlling the molybdenum cofactor resulting in a pleiotropic deficiency in at least three molybdoenzymes, nitrate reductase, xanthine dehydrogenase, and aldehyde oxidase. The mutant was found to lack aldehyde oxidase activity with several substrates including: (a) ABA aldehyde, a putative precursor of ABA; (b) an acetylenic analog of ABA aldehyde; and (c) heptaldehyde. Elevating the growth temperature from 18 to 26°C caused mutant leaves to wilt and brown. Desiccation of mutant leaves was prevented by applying ABA. These results indicate that ABA biosynthesis at some developmental stages is dependent upon a molybdoenzyme which may be an aldehyde oxidase.

The use of mutants in higher plants has led to new information about a number of metabolic pathways including ABA biosynthesis (11, 12, 15, 32, 35) and nitrate reduction (14, 38). Little is known about the specific steps of ABA biosynthesis (35, 40). Both direct and indirect pathways of ABA biosynthesis have been proposed. In the direct pathway it has been suggested that ABA is derived directly from a C_{15} precursor such as farnesyl pyrophosphate (18). For the indirect pathway, it has been postulated that a C_{40} carotenoid is cleaved to xanthoxin which is then oxidized to ABA (4, 27). Definitive proof, however, is not available. Xanthoxin is present in plant leaves (30) and when applied to tomato and bean shoots is converted to ABA (30). Sindhu and Walton (27) have demonstrated that xanthoxin oxidizing activity is present in plant extracts from several species including bean.

Mutants with reduced capacity to produce ABA have been identified in several species including maize, tomato, and *Arabidopsis thaliana*. Most of the maize mutants are viviparous, and the genetic lesions are identified with specific steps in the carotenoid pathway (5). ABA deficient mutants in dicot species are generally 'wilty' and in the case of *Arabidopsis* (11, 12, 15) also have reduced seed dormancy. The biochemical lesions for the three ABA deficient tomato mutants have been proposed to reside in the oxidative cleavage of a C_{40} carotenoid precursor such as violaxanthin to a C_{15} intermediate (*notabilis*), and in the conversion of C_{15} intermediates to ABA (*flacca, sitiens*) (31), although specific enzyme steps have not been identified for any of these mutants. Recently, Sindhu and Walton (28) reported that the extracts of the wilty tomato mutants, *flacca* and *sitiens*, cannot convert ABA aldehyde to ABA and suggested that ABA aldehyde is the final ABA precursor.

Genetic regulation of nitrate reductase has also been extensively studied using induced mutants in higher plants (14, 38). In barley at least seven loci control two nitrate reductases (14). Five of these loci affect the MoCo[2] moiety in nitrate reductase and in other molybdoenzymes. That molybdoenzymes share a common cofactor was proposed by Pateman *et al.* (22) based upon observations that certain mutations had a pleiotropic effect on both nitrate reductase and xanthine dehydrogenase in *Aspergillus nidulans*. Similar MoCo mutants with pleiotropic deficiencies have been isolated in bacteria, fungi and higher plants (14, 38). The MoCo is a molybdopterin and is structurally similar in diverse molybdoenzymes including fungal nitrate reductase and mammalian sulfite and xanthine oxidases (9, 10, 16). The MoCo may be common to all molybdoenzymes except nitrogenase (9).

In this report we show that a barley mutant, originally selected for nitrate reductase deficiency, is also deficient in aldehyde oxidase and xanthine dehydrogenase activities, and that this MoCo mutant has impaired ability to produce ABA.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* L.) genotypes, Steptoe and the molybdopterin cofactor mutant Az34 (*nar*2a), were utilized. Seedlings were grown on vermiculite and provided with a nutrient solution as described by Warner and Kleinhofs (36) or grown hydroponically as described by Rao and Rains (24). For hydroponic studies, seeds were surface-sterilized with 2% (v/v) sodium hypochlorite for 30 min, rinsed three times with water, and then germinated for 24 h in vigorously aerated water at 26°C. Germinated seed were placed on cheesecloth supported by a stainless steel screen positioned 0.5 cm above

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[2] Abbreviation: MoCo, molybdenum cofactor.

aerated quarter-strength Hoagland solution (8). Seedlings were grown at 18°C for 4 d in the dark and then transferred to continuous light (300 $\mu E/m^{-2}/s^{-1}$). Nitrogen treatments and sampling protocol are described in the figure legends.

Cell-Free Extracts

Primary leaves were excised above the coleoptiles and immediately ground in ice-cold 0.25 M Tris-HCl buffer, pH 8.2, containing 1 mM EDTA, 1 μM Na_2MoO_4, 5 μM FAD, 3 mM DTT, 10 μM antipain, 1.0% (w/v) BSA. Leaves were extracted at a ratio of 1 g leaf to 2 mL buffer for aldehyde oxidase and xanthine dehydrogenase assay and at a ratio of 1 g leaf to 6 ml buffer for nitrate reductase assay. Extracts were centrifuged at 27,000 *g* and the supernatants collected.

Enzyme Assays and Detection

Nitrate reductase activity was determined as previously described (13). For detection of xanthine dehydrogenase and aldehyde oxidase activities, native gel electrophoresis of crude extracts was performed (13). Xanthine dehydrogenase activity was visualized with the substrate hypoxanthine (Sigma, St. Louis, MO[3]) according to Mendel and Muller (17). Aldehyde oxidase was measured as described by Vallejos (33) with the substrates heptaldehyde (Aldrich, Milwaukee, WI) ABA aldehyde (92% *cis,trans* and 8% *trans,trans*) and C_4-acetylenic ABA aldehyde (*Z*-5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methylpenta-2-en-4-ynal). The two ABA aldehyde isomers were synthesized and kindly provided to us by Dr. Suzanne Abrams and Angela Shaw, Plant Biotechnology Institute, National Research Council, Saskatoon, Saskatchewan, Canada S7N 0W9.

Water Stress

A 3 cm leaf section was excised from the tip of the primary leaf blade, cut transversely in half and placed in a glass test tube (12 × 75 mm). Leaf sections were dehydrated at room temperature (*ca.* 22°C) by placing the test tubes containing the leaf sections in a desiccator over 25% (v/v) glycerol (2). Water (1 ml) was added to tubes containing control leaves. Fresh weights were determined before and after dehydration.

ABA Extraction and Immunoassay

Fresh leaf tissue (15–25 mg) was weighed before extraction in one ml of methanol (containing 100 mg/L butylated hydroxytoluene and 0.5 g/L citric acid monohydrate). Samples were shaken overnight at 4°C. (+)ABA content of the extracts was measured by indirect ELISA utilizing a monoclonal antibody for (+)ABA as previously described and verified (34). Stability of ABA during extraction and measurement was confirmed by the addition of an internal standard of ^3H(+)ABA (Amersham, Arlington Heights, IL). The ABA-bovine serum albumin-C_4-conjugate utilized in the ELISA assay was prepared according to Weiler (37) as modified by Norman *et al.* (20).

[3] Mention of a specific product name by the U.S. Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

RESULTS

ABA Levels in the MoCo Mutant and Wild Type

Less ABA was accumulated by the MoCo mutant, Az34, during water stress than by the wild type (Fig. 1A). Maximum accumulation of ABA occurred at 12 h water stress in both Az34 and Steptoe. After 12 h ABA sharply declined and

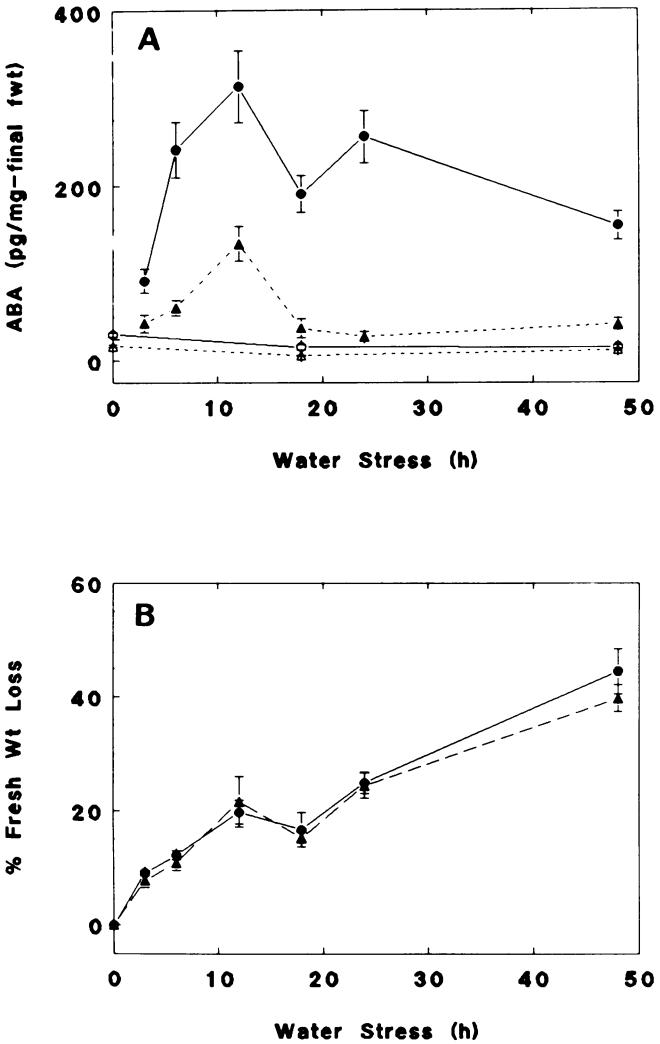

Figure 1. (+)ABA accumulation and fresh weight loss upon water stress of Steptoe or Az34 mutant. Seedlings were grown hydroponically as described in "Materials and Methods." On d 7 KNO_3 was added to the nutrient solution to a concentration of 2 mM. Leaf sections (3 cm) were excised from the tip of 8-d-old seedlings and placed in glass test tubes in the presence (control) or absence (water stress) of water. All tubes were then placed in a desiccator over 25% (v/v) glycerol for the times indicated. A, (+)ABA of water-stressed Steptoe (●——●) and Az34 (▲---▲), and in control Steptoe (○——○) and Az34 (△---△); B, percentage fresh weight loss of water-stressed Steptoe (●——●) and Az34 (▲——▲). No weight loss was observed in control leaves (data not shown). Bars indicate mean ± SE.

remained low in the mutant, but in the wild type ABA levels declined more gradually. Before water stress basal ABA levels were 42% ± 4% (SE) lower in the mutant compared with the wild type. Water loss during water stress was the same for both Steptoe and Az34 (Fig. 1B).

Effect of Seedling Age and Nitrate on ABA Accumulation

ABA accumulation in response to water stress was influenced by seedling age in both genotypes (Fig. 2). Mutant Az34 was able to accumulate ABA in expanding leaves but not in fully expanded leaves. Primary leaves reached full expansion at 7 to 8 d of age. ABA accumulation in fully expanded leaves of Steptoe was also less than in expanding leaves, but still high. Two-day-old germinating seedlings of Az34 did not show an impaired ability to produce ABA upon water stress, and preliminary results indicate that Az34 accumulates wild-type levels of ABA during grain development (data not shown).

The Az34 mutant has low levels of nitrate reductase activity indicating that it is somewhat leaky in the MoCo mutation (Fig 3B). Therefore it would be expected as observed in Figures 1 and 2 that the mutant would also have some capacity to produce ABA during water stress. If MoCo is limiting, then increased competition for MoCo would be expected by induction of nitrate reductase with nitrate, which could limit the MoCo available for other molybdoenzymes. When seedlings were grown in a nutrient solution containing nitrate, reduced ABA accumulation was observed in water stressed leaves of Az34, but not in wild type leaves (Fig. 2).

Temperature Sensitivity of Mutant Az34

Leaf wilting and browning of leaves of Az34 were dependent upon growth temperature. Elevating the growth temperature

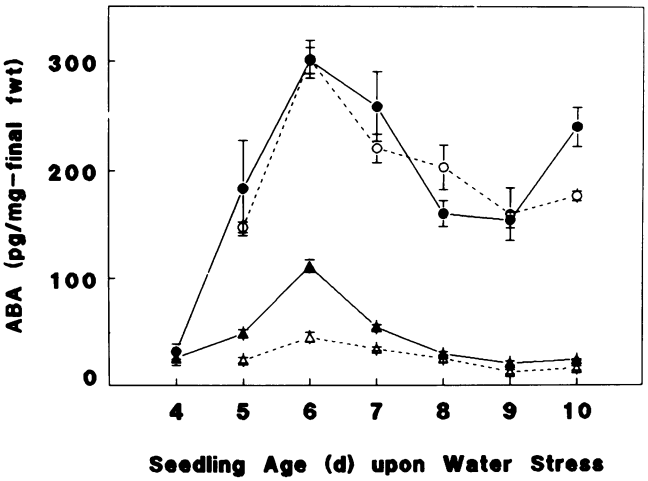

Figure 2. Effect of seedling age and nitrate in the growth medium on ABA accumulation during water stress. Seedlings were grown hydroponically as described in Figure 1. Steptoe (●, ○) and Az34 (▲, △) seedlings were grown in the absence of nitrate (●, ▲) or were provided with 2 mM KNO_3 (○, △) for 24 h prior to excising leaf sections. At the seedling ages indicated leaves were excised and placed in a desiccator for 24 h as described in Figure 1. Bars indicate mean ± SE.

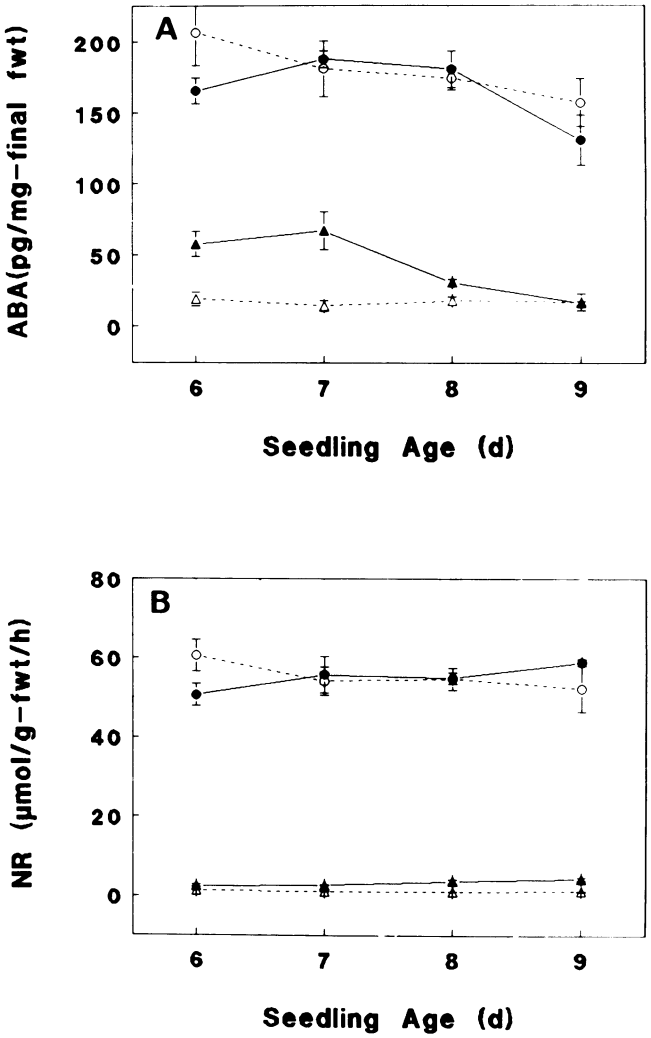

Figure 3. Effect of growth temperature on nitrate reductase (NR) activity and water stress induced ABA accumulation. Steptoe (●, ○) and Az34 (▲, △) seedlings were grown on vermiculite at 18°C (●, ▲) or 26°C (○, △) and harvested at the ages indicated. A, (+)ABA in leaves water stressed for 18 h (as described in Fig. 1); B, nitrate reductase in leaf tissue immediately after harvest. Bars indicate mean ± SE.

from 18°C to 26°C caused leaf wilting and browning in the Az34 plants but had no effect on Steptoe seedlings. The higher growth temperature decreased inducible ABA levels after water stress (Fig. 3A) and endogenous nitrate reductase activity (Fig. 3B) in leaves of the mutant but not in Steptoe. Larger differences were observed in the 6- and 7-d-old mutant seedlings. The older Az34 seedlings (8 and 9 d old) grown at the cooler or warmer temperature had little ability to produce ABA in response to water stress.

Exogenous application of ABA to leaves of the mutant growing at 26°C prevented leaf wilting and browning. Leaves of the Az34 mutant grown at 26°C lost a large amount of leaf moisture in comparison to Steptoe grown at 26°C or Steptoe and Az34 grown at 18°C (Fig. 4). Loss of leaf moisture of the mutant grown at 26°C was prevented by spraying ABA on the leaves (Fig. 4A).

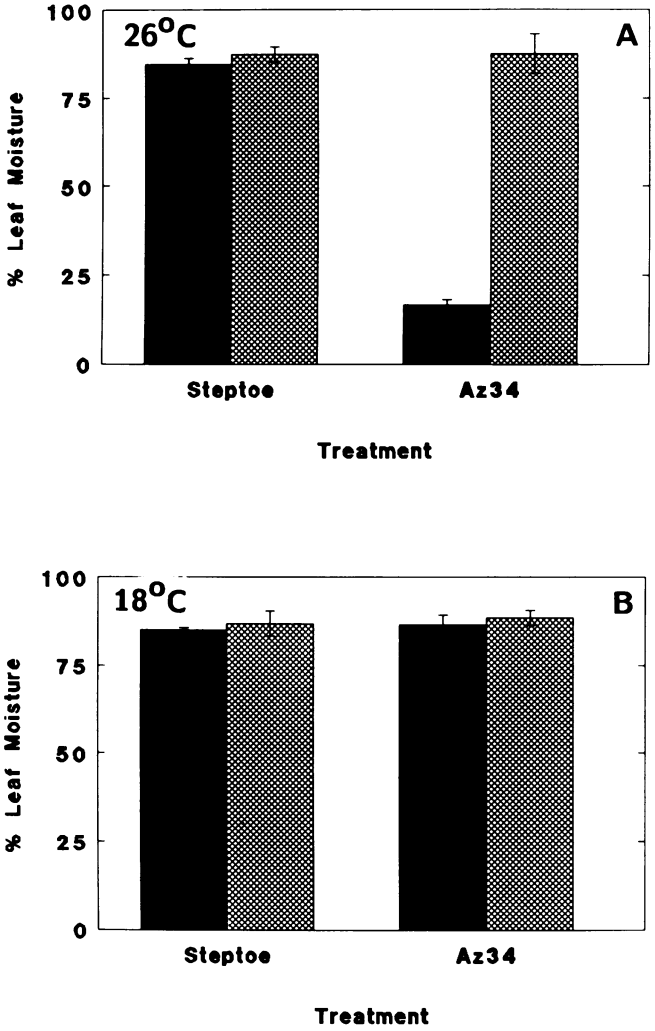

Figure 4. Effect of growth temperature and exogenous ABA on leaf moisture content. Steptoe and Az34 seedlings were grown on vermiculite at 26°C (A) or 18°C (B) as described in Figure 3. Starting at 4 d after planting, leaves were sprayed twice daily with a solution containing 25 μM ABA, 0.5% (v/v) Tween-20, and 0.1% (v/v) ethanol (gridded bar) or the same solution without ABA (solid bar). Percent moistures were determined on 3 cm of the leaf tip of 8-d-old seedlings and are means of three replicates ± SE.

Aldehyde Oxidase Activity in the MoCo Mutant

Aldehyde oxidase activity was measured on zymograms of crude extracts prepared from nonstressed barley leaves. It has been reported previously that MoCo mutants exhibit pleiotropic deficiency in nitrate reductase and xanthine dehydrogenase (14, 22). Leaf extracts of Az34 were deficient in both xanthine dehydrogenase and aldehyde oxidase activities (Fig. 5). Extracts prepared from wild-type barley, Steptoe, showed aldehyde oxidase activity with the substrates heptaldehyde and ABA aldehyde, but crude extracts of Az34 had no detectable aldehyde oxidase activity with either of these substrates (Fig. 5). A similar deficiency in aldehyde oxidase activity in the mutant compared to the wild type was observed with ABA aldehyde analog, C_4-acetylenic ABA aldehyde (data not shown). Aldehyde oxidase activity was less visible in the

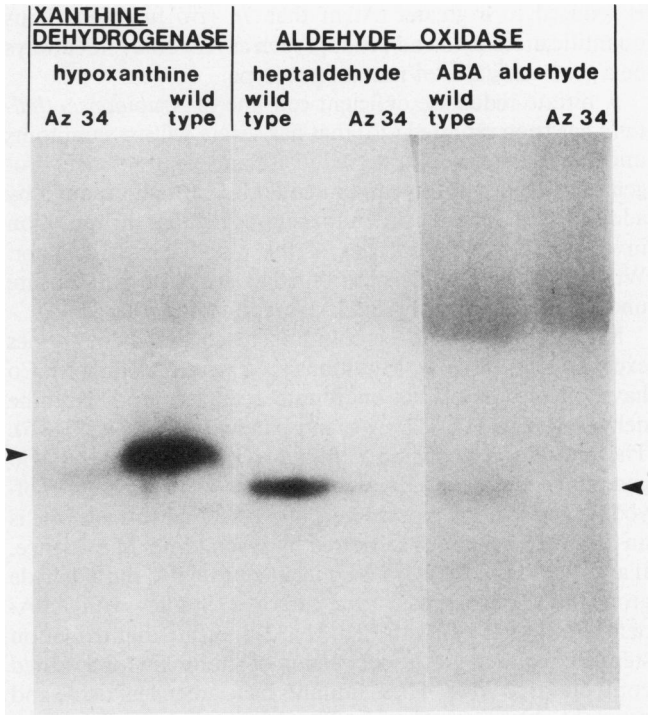

Figure 5. Zymograms of xanthine dehydrogenase and aldehyde oxidase activities in cell free extracts of Steptoe and Az34. Xanthine dehydrogenase was assayed with the substrate hypoxanthine, and aldehyde oxidase was measured with the substrates heptaldehyde and ABA aldehyde as described in "Materials and Methods." Seedlings were grown on vermiculite at 18°C for 8 d as described in Figure 3.

wild type with ABA aldehyde compared to heptaldehyde or C_4-acetylenic ABA aldehyde.

DISCUSSION

The barley mutant Az34 was selected as nitrate reductase deficient and classified as a MoCo mutant based upon pleiotropic deficiencies in nitrate reductase and xanthine dehydrogenase (29). This mutant is also deficient in another molybdoenzyme, aldehyde oxidase (Fig. 5). This mutant meets the criteria described by Taylor (32) for ABA deficient mutants which includes: (a) reduced basal ABA levels (Fig. 1), (b) reduced accumulation of ABA in response to water stress (Fig. 1A), and (c) application of exogenous ABA prevented wilting and browning of Az34 (Fig. 4).

The steady increase in ABA, maintenance of high ABA levels for at least 20 h, and the maximum ABA levels reached in response to water stress in leaves of Steptoe (Fig. 1) were similar to those found in detached, water stressed wheat leaves (7). The greater ability of younger leaves of Steptoe to accumulate ABA in response to water stress than of older leaves (Fig. 2) is similar to other plants (23, 25, 39). Similar responses were observed for the Az34 mutant, but ABA accumulation in the mutant was much less than in the wild type. We know that the mutation in Az34 does not eliminate all nitrate reductase activity (Fig. 3B) indicating that the mutant is slightly leaky. In most leaky mutants *in vitro* enzyme activity

is reduced to a greater extent than *in vivo* function. Thus quantification of an end product such as ABA may not always be a good indicator of mutant genotype.

A nitrate reductase deficient cell line of *Arabidopsis thaliana* has been reported (26) that has severe wilting symptoms under water stress. Nitrate reductase and xanthine dehydrogenase activities of this mutant could be partially restored by addition of excess molybdenum, suggesting that the mutation involved catalytic properties of the molybdenum cofactor. Wilting of the mutant was attributed to lack of stomata closure under water stress, but ABA levels were not measured.

MoCo is thought to be common to all molybdoenzymes except nitrogenase (9). Mutations in genes controlling MoCo have pleiotropic effects on nitrate reductase and xanthine dehydrogenase (14, 22, 38), and aldehyde oxidase (Fig. 5). The reduced capacity of a mutant with a defective MoCo gene to accumulate ABA would appear to implicate a molybdoenzyme in the biosynthesis of ABA. That this enzyme is an aldehyde oxidase is suggested by several lines of evidence. If as postulated xanthoxin is a precursor to ABA, the aldehyde group must be oxidized to a carboxyl. Studies with ABA-deficient tomato mutants (21, 31, 32) indicate that oxidation steps are required for the conversion of xanthoxin (or a related compound) to ABA. These mutants have green leaf tissue and normal levels of carotenoids. Two of the mutants, *flacca* and *sitiens*, are impaired in the conversion of xanthoxin and ABA aldehyde to ABA (21, 28). Two enzymes may be required for the conversion of xanthoxin to ABA with ABA aldehyde as an intermediate (28). Isotope labeling studies indicate that one of the carboxyl O atoms is derived from water (4), which would be expected if xanthoxin or a related aldehyde is oxidized by an aldehyde oxidase.

The barley aldehyde oxidase measured in this study exhibits activity with several substrates including ABA aldehyde, a putative precursor of ABA (Fig. 5). Since most aldehyde oxidases have rather broad substrate specificities (1), it is not possible to predict whether or not the aldehyde oxidase observed in these studies serves to oxidize ABA aldehyde or xanthoxin *in vivo*. It is significant that mutant Az34 lacks this aldehyde oxidase and has impaired ability to accumulate ABA under water stress. However, the involvement of other molybdoenzymes in ABA biosynthesis can not be excluded since the mutation in Az34 would be expected to cause a pleiotropic deficiency of all molybdoenzymes.

ABA-deficient barley mutants will be useful in future studies investigating the biosynthetic pathway for ABA and the survival mechanisms in cereals for protection from water stress. ABA-inducible genes have recently been correlated with elevated ABA levels in dehydrated cereal leaves (3, 6, 19). Further evaluation of mutant Az34, which only produces low amounts of ABA in response to water stress, should increase our understanding of the effects of ABA during dehydration stress in cereals.

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