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Molybdenum Cofactor Mutants, Specifically Impaired in Xanthine Dehydrogenase Activity and Abscisic Acid Biosynthesis, Simultaneously Overexpress Nitrate Reductase[1]

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The molybdenum cofactor is shared by nitrate reductase (NR), xanthine dehydrogenase (XDH), and abscisic acid (ABA) aldehyde oxidase in higher plants (M. Walker-Simmons, D.A. Kudrna, R.L. Warner [1989] Plant Physiol 90: 728–733). In agreement with this, *cnx* mutants are simultaneously deficient for these three enzyme activities and have physiological characteristics of ABA-deficient plants. In this report we show that *aba*1 mutants, initially characterized as ABA-deficient mutants, are impaired in both ABA aldehyde oxidase and XDH activity but overexpress NR. These characteristics suggest that *aba*1 is in fact involved in the last step of molybdenum cofactor biosynthesis specific to XDH and ABA aldehyde oxidase; *aba*1 probably has the same function as *hxB* in *Aspergillus*. The significance of NR overexpression in *aba*1 mutants is discussed.

Selection of mutants is an excellent tool to study metabolic pathways such as nitrate reduction and hormonal regulation in higher plants. Nitrate reduction has been extensively studied by isolation of NR-deficient mutants (Wray, 1988; Kleinhofs et al., 1989; Pelsy and Caboche, 1992). Plant NR (EC 1.6.6.1) has a homodimeric structure. Each subunit contains three domains: a Cyt b_5 domain, a FAD-containing domain, and a MoCo-containing domain. The MoCo is a molybdopterin common to NR and to other molybdoenzymes, XDH, sulfite oxidase (Pateman et al., 1964; Johnson, 1980), and most probably aldehyde oxidase (Walker-Simmons et al., 1989). NR-deficient mutants (NR^-) isolated by selection for chlorate resistance are classified in two main groups on the basis of XDH measurements. NR^-, XDH^+ mutants are apoenzyme-deficient mutants (*nia*). In contrast, those lacking both NR and XDH activity are classified as cofactor-deficient mutants (*cnx*). In *Nicotiana plumbaginifolia*, selection of NR^- mutants (Gabard et al., 1987, 1988) and their biochemical analysis (Marion-Poll et al., 1991) have identified seven loci controlling NR expression: one locus corresponds to *nia*, the structural gene for NR, and six loci, *cnx* A to F, are involved in the biosynthesis of the MoCo. Apart from being impaired in nitrate metabolism, *cnx* mutants are defective in other functions involving the MoCo. Walker-Simmons et al. (1989) have described a barley *cnx* mutant (Az34) with a thermosensitive, wilty phenotype. This phenotype has been attributed to an ABA deficiency due to the absence of activity of a MoCo-dependent aldehyde oxidase, the enzyme involved in the last step of ABA synthesis.

In *Aspergillus nidulans* an exhaustive genetic analysis of nitrate metabolism and purine and nicotinate utilization has been performed (Scazzocchio, 1980). Mutations affecting one of the genes of MoCo synthesis in *A. nidulans* have a pleiotropic effect on the Mo-containing enzymes and result in a simultaneous loss of NR, purine hydroxylase I (XDH I), and purine hydroxylase II (XDH II, nicotinamide hydroxylase). As for higher plants, *cnx* genes specify the steps necessary for the synthesis of a Mo-containing cofactor. But in *A. nidulans*, a mutation at the *hxB* locus results in the loss of both XDH I and XDH II activities without NR impairment (Scazzocchio, 1980; Scazzocchio and Arst, 1989). In such a mutant, XDH-cross-reacting material is detectable and has the same electrophoretic mobility as wild-type XDH. Furthermore, the NADH dehydrogenase partial activity (diaphorase) of both purine hydroxylases is always present (Scazzocchio, 1980). The *hxB* gene probably catalyzes a terminal modification of the MoCo specifically required for the activity of purine hydroxylases but not for NR. This modification might involve a sulfide ion, a terminally inserted ligand of the Mo found only in the purine hydroxylases, as established for the *ma*-1 mutation of *Drosophila* (Wahl et al., 1982).

In search of auxin-resistant mutants selected for resistance to indole-3-butyric acid in *Nicotiana plumbaginifolia*, a mutant with characteristics of an ABA-deficient plant was identified. The mutant, called I 217, had an early-germination, wilty phenotype (Bitoun et al., 1990; Rousselin et al., 1992). This mutant was found to be allelic to a cytokinin-resistant mutant and to an early-germination mutant. The corresponding locus was named *aba*1 (Rousselin et al., 1992). In this paper we describe the further characterization of NR^-*cnx* mutants and the *aba*1 mutant. The different mutants appear to be affected in MoCo biosynthesis and impaired in ABA production.

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Abbreviations: ABA-AG, ABA-alcohol glucoside; ABA-GE, ABA-glucose ester; MoCo, molybdenum cofactor; NR, nitrate reductase; XDH, xanthine dehydrogenase.

MATERIALS AND METHODS

Plant Material

The seeds and plants of *Nicotiana plumbaginifolia* cv Viviani were derived from the haplo-diploidized strain PbH1D (Bourgin et al., 1979). The mutant I 217, one of three mutant alleles at the *aba*1 locus, was isolated by Bitoun et al. (1990). The *cnx* mutants were selected by Gabard et al. (1988). Seeds were harvested from plants grown at the same time in the greenhouse and stored for 1 year at room temperature. They were surface sterilized and then sown aseptically on medium B supplemented with 10 mM diammonium succinate as described by Gabard et al. (1987). Germination was carried out in a controlled culture chamber under the following conditions: 16 h of light (intensity, 75 μE s^{-1} m^{-2}), a constant temperature of 28°C, and a constant 70% RH. These conditions were chosen to increase the differences of germination between the mutants and the wild type. Seeds of the different plants were also tested for their ability to grow on 5 mM hypoxanthine as a sole nitrogen source. Plantlets grown in vitro were transferred to the greenhouse. *cnx* mutants were grafted onto plants of *Nicotiana tabacum* cv Wisconsin 38. All plants were watered with nutrient solution containing nitrate and ammonium (Coïc and Lesaint, 1971). The water loss of leaves was measured by exposing excised leaves from plants grown in vitro to the air stream of a sterile hood at 25°C and 60% humidity, and weight measurements were made at intervals as described by Parry et al. (1992).

Protein Analysis

Extraction and ammonium sulfate precipitation of protein and measurement of NR activity and NR protein by ELISA were performed as previously reported (Deng et al., 1990) from leaves harvested from plants grown in the greenhouse. Soluble proteins were measured on crude extracts as described by Bradford (1976) with BSA as a reference.

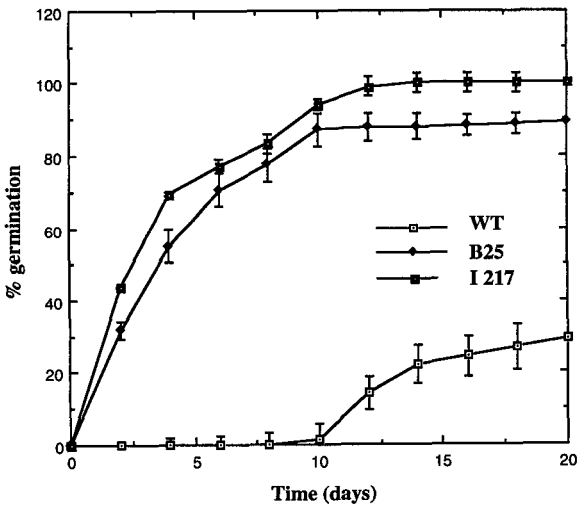

Figure 1. The germination kinetics of *N. plumbaginifolia* seeds: comparison among wild type (WT) and *cnx* A (B25) and *aba*1 (I 217) mutants. Germinated seeds were counted at intervals under a microscope; the numbers are percentages of total seeds incubated. Data are representative of three replicate experiments.

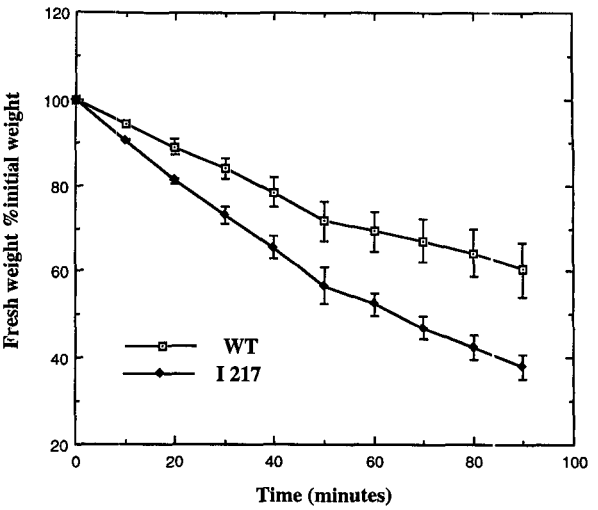

Figure 2. Fresh weight loss upon water stress of detached leaves from *N. plumbaginifolia* wild type (WT) and *aba*1 (I 217) mutants. Plants were grown in vitro under very similar conditions before leaves were collected. The values are an average of weight determinations performed on three leaves, expressed as percentages of the weight of these leaves immediately after excision.

Enzyme Assays on Native Gels

Electrophoresis was carried out as described by Walker-Simmons et al. (1989) using a native 7% polyacrylamide gel. Before the gel was loaded, crude extracts of leaves were concentrated by ammonium sulfate precipitation and dialyzed according to the method of Marusyk and Sergeant (1980). XDH activity was detected according to the procedure of Mendel and Müller (1976), aldehyde oxidase, with heptaldehyde as substrate, was detected as described by Walker-Simmons et al. (1989), and NADH diaphorases were detected as described by Pérez-Vicente et al. (1987).

ABA Assays

Extraction, purification, fractionation, and ELISA quantitation of ABA metabolites were performed according to the method of Julliard et al. (1993) with excised leaves of plants grown in vitro before and after water stress. The characterization of ABA-GE and ABA-AG were performed using a β-glucosidase hydrolysis method as described by Kraepiel et al. (1994).

RESULTS

Physiological Responses

Germination kinetics were compared under conditions indicated in the "Materials and Methods." Mutants I 217 (*aba*1 mutant) and B25 (*cnx* A mutant) germinate more rapidly than the wild type (Fig. 1); 80 to 90% of the seeds were germinated at 10 d when the first wild-type seeds

Table I. *Influence of a 90-min water stress on the levels of ABA and related ABA metabolites in wild-type (WT), cnxA mutant (B25), and hormonal aba1 mutant (I 217) leaves*

The values are expressed in equivalents of ABA (fmol mg^{-1} dry weight). Results are average values ± SE of five replicate assays.

Plant	Treatment	ABA-GE	ABA-AG	ABA
WT	Nonstressed	1900 ± 70	0	900 ± 100
	Water stressed	960 ± 60	0	2900 ± 260
B25	Nonstressed	0	6100 ± 410	130 ± 10
	Water stressed	0	3200 ± 480	120 ± 20
I 217	Nonstressed	0	5300 ± 400	310 ± 25
	Water stressed	0	4000 ± 190	235 ± 30

showed only the first appearance of a root from the seed coat. Mutants grown in the greenhouse, either on their roots (I 217) or grafted (B25), are found to be more prone to wilt than the wild type; therefore, water loss was assayed. The excised leaves from plants grown in vitro were water stressed by incubation under a stream of air, and the loss of their weight was followed for 90 min. Figure 2 shows that there is a significantly higher fresh weight loss of the leaves of mutant I 217 compared to the wild type; on the contrary, the water loss of the B25 mutant is not different from the wild type and not represented in the figure. Therefore, these mutants were characterized by reduced seed dormancy and an increased tendency to water losses for the *aba*1 mutant as previously described (Rousselin et al., 1992).

Levels of ABA and Related Metabolites

The levels of ABA were measured in water-stressed and nonstressed leaves of the different plants. As previously found by Kraepiel et al. (1994), the levels of ABA in the *aba*1 mutants were 10% or less of that found in the wild type (Table I). These levels were not modified in water-stressed plants, as opposed to the wild type in which the level of ABA was increased 3-fold after a water stress. Both *cnx* mutant B25 and *aba* mutant I 217 accumulated ABA-AG as a major metabolite, indicating an ABA-aldehyde oxidase deficiency, whereas the wild-type plants contained ABA-GE as a major immunodetectable metabolite. It can be concluded that these mutants are unable to synthesize ABA and this can account for the excessive water loss rate, at least for the I 217 mutant, as a consequence of the lack of stomatal closure (Davies and Mansfield, 1983).

XDH, Aldehyde Oxidase, and NADH Diaphorase Activities

Since *aba*1 and *cnx* mutants displayed very similar features of ABA-AG accumulation, we decided to study in parallel ABA-aldehyde oxidase and XDH activities in these mutants. These enzyme activities were revealed by native gel electrophoresis of extracts prepared from leaves of plants grown in vitro or in the greenhouse (Fig. 3). Extracts from the wild type possess these two enzymatic activities. Leaf extracts of mutants I 217 and B25 were deficient in both XDH and aldehyde oxidase activities, detected, respectively, with hypoxanthine or heptaldehyde as substrates (Fig. 3A). The specific detection of XDH activity was confirmed by inhibition of tetrazolium staining in the presence of allopurinol, a potent inhibitor of XDH activity. Allopurinol did not impair the detection of aldehyde oxidase (Fig. 3B). This, together with the slightly different electrophoretic mobilities of XDH and aldehyde oxidase

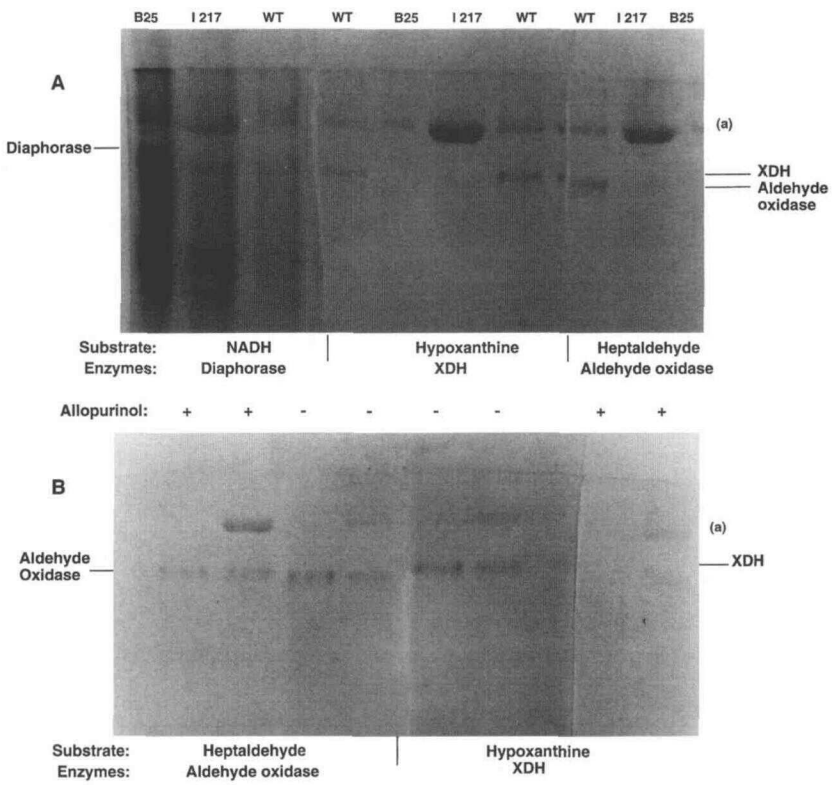

Figure 3. A, Zymograms of diaphorase, XDH, and aldehyde oxidase activities in cell-free extracts of wild type (WT) and *cnx* A (B25) and *aba*1 (I 217) mutants. Diaphorase was assayed with NADH, XDH was assayed with hypoxanthine, and aldehyde oxidase was assayed with heptaldehyde. B, XDH and aldehyde oxidase were detected upon electrophoresis of wild-type extract with or without an inhibitor of the XDH, allopurinol (200 μM). (a), Nonspecific bands are revealed at the top of XDH or aldehyde oxidase lanes; they have a different color than the bands of these enzymes, and their presence is dependent on the concentration of the extracts.

Table II. *Levels of NR activity and NR protein in wild-type (WT), cnxA mutant (B25), and aba1 mutant (I 217) leaves*

The values for NR activity are expressed in nmol NO_2^- min^{-1} g^{-1} fresh weight, for NR protein in ng g^{-1} fresh weight, and for total protein in mg g^{-1} fresh weight. Data are means ± SE of three independent experiments.

Plant	NR Activity	NR Protein	Total Protein
WT	75 ± 3	300 ± 60	3.4 ± 2.2
B25	0	640 ± 90	1.9 ± 1.1
I 217	120 ± 20	440 ± 40	3.7 ± 1.0

activity, confirms that the two different enzymatic activities are present in the wild type and deficient in the mutants. A nonspecific band, also observed by Walker-Simmons et al. (1989), is often revealed as indicated in Figure 3. The band has a different color than the XDH and aldehyde oxidase bands. It appears to be a nonspecific fixation of nitroblue tetrazolium on aggregated proteins in the concentrated extracts.

NADH diaphorase activities were also detected on zymograms using NADH and *p*-nitroblue tetrazolium (Fig. 3A). Among the NADH diaphorases found on the gel, one band has the same mobility as XDH in the wild type and is present in the mutants. This suggests that this diaphorase, corresponding to the partial catalytic activity of the XDH, is maintained in the XDH^- mutants as observed by Scazzocchio (1980) for *A. nidulans*. The lack of functional XDH in the mutants is confirmed by their inability, contrary to the wild type, to grow on hypoxanthine as the sole nitrogen source (data not shown). None of the mutants were able to grow on hypoxanthine; they remained blocked at the two-cotyledon stage of development similar to wild-type seed controls germinated on a nitrogen-free medium. In comparison to this, the wild type grew on hypoxanthine at a slow rate and developed green leaves.

NR Activity and NR Protein of Wild Type and Mutants

cnx A (B25) mutant is deficient in NR activity but has a greater amount of NR protein than wild type (Table II). Other *cnx* groups accumulate lower NR protein (not shown) presumably as a consequence of the degradation of the NR apoenzyme in the absence of bound MoCo (Marion-Poll et al., 1991). As opposed to *cnx* mutants, mutant I 217 exhibits NR activity and NR protein levels measured by ELISA that are significantly higher than levels of the wild type (Table II). This overexpression of NR is highly significant and has been reproducibly observed in a number of independent experiments.

DISCUSSION

In this report we compared the physiological behavior and the biochemical characteristics of two types of mutants from *N. plumbaginifolia* selected either for a hormonal deficiency or a NR deficiency. This comparison was performed on the basis of a common physiological feature: reduced seed dormancy. The two types of mutants were found to have physiological properties of ABA-deficient mutants: early germination (Fig. 1), wilty response to water stress, at least for one of them (Fig. 2), and reduced ABA levels (Table I). They were found to be defective for an enzyme involved in ABA synthesis (aldehyde oxidase). Aldehyde oxidase and XDH share a common MoCo, as suggested by the work of Walker-Simmons et al. (1989). Therefore, we tested the possibility that *aba*1 mutants are MoCo biosynthesis mutants as are the *cnx* mutants. A comparison of XDH activity and of the NADH diaphorase activity linked to XDH in the wild type and the two classes of mutants confirmed that *aba*1 mutants were defective for MoCo. These results suggest that *aba*1 is a locus similar to the locus *hxB* described by Scazzocchio (1980) in *A. nidulans*. The corresponding protein is involved in a biochemical reaction converting the MoCo that is extractable from NR into a MoCo that is catalytically active in XDH and ABA-aldehyde oxidase. NR expression had been described to be stress sensitive (Huffaker et al., 1970; Heuer et al., 1979). The overexpression of NR in the *aba*1-deficient mutant might result from a modified physiology due to the reduced ABA level. This does not appear to be the case, since another class of ABA-deficient mutants was recently identified in our laboratory and found to remain basically unaffected with regard to NR activity (E. Marin, unpublished data). From this, we conclude that NR overexpression results from a defect in MoCo biosynthesis. This can be explained by the fact that there is either a competition by MoCo-carrying enzymes for limiting amounts of MoCo, or a deregulation of MoCo biosynthesis due to the lack of end product of the pathway, or both. The locus *aba*1 defines the first characterized mutation in plants affecting ABA biosynthesis and XDH but not NR activity.

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