Phytochemistry, Vol. 26, No. 8, pp. 2149–2150, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

REGULATION OF MOLYBDENUM COFACTOR OF MAIZE LEAF

WILBUR H. CAMPBELL, DONALD J. DEGRACIA AND ELLEN R. CAMPBELL

Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931, U.S.A.

(*Received* 2 *September* 1986)

Key Word Index—*Zea mays*; Gramineae; maize; molybdenum cofactor; nitrate reductase; nitrogen nutrition; light regime.

Abstract—Molybdenum cofactor and nitrate reductase were extracted from maize leaf and assayed using established methods. While both applied nitrate and light increased nitrate reductase activity, only nitrate was required to increase molybdenum cofactor to its highest level and light had no apparent influence.

INTRODUCTION

Nitrate reductase, xanthine dehydrogenase and all other described molybdenum-containing enzymes, with the exception of nitrogenase, have been found to have their molybdenum bound to the enzyme as a molybdopterin moiety, which is generally referred to as 'Mo-cofactor' [1, 2]. The Mo-cofactor was discovered by Nason and coworkers when they demonstrated that the nitrate reductase activity of the *Neurospora crassa* mutant *nit*-1 could be restored by adding denatured molybdenum-containing enzymes to the extract of the mutant [3]. The chemical nature of Mo-cofactor has been delineated as an oxygen-sensitive, unique phosphorylated pterin and molybdenum by Rajagopalan and coworkers [4]. It was recently shown that Mo-cofactor is present in higher plant nitrate reductase in an amount equal to the enzyme's other cofactors (FAD and heme-Fe) [5]. The availability of Mo-cofactor during the synthesis of apo-nitrate reductase and the appearance of the active enzyme has become a concern in the study of nitrate metabolism and its regulation in higher plants [6, 7]. We report here on the influence of nitrogen nutrition and light regime on Mo-cofactor content of maize leaf.

RESULTS AND DISCUSSION

Molybdenum-cofactor had not been previously assayed in maize leaf extracts but had been measured in barley [8], and we initially applied their approach. Although efficacy of the assay for *nit*-1 complementation to form NADPH–nitrate reductase activity could be shown with Mo-cofactor obtained from purified squash cotyledon NADH–nitrate reductase, no Mo-cofactor activity could be detected in extracts of maize leaf. Varying the additions to the extraction buffer such as type and concentration of reductant, or method of extraction (i.e. grinding without first freezing in liquid N_2) did not help. However, the presence of Mo-cofactor in the resuspended 50% ammonium sulphate precipitates of maize leaf extracts could be demonstrated. Since it was possible to assay nitrate reductase activity in leaf extracts, it seemed likely that a gel filtration treatment of leaf extract might remove inhibitors of the *nit*-1 complementation assay. Indeed, processing leaf extract on a Sephadex G-25 column using the extraction buffer for elution led to detection of levels of Mo-cofactor like those found in barley leaf [8].

One additional change was made from the procedures ref. [8]. After the *nit*-1 extract had been mixed with the denatured Mo-cofactor source, they incubated the mixture for 40 min at 25°, which was stated to be the optimal time for complementation [8]. However, Hawkes and Bray [2] have found that the *nit*-1 extract is unstable at 25° and recommend complementation for 24 hr at 4°, where both the *nit*-1 extract and the holo-nitrate reductase are stable for at least 24 hr. We compared these two approaches to complementing the *nit*-1 extract and Mo-cofactor from squash nitrate reductase. When complementation was allowed to proceed for 24 hr at 4°, about twice as much Mo-cofactor activity was found as compared to an identical complementation done for 40 min at 25°. Thus, we have adopted the former method of complementation [2].

Mendel *et al.* [8] reported that applying nitrate-containing nutrients to barley plants led to measurable increases of Mo-cofactor activity in the leaves. They also observed large increases in nitrate reductase activity in these leaves [8]. We have done similar experiments with maize and also observed an approximate doubling of Mo-cofactor activity in leaves when either ammonium or potassium nitrate was applied to the plants in a nutrient solution (Table 1). Nitrate reductase activity was lowest in maize leaves when the plants had not been given nitrogen and increased by more than 15-fold in the leaves when plants were given nitrate in the light. Thus, the results for maize are basically the same as those reported for barley.

The amount of increase in nitrate reductase activity of maize leaves differs when nitrate is applied to etiolated plants in the dark vs plants transferred to light [7]. Molybdenum-cofactor activity was about the same in leaves of etiolated plants given no nitrogen and those of etiolated, N-starved plants greened in the light for 24 hr (Table 1). Nitrate reductase activity levels were about 5-fold greater in green, nitrogen-starved levels than in etiolated, nitrogen-starved leaves. Molybdenum-cofactor activity increased about 2-fold in leaves of plants treated with either ammonium or potassium nitrate for 24 hr in light or dark. However, leaf nitrate reductase activity levels were 5–10 times greater in plants treated with

Table 1. Molybdenum cofactor and nitrate reductase content of maize leaf

Nutrition	Environment	Mo-cofactor activity (nkat/g leaf)	Nitrate reductase activity (nkat/g leaf)
No Nitrogen	Dark	3.5	0.03
No Nitrogen	Light	3.7	0.17
NH_4NO_3	Dark	8.7	0.50
NH_4NO_3	Light	8.0	2.8
KNO_3	Dark	6.7	0.35
KNO_3	Light	8.0	3.0

These results were obtained using the methods described in the Experimental and are representative of four different experiments.

ammonium or potassium nitrate in the light for 24 hr vs those treated in the dark with these nutrients for 24 hr. Thus, nitrate reductase activity and Mo-cofactor activity in maize leaf are influenced differently by nitrate and light. It has been concluded that both nitrate and light regulate the level of leaf nitrate reductase activity in maize [7], which is also illustrated here in Table 1. Molybdenum-cofactor activity in maize leaves appears to be regulated by only nitrate nutrition and light appears to have little, if any, influence.

The final consideration to be given to the data in Table 1, is the amount of the increase in Mo-cofactor activity as compared to the amount of increase in nitrate reductase activity over the treatment period of 24 hr. Between the N-starved, etiolated plants in the dark and the N-treated plants after 24 hr of light, Mo-cofactor activity has increased by 4.5 nkat/g leaf while nitrate reductase activity has increased about 3 nkat/g leaf. These data indicate that more Mo-cofactor was available in the maize leaf than was used to make nitrate reductase. An over-production of Mo-cofactor relative to nitrate reductase can be calculated from the data previously presented for barley [8]. But Mo-cofactor is found in other enzymes such as xanthine dehydrogenase, which may also be increasing in total activity during this treatment of the maize or barley leaves. However, the present data tend to indicate that the availability of Mo-cofactor may not be a factor limiting the assembly of active nitrate reductase in maize leaf.

EXPERIMENTAL

Maize plants, *Zea mays* L. W64A × W182E, were grown in vermiculite at 30° in the dark and treated with deionized H_2O until they were 4 days old. Three different treatments were applied to the plants: (1) only deionized H_2O, (2) 50 mM NH_4NO_3, and (3) 50 mM KNO_3 with both N-treatments in modified Hoagland nutrient soln. At the time of treatment, some plants were kept in the dark for 24 hr, while other plants were transferred to light at 30° for 24 hr. At the end of the 24 hr treatment, 1 g of leaf for each of the 6 treatments was harvested and ground to a powder in liquid N_2. The powdered leaf was extracted with 10 ml of 50 mM KPi, pH 7.5, 0.5 mM EDTA, 25 mM Na_2MoO_4, 5 mM GSH, and 1 mM dithiothretiol, and centrifuged at 20 000 *g* for 20 min at 4°. NADH-nitrate reductase activity of leaf extracts was assayed as described [7]. A 50% saturated $(NH_4)_2SO_4$ precipitate was prepared by adding 278 mg of the salt/ml of leaf extract. Leaf extract was treated on Sephadex G-25 columns (0.9 × 12 cm) using extraction buffer for elution. For the Mo-cofactor activity assay, 0.125 ml of leaf extract, its 50% $(NH_4)_2SO_4$ precipitate, Sephadex G-25-treated leaf extract or squash NADH-nitrate reductase purified as described [5], was placed in the bottom of an evacuation tube and 0.5 ml of *nit*-1 extract was placed in the upper sidearm. The tube was evacuated and flushed with N_2 alternatively through three cycles, sealed under N_2 and then the Mo-cofactor source was heated for 90 sec at 80°. After the Mo-cofactor source was chilled in ice, the Mo-cofactor source and the *nit*-1 extract were combined while still under nitrogen and allowed to stand under nitrogen for at least 10 min before exposure to the atmosphere. The complementation reaction stood for 24 hr at 4°, before the NADPH-nitrate reductase activity was assayed as described [2, 9]. *N. crassa nit*-1 was grown and extracted as described [1, 9], except that the mycelia were ground in liquid N_2 and the powder extracted in 2 ml of 0.1 M KPi, pH 7.4, 1 mM EDTA, 1 mM dithiothretiol and 1 mg phenylmethylsulphonyl fluoride/g *nit*-1 mycelia. Protein was assayed with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) using BSA as the standard.

Acknowledgements—Supported by U.S. Department of Agriculture, Competitive Research Grants Office, Grant No. 86-CRCR-1-2071, and the U.S. National Science Foundation, Grant No. DMB 85-02672. We thank John Smarrelli, Jr and Annette Miller for assistance with *N. crassa nit*-1.

REFERENCES

1. Kramer, S., Hageman, R. V. and Rajagopalan, K. V. (1984) *Arch. Biochem. Biophys.* **233**, 821.
2. Hawkes, T. R. and Bray, R. C. (1984) *Biochem. J.* **219**, 481.
3. Nason, A., Lee, K.-Y., Pan, S.-S., Ketchum, P. A., Lamberti, A. and DeVries, J. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 3242.
4. Johnson, J. L. and Rajagopalan, K. V. (1982) *Proc. Nat. Acad. Sci. U.S.A.* **79**, 6856.
5. Redinbaugh, M. G. and Campbell, W. H. (1985) *J. Biol. Chem.* **260**, 3380.
6. Mendel, R. R., Alikulov, Z. A. and Muller, A. J. (1982) *Plant Sci. Letters* **25**, 67.
7. Remmler, J. L. and Campbell, W. H. (1986) *Plant Physiol.* **80**, 442.
8. Mendel, R. R., Kirk, D. W. and Wray, J. L. (1985) *Phytochemistry* **24**, 1631.
9. Amy, N. K. and Rajagopalan, K. V. (1979) *J. Bacteriol.* **140**, 114.