Journal of Experimental Botany, Vol. 58, No. 9, pp. 2289–2296, 2007
Nitrogen Nutrition Special Issue
doi:10.1093/jxb/erm024 Advance Access publication 9 March, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

SPECIAL ISSUE PAPER

Biology of the molybdenum cofactor

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Received 1 December 2006; Revised 23 January 2007; Accepted 23 January 2007

Abstract

The transition element molybdenum (Mo) is an essential micronutrient for plants where it is needed as a catalytically active metal during enzyme catalysis. Four plant enzymes depend on molybdenum: nitrate reductase, sulphite oxidase, xanthine dehydrogenase, and aldehyde oxidase. However, in order to gain biological activity and fulfil its function in enzymes, molybdenum has to be complexed by a pterin compound thus forming the molybdenum cofactor. In this article, the path of molybdenum from its uptake into the cell, via formation of the molybdenum cofactor and its storage, to the final modification of the molybdenum cofactor and its insertion into apo-metalloenzymes will be reviewed.

Key words: Aldehyde oxidase, *Arabidopsis thaliana*, molybdenum cofactor, nitrate reductase, sulphite oxidase, xanthine dehydrogenase.

Introduction

The transition element molybdenum (Mo) is an essential micronutrient for plants, animals, and most micro-organisms (Sigel and Sigel, 2002). In soils, the molybdate anion is the only form of Mo that is available for plants and bacteria (Stiefel, 2002). To this end, more than 50 enzymes are known to contain Mo and most of them occur in bacteria, while in plants only four were found, with nitrate reducatse (NR) as the most prominent one (Sigel and Sigel, 2002). For this journal, molybdenum metabolism in plants was reviewed 5 years ago (Mendel and Hansch, 2002). Therefore, the progress that has been reached since then will be discussed here.

In all organisms, Mo has to be complexed by a pterin compound, thereby forming the molybdenum cofactor (Moco), in order to gain biological activity. This pterin compound is a unique pterin named molybdopterin or metal-containing petrin (MPT). With the exception of nitrogen-fixing nitrogenase, all Mo-containing enzymes characterized to date contain the pterin-type cofactor (Hille, 1996). As depicted in Fig. 1, Moco is a tricyclic pterin that co-ordinates the metal via a dithiolene group at the third (pyrano) ring. The task of the cofactor is to position the catalytic metal Mo correctly within the active centre, to control its redox behaviour, and to participate with its pterin ring system in the electron transfer to or from the Mo atom. The pterin, with its several possible reduction states as well as different structural conformations, might also be important in channelling electrons from or to other prosthetic groups (Hille, 2005).

Nitrate reductase needs Moco

Eukaryotic NR is a homodimeric enzyme. The monomer of plant NR consists of three functional domains: the N-terminal domain associated with Moco, the central haem-binding cytochrome b_5 domain, and the C-terminal FAD-binding domain, each redox active prosthetic group bound to the monomer in a ratio of 1:1:1. Dimerization is dependent on the presence of Moco (Campbell, 2001). The domains are connected by solvent-exposed and protease-sensitive hinge regions which had already been observed in the 1980s when defined degradation products of the NR monomer generated by limited proteolysis were detected on SDS-PAGE gels. The three domains form three redox centres catalysing the transfer of electrons from the reductant NAD(P)H via FAD, haem, and Moco to nitrate.

The atomic structure of holo-NR is still unsolved, probably because the purification of large amounts of native, non-degraded plant NR sufficient for crystallization experiments was principally possible, yet there was no success in obtaining diffracting crystals. Also, the

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Abbreviations: AO, aldehyde oxidase; MCP, molybdenum cofactor carrier protein; Mo, molybdenum; Moco, molybdenum cofactor; MPT, molybdopterin; NR, nitrate reductase; SO, sulphite oxidase; XDH, xanthine dehydrogenase.

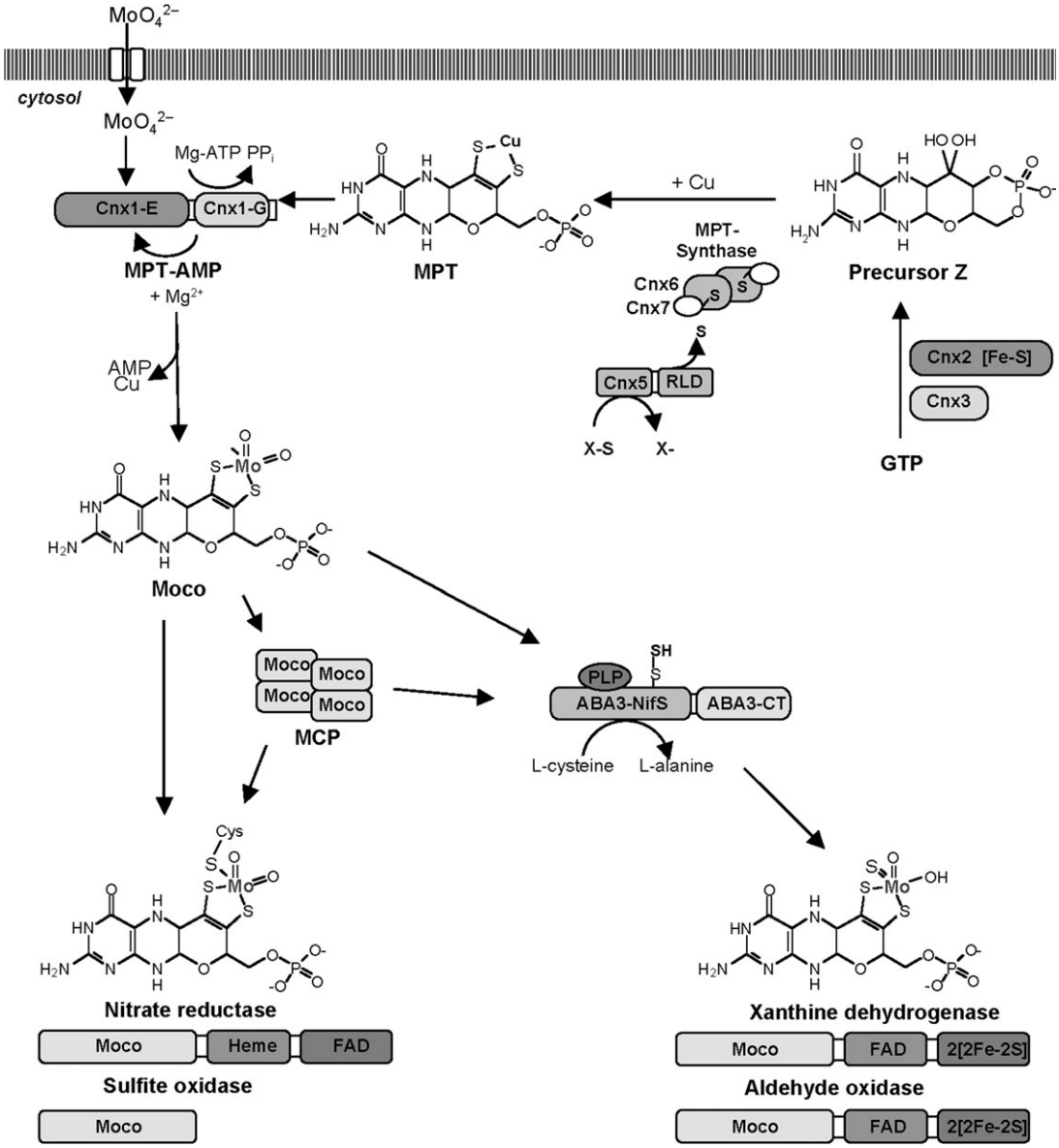

Fig. 1. Biosynthesis and distribution of Moco in plant cells. The basic steps of Moco biosynthesis are shown, starting from GTP (right upper part) to Moco, including Moco biosynthesis enzymes. MPT-synthase, consisting of Cnx6 and Cnx7, is sulphurated by Cnx5, with the primary sulphur donor of Cnx5 (X-S) being unknown. It is assumed that copper (Cu) is inserted directly after dithiolene formation. Mature Moco can be bound either to a Moco carrier protein (MCP), to NR and SO, or to the ABA3 protein. ABA3 is known to generate a protein-bound persulphide, which is the source of the inorganic sulphur ligand of Moco in enzymes of the XDH/AO family. The domain structure of the Mo-enzyme monomers is given in the lower part of the figure. Like Cnx2, XDH and AO also depend on iron–sulphur clusters [Fe–S] supplied by the mitochondria. Clearly, the formation of active Mo-enzymes depends not only on the availability of Mo but also on the presence of the two metals iron and copper.

recombinant expression of holo-NR in *Escherichia coli* yielded only inactive NR proteins, while expression in the methylotrophic yeast *Pichia pastoris* was more promising. So use was made of the linear domain arrangement of the NR monomer to express recombinantly the FAD-domain and the haem-domain separately and to solve their structures (Dwivedi *et al*., 1994; Lu *et al*., 1995). Finally, the structure of the Mo-binding domain of the yeast *Pichia angusta* was solved (Fischer *et al*., 2005). This structure revealed a unique slot for binding nitrate in the active site and also showed that the Mo-domain can be further subdivided into a Moco-binding catalytic domain and a C-terminal domain representing the dimerization interface of holo-NR. It is remarkable that the Moco is completely buried inside the protein and a funnel-like structure leads from the surface of the protein to the active site.

Molybdenum uptake

Biologically, Mo belongs to the group of trace elements, i.e. the organism needs it only in minute amounts.

However, unavailability of Mo is lethal for the organism. However, even if Mo is available for the cell, it seems to be biologically inactive until it becomes complexed to form Moco, thus gaining biological activity. Uptake of Mo as molybdate anion requires specific uptake systems to scavenge molybdate in the presence of competing anions. Eukaryotic molybdate transport is still poorly understood. There are reports that sulphate (Alhendawi *et al*., 2005) as well as phosphate (Heuwinkel *et al*., 1992) starvation of plants enhances Mo uptake up to 10-fold which means that sulphate transporters and phosphate transporters might co-transport molybdate anions. In the alga *Chlamydomonas reinhardtii*, genetic evidence suggested the existence of a distinct molybdate uptake system (Llamas *et al*., 2000) and very recently a molybdate transporter has been cloned from this organism (E Fernandez, personal communication). In parallel, in *Arabidopsis thaliana*, a molybdate transporter has been cloned and characterized (T Fujiwara, personal communication). In bacteria, high-affinity molybdate transporters are described, driven by ATP hydrolysis. In some bacteria, specific molybdate-binding proteins are known with a capacity of up to eight anions (Pau and Lawson, 2002) that store molybdate until further use by the cell; however, these proteins have not been found in eukaryotes yet.

Moco biosynthesis

In pre-genomic times, the detailed characterization of NR-deficient mutants contributed substantially to our understanding of the genetics and biochemistry of Moco biosynthesis in plants. These NR-deficient mutants fell into two groups: those with a defect in the NR-apoprotein and others which were defective in Moco. The identification of several genetic complementation groups among Moco-deficient mutants and the conserved structure of Moco provided a basis on which to propose an evolutionary multi-step biosynthetic pathway (Mendel, 1992). Among eukaryotes, the molecular, biochemical, and genetic analysis of Moco mutants was most advanced in higher plants. These results formed the basis to decipher Moco biosynthesis in humans as well, where Moco deficiency is a severe genetic disease with fatal consequences for the affected individuals (Reiss and Johnson, 2003).

In plants and in all organisms studied so far, Moco is synthesized by a conserved biosynthetic pathway that can be divided into four steps, according to the biosynthetic intermediates precursor Z, MPT, adenlyated MPT, and Moco (Fig. 1). In plants, six gene products participate in Moco biosynthesis (Schwarz and Mendel, 2006). These genes are homologous to their counterparts in humans, fungi, and bacteria. Genes and gene products were named in plants according to the *cnx* nomenclature (cofactor for nitrate reductase and xanthine dehydrogenase).

Step 1: conversion of GTP into precursor Z

A guanosine derivative (probably GTP) is transformed into a sulphur–free pterin compound, the precursor Z (Fig. 1). GTP labelling studies and NMR demonstrated that each carbon atom of the ribose and of the guanine ring is incorporated into precursor Z (Wuebbens and Rajagopalan, 1995; Rieder *et al*., 1998). This reaction is catalysed by Cnx2 and Cnx3, one of them (Cnx2) containing iron–sulphur clusters and belonging to the superfamily of *S*-adenosylmethionine-dependent radical enzymes (Hanzelmann *et al*., 2004).

Step 2: synthesis of molybdopterin

In the second stage, sulphur is transferred to precursor Z in order to generate MPT. This reaction is catalysed by the enzyme MPT synthase. After MPT synthase has transferred the two sulphurs to precursor Z, it has to be resulphurated in order to reactivate the enzyme for the next reaction cycle of precursor Z conversion (Fig. 1). This resulphuration is catalysed by Cnx5 involving an adenylation of MPT synthase followed by sulphur transfer (Matthies *et al*., 2004, 2005). Cnx5 is a two-domain protein consisting of an N-terminal domain responsible for adenylating MPT synthase and a C-terminal rhodanese-like domain where the sulphur is bound to a conserved cysteine in the form of persulphide (Matthies *et al*., 2005). The identity of the donor for the reactive mobile sulphur is as yet unknown.

Step 3: adenlyation of molybdopterin

In the third step, Mo has to be transferred to MPT in order to form Moco, thus linking the molybdate uptake system to the MPT pathway. In plants, this step is catalysed by the two domain-protein Cnx1. Its C-terminal domain (= Cnx1-G) was known to bind MPT tightly (Schwarz *et al*., 1997), yet its crystal structure (Kuper *et al*., 2004) revealed an unexpected finding: a novel reaction intermediate, adenylated MPT (MPT-AMP) (Fig. 1), was found. Subsequently it was demonstrated that Cnx1-G adenylates MPT in an Mg^{2+}- and ATP-dependent way and forms MPT-AMP that remains bound to Cnx1-G (Llamas *et al*., 2004).

Step 4: molybdenum insertion

The crystal structure of the Cnx1-G revealed another unexpected finding, namely a copper bound to the MPT dithiolate sulphurs. Up to now, the function of this novel MPT ligand is unknown but copper might play a role in sulphur transfer to precursor Z, in protecting the MPT dithiolate from oxidation, and/or presenting a suitable leaving group for Mo insertion. The origin of this copper is still unclear, but it is reasonable to assume that it binds to the dithiolate group just after the latter has been formed, i.e. at the end of step 2 of Moco biosynthesis. In the final step of Moco biosynthesis, MPT-AMP is transferred to the

N-terminal domain of Cnx1 (=Cnx1-E) that cleaves the adenylate in a molybdate-dependent way (Llamas *et al*., 2006), releases copper, and inserts Mo, thus yielding active Moco (Fig. 1).

Is there storage of Moco?

Moco is highly unstable once liberated from proteins; it loses the Mo atom and undergoes rapid and irreversible loss of function due to oxidation (Rajagopalan and Johnson, 1992). Therefore, it was assumed that Moco does not occur free in the cell, rather Moco should be transferred immediately after biosynthesis to an apo-target-enzyme, or Moco could be bound to a carrier protein that protects and stores it until further use (Fig. 1). The availability of sufficient amounts of Moco is essential for the cell to meet its changing demand for synthesizing Mo enzymes, therefore the existence of a Moco carrier protein (MCP) would provide a way to buffer supply and demand of Moco. Among eukaryotes, an MCP was described first in the green alga *C. rheinhardtii* (Aguilar *et al*., 1992). It is able to bind and protect Moco against oxidation (Witte *et al*., 1998; Ataya *et al*., 2003), and its crystal structure shows that it is a homotetramer (Fischer *et al*., 2006). It is still open whether MCP represents the default pathway for Moco allocation to its users or whether MCP is only a buffer, and the default pathway goes directly from Cnx1 to the appropriate apo-enzymes. Among higher plants, proteins with homologies to *C. reinhardtii* MCP are found that form a multi-gene familiy in *A. thaliana* (R Mendel, unpublished data).

How is Moco assembled into apo-nitrate reductase?

How do Moco, terminal sulphur, FAD, iron–sulphur clusters and haem become assembled into the apoproteins of Mo-enzymes? Nothing is known about how and when FAD, iron–sulphur centres, and haem are bound to the proteins. For Moco, however, the first crystallographic analyses of Mo-enzymes showed that the cofactor is deeply buried within the holo-enzyme so that Moco could only have been incorporated prior to or during completion of folding and dimerization of the apoprotein monomers. For the insertion of Moco into the target apo-enzymes as occurs in the living cell, either (still unknown) chaperone proteins would be needed or the Moco carrier proteins could become involved at this stage. For some bacterial Mo-enzymes, system-specific chaperones are required for Moco insertion and protein folding, for example NarJ for *E. coli* nitrate reductase (Vergnes *et al*., 2006).

Having been introduced into the apoprotein, Mo is coordinated by additional ligands present in the active centre of the respective Mo-enzyme. Moco forms an important part of the active centre but it is not the only constituent of the centre because the additional ligands supplied by the apoprotein are equally important for the specificity of the reaction.

Like every other protein, Mo-enzymes are finally degraded after having fulfilled their tasks. In particular, the pool of NR is degraded to a large extent once a day during its diurnal rhythm. Nothing is known about the fate of Moco and Mo after hydrolysis of the apoprotein. It can be assumed, however, that Moco is degraded as well, because free Moco released from proteins is extremely labile and sensitive to oxidation.

Nitrate reductase and sulphite oxidase are sister enzymes

Five years ago, the gene for plant sulphite oxidase (SO) was cloned from *A. thaliana* (Eilers *et al*., 2001) exhibiting high sequence similitaries to the Moco-domain of the NR gene. The SO gene is found to be widespread and highly conserved within the plant kingdom. It encodes the smallest (43 kDa for the monomer) and simplest eukaryotic Mo-enzyme that possesses only Moco as redox centre (Fig. 1). Recently, the crystal structures of SO and of the Moco-domain of NR were also solved (Schrader *et al*., 2003; Fischer *et al*., 2005), revealing a high degree of structural conservation between both proteins with only few differences in the boundaries of the enzymes. Substrate binding is proposed to be very similar between SO and NR due to three overall conserved residues in the active sites. Yet, there are also important changes within the active site that are believed to determine substrate specificity and catalytic properties. Consequently, the question might be raised of whether or not it is possible to convert the nitrate-reducing Moco-domain of NR into a sulphite-oxidizing domain, and vice versa.

Oxygen serves as the terminal electron acceptor for plant SO (EC 1.8.3.1) and becomes reduced to hydrogen peroxide (Hänsch *et al*., 2006). The latter finding explains the peroxisomal localization of plant SO (Nowak *et al*., 2004). Since plant SO is not found in the chloroplasts, it can be assumed that the function of SO is not related to the chloroplast-based sulphur assimilation pathway (Saito, 2004). Rather it was recently found that SO has a sulphite-detoxifying function (Lang *et al*., 2007); sulphite is a highly reactive metabolite that can react with a wide variety of cellular components. Therefore it has to be removed in order to protect the cell against a surplus of sulphite derived from SO_2 gas in the atmosphere (acid rain) (Heber and Hüve, 1998) or during the decomposition of sulphur-containing amino acids. The compartmentalization of sulphur assimilation and sulphite oxidation in different organelles allows plants to co-regulate these opposing metabolic demands. As SO is capable of producing hydrogen peroxide, its function might also be connected to reactive oxygen metabolism.

What other enzymes need molybdenum in plants?

There are more than 50 different enzymes known that need Moco (Sigel and Sigel, 2002), but most of them occur in bacteria and only very few are found in eukaryotes. In addition to NR and SO, only two additional Mo-enzymes are described in plants: xanthine dehydrogenase (XDH) and aldehyde oxidase (AO).

Xanthine dehydrogenase

Xanthine dehydrogenase (XDH; EC 1.1.1.204) is a key enzyme of purine degradation that oxidizes hypoxanthine to xanthine and xanthine to uric acid by simultaneous release of electrons from the substrate. Two genes were found in *Arabidopsis*, located side by side in the genome, encoding the isoenyzmes XDH1 and XDH2 (Hesberg *et al*., 2004). XDH is active as a homodimer of two identical subunits (150 kDa for the monomer), each being subdivided into three distinct domains (Fig. 1): an N-terminal domain with a size of 20 kDa for binding of two [2Fe-2S] clusters, a 40 kDa domain harbouring an FAD-binding site, and a C-terminal domain required for Moco binding and dimerization. Electrons derived from substrate oxidation are either transferred to NAD^+ to form NADH, or they are transferred to molecular oxygen to yield superoxide anions. Plant XDH also has an NADH oxidase activity (Yesbergenova *et al*., 2005) and might, therefore, have additional physiological functions in reactive oxygen metabolism because increasing XDH activities and simultaneous ROS production were observed upon plant–pathogen interactions (Montalbini, 1992*a*, *b*), hypersensitive response (Montalbini and Della Torre, 1996), drought stress (Yesbergenova *et al*., 2005), and natural senescence (Pastori and Rio, 1997; Hesberg *et al*., 2004). The subcellular localization of XDH is still not clear as both a cytosolic (Datta *et al*., 1991) and a peroxisomal localization (Sandalio *et al*., 1988) were reported.

Aldehyde oxidase

Aldehyde oxidase (AO; EC 1.2.3.1) enzymes are very similar to XDH enzymes (Fig. 1) as they share a high degree of sequence homology, have nearly identical molecular mass, bind the same cofactors, form dimers, and also act as hydroxylases. AO enzymes can be aligned along their entire length with XDH enzymes, and phylogenetic analysis has shown that AO proteins have derived from XDH after ancient gene duplications (Rodriguez-Trelles *et al*., 2003). *Arabidopsis thaliana* has four AO genes, *aao1–aao4*, whose products form homodimers as well as heterodimers, thus leading to altered substrate specificities of the respective isoenzymes. AO enzymes are strict oxidases that are unable to bind NAD^+ and exclusively use molecular oxygen as an electron acceptor, thus producing hydrogen peroxide (Badwey *et al*., 1981; Yesbergenova *et al*., 2005). One AO isoform (AAO3) acts best with abscisic aldehyde as a substrate. Abscisic aldehyde is the native precursor of the plant hormone absicisic acid which is essential for many developmental processes as well as for a variety of abiotic and biotic stress responses (Seo and Koshiba, 2002; Mauch-Mani and Mauch, 2005; Verslues and Zhu, 2005). Hence, AO enzymes in plants are essential for many physiological processes that require the involvement of absicisic acid and perhaps also of auxins.

A novel plant Mo-enzyme?

Very recently, a novel Mo-enzyme in mammals has been found that forms part of the amidoxime reductase complex (Havemeyer *et al*., 2006), for which the homologous sequence from *Arabidopsis* has been cloned (F Bittner, R Mendel, unpublished data), so that it might well be that plants possess a fifth Mo-enzyme.

Post-translational activation of AO and XDH

AO and XDH require a final step of maturation during or after insertion of Moco, i.e. the addition of a terminal inorganic sulphur to the Mo-centre in order to gain enzymatic activity. This sulphur ligand does not originate from the apoprotein nor does it come from the Moco moiety (Wahl and Rajagopalan, 1982), but it is added by a separate enzymatic reaction catalysed by the Moco-sulphurase ABA3 (Bittner *et al*., 2001). ABA3 is a two domain protein acting as a homodimer (Fig. 1). In a pyridoxal phosphate-dependent manner, the N-terminal domain of ABA3 decomposes L-cysteine to yield alanine and elemental sulphur, the latter being bound as a persulphide to a highly conserved cysteine residue of ABA3 (Heidenreich *et al*., 2005). As the C-terminal domain of ABA3 recently was found to bind sulphurated Moco efficiently (S Wollers, F Bittner, unpublished data), it appears likely that the persulphide sulphur is transferred from the N-terminal domain to Moco bound at the C-terminal domain prior to activation of the respective target enzyme. It is still unclear whether ABA3 finally activates its target enzymes by transferring only the sulphur, which is bound to the Moco of the C-terminal domain, or whether it transfers the entire sulphurated Moco of the C-terminus in order to activate XDH and AO. Under physiological aspects the terminal sulphuration step provides an efficient way of regulating the amount of active XDH and AO enzymes in the cell.

Molybdenum deficiency

The Mo content of plants is directly correlated to the bioavailability of Mo in the soil. The lower the soil pH, the less available is Mo (Mengel and Kirkby, 2001), thus causing Mo deficiency in plants. Mo-deficient plants

develop a characteristic phenotype including lesions and altered morphology of leaves (referred to as 'whiptail') that was first described by Arnon and Stout (Arnon and Stout, 1939) and later analysed in detail by Hewitt's group (Fido *et al*., 1977). In contrast, Mo toxicity in plants under most agricultural conditions is rare. There is an extensive literature about Mo nutrition in field-grown plants that was recently reviewed (Kaiser *et al*., 2005).

Mo deficiency could also be caused by a mutation in the Mo-specific uptake system. As the Mo-transporter of higher plants is still not published, its loss cannot be discussed here. Particularly this point, however, would be interesting because knock-out plants in the Mo transporter could be used as a tool to study the assumed contribution of the sulphate and phosphate uptake systems to the Mo supply of higher plants.

Finally, Mo deficiency can be caused by a defect in Moco biosynthesis which has dramatic consequences for the cell because pleiotropically all Mo-enzyme activities are lost or strongly reduced. The loss of which Mo-enzyme is most severe for the plant? This question can be answered by comparing the phenotypes of *Arabidopsis* knock-out mutants in one of the four Mo-enzyme types described to this end in plants. (i) NR-mutants with complete loss of NR-activity have been known about for many years; the loss of NR activity is lethal if these mutants are cultured with nitrate as the sole nitrogen source (Gabard *et al*., 1988; Müller and Mendel, 1989). (ii) The loss of SO has no phenotype provided the plants are not challenged to an atmosphere with high sulphur dioxide (Lang *et al*., 2007). (iii) The loss of XDH1 has no lethal consequences although the phenotype of the plants is slightly, but not dramatically changed (F Bittner, R Mendel, unpublished results). (iv) For AO, only a mutation in AAO3 has been documented (Seo *et al*., 2000). AAO3 is important for the conversion of abscisic aldehyde into abscisic acid, hence its loss leads to a wilty phenotype which is severe for the survival of the plant. In summary, the complete loss of Moco as occurs in *cnx*-mutants is lethal and leads to the death of plants when they are grown in soil. In cell culture, however, these mutants can be kept alive when grown on media with reduced nitrogen as N-source.

Outlook

Recent years have brought rapid progress in our understanding of the role and the function of Mo in plants. Clearly, research concentrates both on the detailed enzymology of Moco biosynthesis/allocation and on studying the structure–function relationships of Mo-enzymes. Yet there is still a large number of unresolved questions that need to be answered. How is the molybdate transporter organized in detail? What is the role of copper in Moco synthesis? How is the multi-enzyme complex for Moco biosynthesis organized? What is the mechanism of Moco insertion into apo-enzymes? In which cellular compartment is Moco synthesized and how is Moco biosynthesis regulated to meet the changing demands of the cell for Moco? The coming years will bring insight into these and perhaps novel aspects of Mo within the metabolic and physiological network of the cell.

Acknowledgements

I thank the many people who have worked with me over the years on molybdenum. I am grateful to Steffen Rump for help with the figures. My research was consistenly supported by the Deutsche Forschungsgemeinschaft which is gratefully acknowledged. Financial support also came from the European Union and the Fonds der Chemischen Industrie.

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