Gene expression related to molybdenum enzyme biosynthesis in response to molybdenum deficiency in winter wheat

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Abstract

Molybdenum (Mo) is an essential trace element for higher plants. Mo enzyme biosynthesis is regulated by several plant genes. A hydroponic trial was conducted to investigate the effect of Mo deficiency on the expression of genes that are related to Mo enzyme biosynthesis in winter wheat. The results showed that Mo deficiency decreased the activities of nitrate reductase (NR), sulphite oxidase (SO), aldehyde oxidase (AO) and xanthine dehydrogenase (XDH) in the leaves or roots of winter wheat by regulating the expression of *NR*, *SO*, *AO* and *XDH* genes. Mo deficiency induced the expression of *TaCnx2* and *TaCnx5*, which might result in the accumulation of cyclic pyranopterin monophosphate (cPMP) and molybdopterin (MPT) in wheat. Mo deficiency significantly decreased *TaCnx1* expression, which is expected to reduce molybdenum cofactor (Moco) formation. Moco shortage induced the expression of *TaAba3* in winter wheat. The blockade of Moco biosynthesis under conditions of Mo deficiency might decrease the activities of Mo enzymes.

Keywords: Molybdenum deficiency, molybdenum enzyme biosynthesis, gene expression, winter wheat

Abbreviations: Mo, molybdenum; NR, nitrate reductase; SO, sulphite oxidase; AO, aldehyde oxidase; XDH, xanthine dehydrogenase; Moco, molybdenum cofactor; GTP, guanosine 5'-triphosphate; cPMP, cyclic pyranopterin monophosphate; MPT, molybdopterin; MPT-AMP, adenylated molybdopterin; ABA, abscisic acid.

1. Introduction

The trace element Molybdenum (Mo) is essential for plant growth. More than 50 Mo enzymes have been found in all organisms and catalyse a variety of redox reactions, but only four (nitrate reductase (NR), sulphite oxidase (SO), xanthine dehydroge-

nase (XDH), and aldehyde oxidase (AO)) have been found in plants thus far (Mendel and Leimkühler, 2015). Mo enzymes participate in various metabolic processes, such as nitrate assimilation, sulphite detoxification, phytohormone synthesis and purine catabolism, in plants (Mendel and Leimkühler, 2015).

Mo deficiency induces the yellowing and whiptail of leaves, as well as depressed photosynthesis, cold resistance, and drought resistance, characteristics that are thought to be related to the inactivation of Mo enzymes under Mo deficiency (Kaiser *et al.*, 2005).

Mo uptake and transport in plants require the involvement of some proteins, such as MOT1 and MOT2. The specific transporter MOT1 and MOT2 are required for efficient uptake, translocation and storage of molybdate. Mo deficiency reduced *MOT1* expression and induced *MOT2* gene in plant shoot, and then decreased Mo uptake and transfer from root to shoot (Baxter *et al.*, 2008; Gasber *et al.*, 2011). After being taken up by plants, Mo exhibits no bioactivity unless it is combined with a ubiquitous pterin-based Mo cofactor (Moco) (Zaman *et al.*, 2014), which forms part of the active centre of all Mo enzymes in plants (Mendel and Leimkühler, 2015). Moco biosynthesis includes three steps (Mendel and Leimkühler, 2015). In the first step, guanosine 5'-triphosphate (GTP) is transformed into the sulphur-free pterin compound, cyclic pyranopterin monophosphate (cPMP, also known as precursor Z), a reaction that is catalysed by two proteins, CNX2 and CNX3, which are located in the mitochondria (Teschner *et al.*, 2010). The second step is the synthesis of molybdopterin (MPT), which is catalysed by MPT synthase, comprising CNX6 and CNX7. The formation of MPT is not a catalytic reaction; therefore, MPT synthase needs to be reactivated for the following cPMP conversion cycle after each reaction step. MPT-synthase sulphurase, CNX5, catalyses this separate resulphuration via sulphur transfer. Finally, Mo is transferred into the MPT and forms Moco. CNX1 adenylates the Moco precursor MPT to MPT-AMP and subsequently catalyses the insertion of molybdate into MPT, generating Moco (Llamas *et al.*, 2006). ABA3 is a Moco sulphurase that is required for the activation of AO and XDH; thus, it plays an important role in the biosynthesis of AO and XDH

(Bittner, Oreb, and Mendel, 2001). Mo enzymes are activated after insertion of Moco. The activity or protein synthesis of these enzymes are regulated by their genes. For example, the transcript of *NR* regulates its activity and synthesis (Miller and Cramer, 2005). The functions of *SO* and *AO* gene are to encode the SO and AO involving in sulfur metabolism and hormone biosynthesis, respectively (Eilers *et al.*, 2001; Yesbergerova *et al.*, 2005). Like AO, XDH activity requires a post-translational of the holoenzyme, but it is also regulated by transcript of XDH gene especially in environment-stressed plants (Hesberg *et al.*, 2004). In principle, the transcript level of Mo enzyme gene affects biosynthesis amounts of the proteins directly. The gene expression of the four Mo enzymes could be regulated by environmental factor, such as light, low temperature, drought and so on.

Previous studies have found that Mo deficiency slightly induced the expression of *CNX2*, *CNX5*, *CNX6*, *ABA3*, and *MOT2* in a *Arabidopsis thaliana* T-DNA inserted mutant, *mot1-1* (Ide *et al.*, 2011). In addition, several reports also show Mo deficiency can decrease the activities of Mo enzyme such as NR, AO and XDH in plant (Sun *et al.*, 2009; Yesbergerova *et al.*, 2005). We speculated Mo deficiency might affect the biosynthesis of Moco and Mo enzymes through regulating the expression of above genes in winter wheat. To get a better understanding of the reason for the decrease in Mo enzyme activities, we investigated the expression of genes related to the biosynthesis of Mo enzymes in winter wheat.

2. Materials and Methods

2.1. Plant material

Winter wheat (*Triticum aestivum*) 'Jing852' seeds were disinfected in a solution of 0.5% NaClO before being germinated in deionized water (resistivity > 18.25

MΩ.cm at 25 °C) at 25°C for 5 d. After germination, the seedlings were transferred to 10-L plastic boxes containing nutrient solutions including 4 mmol L⁻¹ Ca(NO₃)₂·4H₂O, 6 mmol L⁻¹ KNO₃, 1 mmol L⁻¹ NH₄H₂PO₄, 2 mmol L⁻¹ MgSO₄·7H₂O, 100 μmol L⁻¹ EDTA-Fe, 46 μmol L⁻¹ H₃BO₃, 9 μmol L⁻¹ MnCl₂·4H₂O, 0.8 μmol L⁻¹ ZnSO₄·7H₂O, and 0.3 μmol L⁻¹ CuSO₄·5H₂O, adjusted to a pH of 6.0. Mo was added to the nutrient solutions as Na₂MoO₄·2H₂O at two rates: 0 (-Mo) and 1 μmol L⁻¹ (+Mo). Each treatment was replicated three times. Quarter- and half-strength nutrient solutions were used in the first and second weeks, respectively, and full-strength solutions were then used until the seedlings were sampled. The solutions were refreshed every three days. All vessels that were used in the experiment were dipped in 5% HCl for one week and then washed with deionized water a minimum of 3 times. To minimize possible Mo contamination, the water used to prepare the nutrient solutions was deionized, and pure reagents were used. The plants were grown for 30 days in a glasshouse under controlled environmental conditions using a light/dark regime of 12/12 h, corresponding air temperatures of 22/18°C, a photon flux density of approximately 500 μmol m⁻² s⁻¹ and a relative humidity of approximately 65%.

2.2. Assay of Mo-enzyme activities

NR activity was assayed according to the method described by Evans and Nason (1953). The leaves or roots (0.5 g) were homogenized in 4 mL ice-cold extraction medium (pH 8.7, 0.025 mol L⁻¹ potassium phosphate, 1 mmol L⁻¹ EDTA, 10 mmol L⁻¹ L-cysteine), and the homogenate was then centrifuged at 4,000 × g for 15 min at 4 °C. The assay mixture contained 1.2 mL of 0.1 mol L⁻¹ potassium phosphate buffer (pH 7.5) containing 0.1 mol L⁻¹ KNO₃, 0.4 mL of 2.62 mmol L⁻¹ NADH, and 0.4 mL of enzyme extract. The mixture was incubated at 25 °C for 15 min,

after which the reaction was stopped by adding 1 mL of 1% w/v sulphanilamide in 3.0 mol L⁻¹ HCl. N-(1-naphthyl) ethylene diamine hydrochloride reagent was then added (1 mL, 0.02% w/v), and the contents were mixed by inverting the tubes. The colour was allowed to develop for 15 min before the mixture was centrifuged at 4,000 × g for 5 min to remove turbidity. The absorbance was determined by reading each sample against its own blank (a complete reaction except that NADH was absent) in a spectrophotometer at 540 nm. AO and XDH activities were assayed according to the methods described by Yesbergenova *et al.* (2005). Tissues were homogenized in ice-cold extraction medium containing 250 mmol L⁻¹ Tris-HCl (pH 8.5), 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ DTT, 3 mmol L⁻¹ GSH, and 3% (w/v) PVPP. Samples comprising 1 g of leaves or roots were extracted in 3 or 2 mL of buffer (1:3 and 1:2, w/v), respectively. The homogenates were then centrifuged at 12,000 × g for 20 min at 4 °C. AO activity was assayed by monitoring the decrease of absorbance at 600 nm using a UV 2100 spectrophotometer (Shimadzu, Kyoto, Japan); DCIP was used as an electron donor. The AO reaction mixture (1 mL) contained 100 μL of enzyme extract, 50 mmol L⁻¹ potassium phosphate (pH 7.4), 0.002% (w/v) DCIP, 0.1 mmol L⁻¹ PMS, and 2 mmol L⁻¹ indole-3-aldehyde. The XDH reaction mixture (1 mL) contained 100 μL of enzyme extract, 50 mmol L⁻¹ Tris-HCl (pH 8.48), 0.002% (w/v) DCIP, 0.1 mmol L⁻¹ PMS, and 0.6 mmol L⁻¹ hypoxanthine. AO and XDH activities were expressed as nanomoles DCIP reduced per milligram of protein per minute.

SO activity was assayed according to the method described by Eilers *et al.* (2001). The leaves or roots (1 g) were homogenized in 3 mL ice-cold extraction medium containing 100 mmol L⁻¹ HEPES (pH 7.3), 1 mmol L⁻¹ EDTA, 5% (v/v) glycerol, 1 mmol L⁻¹ Na₂MoO₄, and 1 mmol L⁻¹ PMSF. The homogenate was centrifuged at 21,000 × g for 20 min at 4 °C. SO

activity assay was monitored by following the reduction of ferricyanide at 420 nm using a UV 2100 spectrophotometer (Shimadzu, Kyoto, Japan). Sodium sulphite (4 μL , 0.1 mol L^{-1}) was added to a cuvette containing 20 μL of the enzyme extract, 20 mmol L^{-1} Tris-HCl (pH 8.0), 0.1 mmol L^{-1} EDTA, and 0.4 mmol L^{-1} ferricyanide. One unit of SO activity was defined as the conversion of 1 μmol of sulphite per milligram of protein per minute.

Soluble protein concentrations in the assays were measured using crystalline BSA as a reference (Bradford, 1976).

2.3 Determination of gene expression levels

Total RNA was extracted from the roots using Trizol reagent (Invitrogen, USA). First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV

Reverse Transcriptase (Promega, USA) according to the manufacturer's instructions, and the product was diluted to 1000 ng μL^{-1} . cDNA was used as the template in a real-time PCR reaction using a CFX⁹⁶ Real-Time PCR Detection System (Bio-Rad, USA) and SYBR green for product detection at the end of each amplification cycle (Karsai *et al.*, 2002). Real-time PCR was carried out according to Van Riet *et al.* (2006). Primers for the genes of interest were designed by Primer Premier 5.0 based on wheat gene sequences derived from NCBI (<http://www.ncbi.nlm.nih.gov/>) or CBFG (L) (<http://compbio.dfci.harvard.edu/tgi/>) according to Quackenbush *et al.* (2001), as listed in Table 1. The wheat *Actin* gene was used as a reference for all genes of interest. The relative quantities of the transcripts were calculated according to the method of Pfaffl (2001).

Table 1. Primers used for real-time PCR amplification

Gene	Gene Index No.	Strand	Primer sequence 5' to 3'
TaCnx2	TC381184	Forward	GCCCCTCAGAGGTGAGCTTG
		Reverse	TGTCACGCAGGGAAGAAGGA
TaCnx5	TC419777	Forward	TTGGATGTACGGCTGTCTCA
		Reverse	GGACAGCGCTCTGTGAGTCG
TaCnx1	TC385680	Forward	AGGGAAGCCATTGACATTTG
		Reverse	TCACTGCACGATGAACTCC
TaAba3	TC385542	Forward	TTGGATGTACGGCTGTCTCA
		Reverse	GGACAGCGCTCTGTGAGTCG
NR	TC457165	Forward	TCAACGAGCTCAACACCAAC
		Reverse	CAGAAGCACCAGCACCAGTA
SO	TC375951	Forward	CCGTGTGGTCGTACCTGGTG
		Reverse	CACATCTTCCAGGGGTGCAA
AO	TC454526	Forward	TGAAGCAGATGACGGCGTTC
		Reverse	AAGAGCACGGTGCACGACTG
XDH	TC409845	Forward	CGCGGCATCTGTAGCTTCCT
		Reverse	GCACGCTCCAAGACTGCAAG
TaActin	AB181991.1*	Forward	ACTGGGATGACATGGGGAA
		Reverse	ACCGCTGGCATAACAAGGAC

Note: *Sequences derived by cDNA/genomic DNA isolation and sequencing; TC gene index numbers were derived from TIGR gene indices (Quackenbush *et al.*, 2000).

2.4. Analysis of Mo concentration

Plant samples were ground, carbonized and ashed. Mo concentrations were determined using polarographic catalytic wave analysis using a JP-2 oscilloscope polarograph according to Wan *et al.* (1988).

2.5. Statistical analysis

Data are presented as the averages of three replicates. The results were statistically analysed using the Duncan multiple comparison as implemented

in SPSS 18 software. All statistically significant differences were tested at $P < 0.05$.

3. Results

3.1. Mo concentration

Pronounced differences were observed for Mo concentrations in the tissues of winter wheat. Mo concentrations in the shoots and roots were significantly lower after -Mo treatment than after +Mo treatment (Table 2).

Table 2 Molybdenum concentrations in the shoots and roots of winter wheat. -Mo and +Mo treatments represent plants that were fertilized with 0 and 1 $\mu\text{mol Mo L}^{-1}$ $[\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}]$, respectively. Data are the means of three replicates ($\pm$ se) with ten plants in each replicate per treatment. Different letters in a column indicate significant differences at $P 0.05$ as determined by ANOVA followed by Duncan's test.

Treatments	Mo concentration ($\mu\text{g g}^{-1}$)	
	Shoot	Root
-Mo	0.084 $\pm$ 0.009 ^b	0.293 $\pm$ 0.017 ^b
+Mo	4.439 $\pm$ 0.406 ^a	5.055 $\pm$ 1.150 ^a

3.2. Mo enzyme activities and gene expression

Mo deficiency decreased NR activity in the leaves and roots of winter wheat 2.5-fold and 2.6-fold, respectively (Figure 1A). There was no perceptible difference in the SO activity of leaves between treatments, but the activity in roots was approximately 1.7-fold lower under Mo deficiency (Figure

1B). Similarly, Mo deficiency had no significant influence on AO activity in leaves but significantly decreased AO activity in roots 3.3-fold (Figure 1C). XDH activity was significantly decreased (1.2-fold) in leaves of winter wheat in response to Mo deficiency, but no significant difference was observed in roots between the treatments (Figure 1D).

Figure 1 Activities of NR (A), SO (B), AO (C) and XDH (D) in the leaves and roots of winter wheat. -Mo and +Mo treatments represent plants that were fertilized with 0 and 1 $\mu\text{mol Mo L}^{-1}$ [$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$], respectively. Data are the means of three replicates ($\pm\text{se}$) with four plants in each replicate per treatment. * and ** indicate differences between treatments as determined by ANOVA followed by Duncan's test (*: $P < 0.05$, **: $P < 0.01$).

Mo deficiency significantly induced the expression of *NR* in leaves of winter wheat (12-fold), but no significant difference was found in roots (Figure 2A). The expression of genes encoding other Mo enzymes were also tested; *SO* in leaves, *AO* in leaves and roots and *XDH* in roots were all significantly decreased (approximately 3.0-fold, 3.8-fold,

5.5-fold and 15-fold, respectively) under Mo deficiency (Figure 2B, C and D). However, no significant difference was found between -Mo and +Mo treatments regarding *SO* expression in the roots and *XDH* expression in the leaves of winter wheat (Figure 2B and D).

Figure 2 Expression of *NR* (A), *SO* (B), *AO* (C) and *XDH* (D) in the leaves and roots of winter wheat. -Mo and +Mo treatments represent plants that were fertilized with 0 and 1 $\mu\text{mol Mo L}^{-1}$ [$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$], respectively. Data are the means of three replicates ($\pm$ se) with four plants in each replicate per treatment. * and ** indicate differences between treatments as determined by ANOVA followed by Duncan's test (*: $P < 0.05$, **: $P < 0.01$).

3.3. Expression of genes involved in the biosynthesis and sulphonation of *Moco*

Compared with the +Mo treatment, *TaCnx2* mRNA levels in leaves and roots were increased 2.6-fold and 3.1-fold, respectively, under -Mo treatment (Figure 3A). Mo deficiency similarly induced in-

creased *TaCnx5* mRNA levels in leaves and roots, as shown in Figure 3B. *TaCnx1* expression in leaves and roots was decreased by Mo deficiency (approximately 1.3-fold and 1.8-fold, respectively; Figure 3C). *TaAba3* expression in leaves and roots was induced by Mo deficiency (approximately 1.2-fold and 2.0-fold, respectively; Figure 3D).

Figure 3. Expression of *TaCnx2* (A), *TaCnx5* (B), *TaCnx1* (C) and *TaAba3* (D) in the leaves and roots of winter wheat. -Mo and +Mo treatments represent plants that were fertilized with 0 and 1 μmol Mo L⁻¹ [Na₂MoO₄•2H₂O], respectively. Data are the means of three replicates (± se) with four plants in each replicate per treatment. * and ** indicate differences between treatments as determined by ANOVA followed by Duncan's test (*: P<0.05, **: P<0.01).

4. Discussion

A reasonably proportional relationship exists between enzyme activity and Mo concentration in plants (Hew and Chai, 1984). In our experiment, Mo deficiency significantly decreased the activities of NR, SO, AO and XDH in the tissues of winter wheat (Figure 1); this was due to decreased Mo concentrations in the tissues (Table 2). These results are highly consistent with our earlier results (Sun *et al.*, 2009).

As previously mentioned, Mo enzymes have been identified in plants, including NR, SO, AO and XDH (Mendel and Leimkühler, 2015). NR is involved in primary nitrogen assimilation (Márquez-Quiroz *et al.*, 2014). The activity of NR can be regulated by mRNA (transcription) and protein

(translation) synthesis level, as well as post-translation modifications (Miller and Cramer, 2005). NR mRNA is induced by nitrate, light, and downstream N assimilation products such as amino acids or C products. NR protein synthesis and post-translation modifications are controlled by the reversible phosphorylation of the protein, and other environmental conditions such as light/dark transitions and CO₂ removal. It revealed that there was a complicated process in regulating the activity of NR (Miller and Cramer, 2005). In our experiments, Mo deficiency induced NR gene expression but decreased NR activity in the leaves of winter wheat (Figure 1A and 2A). The observed increase in NR gene expression might be a compensatory response to the decrease in nitrate assimilation that occurs under Mo deficiency, and the observed decrease in

NR activities might be related to lack of Moco or post-translation modifications under Mo deficiency (Ide *et al.*, 2011). Mo deficiency decreases NR activity, thereby depressing nitrogen metabolism (Hu, Wang, and Wei, 2002). SO oxidizes sulphite to sulphate and protects plant cells from the toxic effects of endogenously arising sulphite (Lang *et al.*, 2007). Mo deficiency decreased SO activity and its expression (Figure 1B and 2B), indicating that sulphite detoxification was inhibited. AO has been shown to catalyse the synthesis of abscisic acid (ABA) (Kaiser *et al.*, 2005). The observed decrease in AO mRNA levels in the roots was consistent with the significant decrease in AO activity in the roots under Mo deficiency (Figure 1C and 2C) and with the results of Tiedge *et al.* (1997) and Hesberg *et al.* (2004), who reported that enzyme activities are determined by mRNA levels. Mo deficiency decreases AO activity and endogenous ABA levels in winter wheat (Sun *et al.*, 2009), thereby affecting cold resistance in winter wheat (Li *et al.*, 2001). XDH is involved in purine catabolism and activated oxygen metabolism during senescence (Zimmer and Mendel, 1999). Mo deficiency resulted in a decline in XDH activity and XDH gene expression (Figure 1D and 2D), suggesting that Mo deficiency inhibits purine catabolism and accelerates senescence. In short, Mo deficiency might depress nitrate assimilation, sulphite detoxification, ABA biosynthesis and purine catabolism by regulating Mo enzyme activities and their gene expression.

Moco is the active compound at the catalytic site of all Mo enzymes in nature except nitrogenase (Mendel and Leimkühler, 2015). CNX2, CNX5 and CNX1 are involved in Moco biosynthesis. CNX2 induces the transformation from GTP to cPMP (Balk and Lobréaux, 2005). Mo deficiency increased *TaCnx2* mRNA levels in leaves and

roots, which is expected to enhance the transformation from GTP to cPMP. CNX5 catalyses the resulphuration of MPT synthase to continuously generate MPT (Mendel and Leimkühler, 2015). Mo deficiency induced the expression of *TaCnx5* (Figure 3B), indicating that Mo deficiency increases the regeneration of MPT synthase and the formation of MPT. Therefore, it was speculated that Mo deficiency induced the accumulation of cPMP and MPT by regulating the expression of *TaCnx2* and *TaCnx5*. Ide *et al.* (2011) also reported that Mo deficiency slightly induced the expression of *CNX2* and *CNX5* in *Arabidopsis thaliana mot1-1* shoots. CNX1 catalyses the insertion of molybdate into MPT, which is the last step in Moco biosynthesis (Kaufholdt *et al.*, 2016). Mo deficiency significantly decreased the expression of *TaCnx1* in winter wheat leaves (Figure 3C), which might inhibit the production of Moco and result in the accumulation of cPMP and MPT in wheat (Figure 3C and Table 2). ABA3 catalyses the sulphonation of Mo-cofactor, which is essential for the biosynthesis of members of the XDH/AO family (Bittner, Oreb, and Mendel, 2001). Mo deficiency induced the expression of *TaAba3* in the leaves and roots of winter wheat (Figure 3D), indicating that higher transcript levels of *ABA3* are needed under Moco deficiency.

5. Conclusions

The present study provides evidence that Mo deficiency affects the expression of genes that are related to Mo enzyme biosynthesis in winter wheat. Compared with Mo application treatment, Mo deficiency decreases Mo concentrations by 52.8-fold and 17.3-fold in leaves and roots, respectively. Mo deficiency accordingly decreased NR activities by 2.5-fold and 2.6-fold in leaves and roots, and decreased SO

activity by 1.7-fold in leaves, AO activity by 3.3-fold in roots, XDH activity by 1.2-fold in leaves, respectively. Similarly, the expression of *SO*, *AO*, *XDH* and *TaCnx1* were decreased from 1.3-fold to 15.0-fold in leaves or roots. But *NR*, *TaCnx2*, *TaCnx5* and *TaAba3* were induced from 1.2-fold to 3.0-fold in leaves or roots in response to Mo deficiency. These results indicated Mo can regulated the mo-cofactor biosynthesis, and then affected the Mo-enzymes activities under a certain degree.

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