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Effects of tungsten on uptake, transport and subcellular distribution of molybdenum in oilseed rape at two different molybdenum levels

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ABSTRACT

Due to the similarities of molybdenum (Mo) with tungsten (W) in the physical structure and chemical properties, studies involving the two elements have mainly examined their competitive relationships. The objectives of this study were to assess the effects of equimolar W on Mo accumulation, transport and subcellular distribution in oilseed rape at two Mo levels with four treatments: Mo_1 (1 μmol/L Mo, Low Mo), $Mo_1 + W_1$ (1 μmol/L Mo + 1 μmol/L W, Low Mo with Low W), Mo_{200} (200 μmol/L Mo, High Mo) and $Mo_{200} + W_{200}$ (200 μmol/L Mo + 200 μmol/L Mo, High Mo with high W). The fresh weight and root growth were inhibited by equimolar W at both low and high Mo levels. The Mo concentration and accumulation in root was increased by equimolar W at the low Mo level, but that in the root and shoot was decreased at the high Mo level. Additionally, equimolar W increased the Mo concentrations of xylem and phloem sap at low Mo level, but decreased that of xylem and increased that of phloem sap at the high Mo level. Furthermore, equimolar W decreased the expression of *BnMOT1* in roots and leaves at the low Mo level, and only decreased its expression in leaves at the high Mo level. The expression of *BnMOT2* was also decreased in root for equimolar W compared with the low Mo level, but increased compared with high Mo level. Moreover, equimolar W increased the proportion of Mo in cell wall fraction in root and that of soluble fraction in leaves when compared with the low Mo level. The results suggest that cell wall and soluble fractions might be responsible for the adaptation of oilseed rape to W stress.

1. Introduction

Molybdenum, an essential microelement in plant, plays a very important role in nitrate assimilation, sulphite detoxification, purine metabolism and the synthesis of abscisic acid, auxin and glucosinolates [1]. Tungsten (W), a twin element of molybdenum (Mo), is considered as a toxic element for plants [2]. The morphology and physiology of W effects on the growth of various plant species generally include: (1) the decrease of primary root length and plant biomass in growth rate, shorter leaves of seedlings with unfolding and dark-colored roots with fewer lateral roots [3]; (2) disruption of cortical microtubule array, nucleus morphology, mitosis and cytoskeleton of *Pisum sativum* root cells, the decrease of the cell length in the elongation zone and disruption in the actin microfilament [4,5], breakage of phosphodiester bonds in native DNA at a limited number of sites in wheat embryos after a biolistic transformation [6] and programmed cell death [7]; (3) inhibition of the Mo-enzyme activity due to the competition of tungstate with molybdate for incorporation into MoCo in plants including nitrate reductase, sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase, further influencing the metabolism of carbon, sulphur and nitrogen [8].

Due to the similar physical and chemical properties between Mo and W, W can compete with Mo for incorporation into the center of molybdenum cofactor, resulting in inactivation of molybdoenzymes. However, the research results about the possible effects of W on Mo uptake in plants are inconsistent with each other. Quin et al. reported that W addition increased the uptake of Mo below 50 mg/L W concentration in clover [9]. Jiang et al. [10] observed that the Mo concentrations in leaves were increased even when the W concentration reached 500 μmol/L in barley. Mo contents in plant tissues showed an increase with an increase of W contents in

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wheat tissue up to a threshold of 81 mg/kg, after which it showed an abrupt decrease [11]. In all these experiments, high W to Mo ratios (approximately 100:1) were considered necessary for inhibition of growth or Mo utilization; it was also found that decreasing the ratio to approximately unity by the addition of more Mo invariably reversed the tungsten inhibition. However, to our knowledge, currently little information is available on Mo homeostasis in response to equimolar W in plants. The objective of the present work was to determine the effects of equimolar W on Mo uptake, transport and subcellular distribution in plants at low and high Mo levels.

2. Materials and methods

2.1. Experimental design

Seeds of oilseed rape (*Brassica napus* L.) were disinfected in 5% sodium hypochlorite for 30 min before germination in polyethylene boxes filled with distilled water. 7-day-old seedlings of uniform size were transferred to plastic container (55 cm × 35 cm × 7 cm; 20 seedlings/container) filled with 10 L 1/2-strength mineral solution for 6 days and subsequently full-strength solution. The concentrations of macronutrients consisted of 4 mmol/L $Ca(NO_3)_2{\cdot}4H_2O$, 6 mmol/L KNO_3, 1 mmol/L $NH_4H_2PO_4$, 2 mmol/L $MgSO_4{\cdot}7H_2O$, and those of micronutrients were composed of 46.2 μmol/L H_3BO_3, 9.1 μmol/L $MnCl_2{\cdot}4H_2O$, 0.8 μmol/L $ZnSO_4{\cdot}7H_2O$, 0.3 μmol/L $CuSO_4{\cdot}5H_2O$, 100 μmol/L FeNaEDTA, and 1 μmol/L $Na_2MoO_4{\cdot}2H_2O$. The pH of the solution was adjusted to 6.7 ± 0.1 using NaOH or HCl. Nutrient solutions were replaced every 4 days. After an initial growth period of 20 days, the following four treatments were applied to the seedling: Mo_1 (1 μmol/L Mo), $Mo_1 + W_1$ (1 μmol/L Mo + 1 μmol/L W), Mo_{200} (200 μmol/L Mo) and $Mo_{200} + W_{200}$ (200 μmol/L Mo + 200 μmol/L W). The used reagents were $Na_2MoO_4{\cdot}2H_2O$ and $Na_2MoO_4{\cdot}2H_2O$. Each treatment was replicated four times. Plants were grown for a further 10 days in a greenhouse maintained with a 16 h photoperiod and the temperature controlled at 24–28 °C. Plant fresh weight (FW) was determined and samples were separated into roots, stalks, and leaves. Separated tissues were dried at 70 °C until constant weight.

2.2. Root parameters

Root images of the oilseed rape plants were captured using a flatbed scanner (Scanjet 3670, USA). Images were loaded into ImageJ software and the root parameters were calculated [12].

2.3. Exudation techniques

The xylem sap was collected using the method of Wu et al. [13]. Briefly, plant shoots (2 plants) were cut at 3 cm above the roots and the xylem sap was collected from the cut surface by the root pressure method (the initial 1–2 μL of exudates were discarded). The volume of xylem sap was recorded and 4 mL xylem sap was placed in a 50 mL volumetric flask and dried on a hot plate for Mo determination as described below. The phloem sap was collected as described by Dinant and Kehr [14]. Shoots were incubated in conical flask filled with 65 mL 25 mmol/L EDTA-Na_2 in a growth chamber at 20 °C in the dark and 95% humidity for 24 h. The collected solution was dried on a hot plate for Mo determination as described below.

2.4. Isolation of organelles

Cell walls, organelles, and soluble fraction were isolated as described by Wu et al. [15]. Fresh leaves (1 g) were homogenized using a mortar and pestle in 10 mL buffer containing 250 mmol/L sugar, 50 mmol/L tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, and 10 mmol/L cysteine using a chilled mortar and pestle. The extract was centrifuged at 2057 × *g* for 10 min with a refrigerated centrifuge (Eppendorf 5810 R, Germany), and the precipitate was designated as the cell wall fraction mainly consisting of cell walls and cell wall debris. The supernatant was re-centrifuged at 12,857 × *g* for 50 min, and the precipitate was designated as the organelle fraction which mainly included membrane and organelle components, and the resultant supernatant solution was defined as the soluble fraction which was mainly composed of vacuoles and cytoplasm. All steps were performed at 4 °C.

Table 1
Sequences of primers used for RT-PCR.

Target	Accession no.	Primer
BnMOT1	BnaA09g40790D	F-5′-AGTTAGTTAGAATGAACAAGGCAG-3′ R-5′-TCAGGGAATAAGTCGTAAGAAAG-3′
BnMOT2	BnaC06g40650D	F-5′-GCTTCCAAAGATATGAACACGA-3′ R-5′-CAAGTAAAGAACAACGCCACAC-3′
Actin	AF111812.1	F-5′-ACAGTGTCTGGATCGGTGGTTC-3′ R-5′-TGCCTCATCATACTCAGCCTTG-3′

2.5. Determination of Mo in plant tissues and sap

Plant material (0.3000 g) and dried xylem and phloem sap samples were digested with 6 mL $HNO_3/HClO_4$ in a 4:1 (v/v) mixture at 190 °C for 2 h and then at 205 °C. The samples were then dissolved in 10 mL deionized water and Mo concentrations were determined using a graphite furnace atomic absorption spectrometer (Z-2000 series, Hitachi, Japan) method [16].

2.6. Determination of genes expression levels

The total RNA was extracted from root and leaf using TRIzol reagent (Invitrogen, USA). The quality and concentration of the RNA preparations were measured using a NanoDrop 2000 spectrophotometer (Thermo scientific, USA). First-strand cDNA was synthesized from 1 μg of total RNA using M-MLVRTase (Promega, USA) according to the manufacturer's instructions and the product was diluted to concentration of 1000 ng mL^{-1}. Real-time PCR was carried out according to the following cycling program: 30 s denaturation at 95 °C, and then 40 cycles of 5 s at 95 °C, 10 s at 57.4 °C and 15 s at 72 °C. A total volume of 20 mL solution containing 2 mL of the synthesized cDNA, 0.8 mL of each primer, 6.4 mL dd H_2O and 10 mL of the SYBR Green PCR Master Mix Kit (TOYOBO, Japan) was used for each real-time PCR reaction. As an internal control for calibration of relative gene expression level, expression of Actin was monitored as reported by Wang et al. [17]. Primers for the genes were designed by Primer Premier 5.0 and shown in Table 1. Real-time PCR experiments were conducted on four biological replicates, with three technical replicates for each sample. The relative quantities of the transcripts were calculated by the following formula according to Pfaffl [18].

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T,\ \text{target(calibrator}-\text{test)}}}{(E_{\text{ref}})^{\Delta C_T,\ \text{ref(calibrator}-\text{test)}}}$$

2.7. Statistical analysis

The data were statistically analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Values were reported as the means ± SE of four replicates. The significance of differences was determined at 0.05 level of probability, and differences among treatment means were evaluated by two-way analysis of variance and least significant difference (LSD) multiple range tests. Sigma Plot v12.0 was used for the graphs.