

An Assay to Test Manganese Tolerance in *Arabidopsis*

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[Abstract] Manganese (Mn) is an essential nutrient required for the catalytic or regulatory function of several cellular enzymes. However, excessive Mn concentrations in plant tissues are toxic to plant cells as they negatively affect enzymatic activities, lead to oxidative stress and disturb the uptake and distribution of other essential mineral elements (Ca, P, Mg or Fe). Plants have developed multiple mechanisms to avoid heavy metals (including Mn) toxicity, including transport across the plasma membrane or tonoplast. The genes encoding transporters involved in Mn detoxification are now being identified in different plant species, and functional characterization of genes isolated from species can be easily carried out in *Arabidopsis*.

Here we provide a method to evaluate the tolerance to excess Mn of *Arabidopsis* lines transformed with empty vector pMDC43 or the same vector carrying cucumber gene *CsMTP8* encoding putative manganese transporter localized in the vacuolar membrane. We analyzed the growth and developmental phenotypes of plants grown in controlled conditions (phytotrone) on sterile plates containing different concentrations of MnSO_4 during a 16 days period. Mn accumulation was measured in the same plants grown in liquid medium supplemented or not (control) with toxic Mn concentration.

Materials and Reagents

1. The seeds of *Arabidopsis thaliana* ecotype *Columbia* (*Col-0*) transformed with empty vector (pMDC43) or with vector pMDC43 carrying the coding sequence of *CsMTP8* gene under 35S CaM promoter (*35S::CsMTP8*)
2. 96% ethanol
3. 5% sodium hypochlorite (NaClO) (commercial detergent ACE produced by Procter and Gamble)
4. Sterile water
5. 1 M KOH (for pH establishment)
6. Murashige & Skoog Basal Medium (MS) (Sigma-Aldrich, catalog number: M5519)
7. Phytigel (Sigma-Aldrich, catalog number: P8169)
8. Salts for media preparation:

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- Ca(NO₃)₂ (POCH, catalog number: 874582797)
 - MgSO₄·7H₂O (POCH, catalog number: 613780111)
 - KH₂PO₄ (POCH, catalog number: 742020112)
 - K₂HPO₄ (POCH, catalog number: 742100117)
 - HNO₃ (POCH, catalog number: 5296041)
 - KNO₃ (POCH, catalog number: 738910115)
 - FeSO₄·7H₂O (POCH, catalog number: 902840115)
 - MnSO₄·H₂O (POCH, Poland, catalog number: 616940119)
 - H₃BO₃ (POCH, catalog number: 531360115)
 - CuSO₄·5H₂O (POCH, catalog number: ACRS42361)
 - ZnSO₄·7H₂O (POCH, catalog number: Ph. Eur. 6-265762730)
 - (NH₄)₆Mo₇O₂₄·4H₂O (POCH, catalog number: 139000115)
 - Na₂EDTA·2H₂O (BioShop, catalog number: OM19432)
9. Medium composition for growing plants on plates (see Recipes)
 10. Medium composition for growing plants in liquid solution (see Recipes)
 11. 10 mM Na₂EDTA (see Recipes)
 12. 65% HNO₃ (see Recipes)

Equipment

1. Square (120 x 120 mm) petri dishes polystyrene sterile
2. Growth chamber or phytotrone (16/8 h photoperiod at 250 μmol·m⁻²·s⁻¹ and 23 °C during the day and 22 °C during the night)
3. Fume hood
4. Laminar flow cabinet
5. Autoclave
6. Shaker
7. Forceps
8. Tubes 1.5 ml (Axygen)
9. Heating digester with closed vessels (mineralizator) (Kiejdal Digestion Unit DK-20, VELP Scientifica, catalog number: F30100350)
10. Atomic absorption spectrophotometer (Perkin Elmer, model: AAS 3300)
11. Image capturing device (regular digital SLR-single lens reflex camera, e.g. Nikon D40 camera)
12. Precision balance (± 0.0001)

Procedure

A. Sterilization and germination of seeds

1. Seeds (10-15 mg) were surface sterilized in 1.5 ml tubes in a laminar flow cabinet by washing in 3% sodium hypochlorite dissolved in 96% ethanol for 5 min, and then by washing five to six times in 96% ethanol. After sterilization, the seeds were dried in laminar for at least two hours.
2. Sterilized seeds were sown on Petri dishes (20 seeds per Petri dish: 10 seeds of control seeds with empty vector and 10 seeds carrying *CsMTP8*) containing 0.5 MS solid medium supplemented or not (control) with 2 mM MnSO_4 in laminar flow cabinet (Figure 1A-C). The dishes with seeds were then kept in the dark at 4 °C for 2 days for stratification.

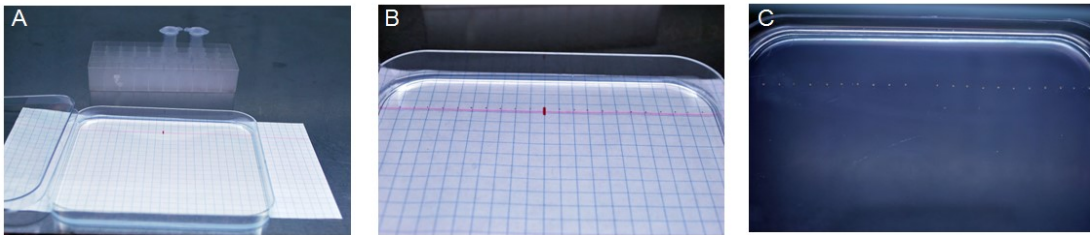


Figure 1. View of seed sowing on petri dishes. The seeds were sterilized in 1.5 ml tubes A. The sterile plate was placed on a squared paper to facilitate the precise sowing and the seeds were placed along the line marked on the paper. B. The space between seeds was 0.5 cm. C. The view of the petri dish after sowing.

B. Growth tests of *A. thaliana* plants in manganese excess

1. Following stratification, the dishes with seeds were transferred to the culture chamber or phytotrone (22 °C under 16/8 h light/dark photoperiod) and grown for 16 days in vertical position (Figure 2A-B). Then, the plant images were captured using a Nikon D40 camera and the weight of the plants was measured. The experiment was repeated three times with three replicates made for each treatment.

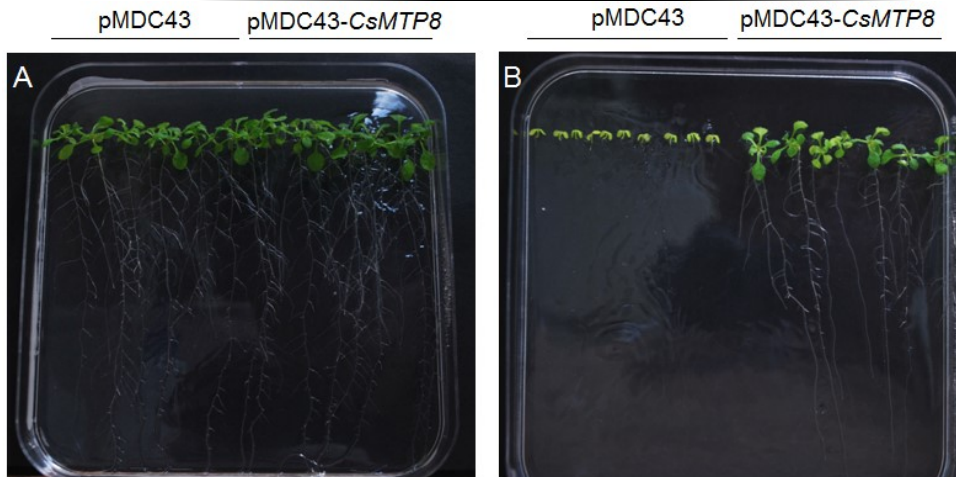


Figure 2. Example of measured effects of Mn excess on *Arabidopsis* growth in lines transformed with empty vector or vector carrying *CsMTP8* (Migocka *et al.*, 2014). Plants were grown in control 0.5 MS (A) or in 0.5 MS supplemented with 2 mM MnSO₄ (B) for 16 days in phytotrone (22 °C under 16/8 h light/dark photoperiod).

2. Measurement of plant fresh weight. Whole 16 day-old-plants grown in MS medium or MS medium supplemented with 2 mM MnSO₄ were collected from a Petri dish using forceps and weighed using a precision balance (Figure 3A-B). Beside the estimation of plant weight, other phenotypes could be assessed in this assay, *e.g.* root length, leaf number and area or chlorophyll quantity.
3. The obtained data were statistically analyzed using student's t tests and ANOVA (Excel) ($P < 0.05$) (Figure 3C).



Figure 3. The estimation of plant fresh weight. 16-day-old plants were carefully taken from petri dish and weighed with a precision balance (A-B). Data are expressed as means \pm standard deviations of three independent experiments with at least 30 plants each (C). Different letters indicate significant differences between control and stress conditions (a) or between the line transformed with empty vector and two lines carrying *CsMTP8* (b) ($P < 0.05$; ANOVA Student-t tests).

C. Measurement of manganese accumulation in plants

1. To assess Mn accumulation in plants, the 16 day-old-plants grown on petri dishes containing control 0.5 MS were carefully transferred into liquid media of the composition described earlier (Morel *et al.*, 2009), supplemented or not with 50 μM MnSO_4 and further grown for five days (Figure 4A-B). Briefly, five plants were gently wrapped with a cotton wool and placed into a 15-ml Falcon tube containing liquid medium. After five days, plants were carefully harvested, washed in 10 mM Na_2EDTA (incubated in a Na_2EDTA solution for 5 min on bench, Figure 4C) and dried at 50 °C.

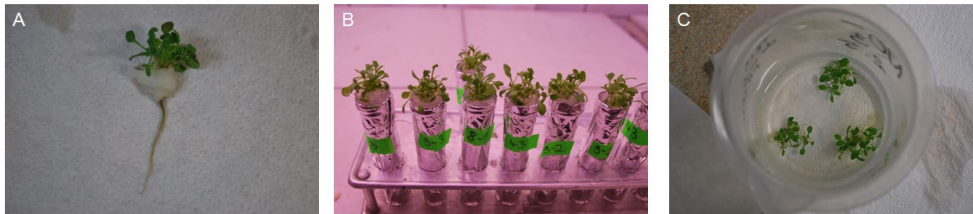


Figure 4. Details of plant preparation for measurement of Mn accumulation. 16-day-old plants were carefully taken from petri dish, wrapped with a cotton wool (A) and transferred into 15-ml Falcon covered with aluminum foil (B; 5 plants per Falcon) containing liquid medium (Morel *et al.*, 2009). After 5 days in phytotrone, the plants were washed in 10 mM Na_2EDTA (C) for 5 min and dried.

2. The dried plants collected from liquid media supplemented or not with MnSO_4 were ground to the powder in mortar with pestle.
3. 100 mg of the ground tissue was placed in the glass vessel (Figure 5A) and then digested overnight with 10 ml of the concentrated (65%) HNO_3 at room temperature under the fume hood.
4. After that, plant samples were boiled in the same solution at 150 °C for 12 h under the fume hood (Figure 5A). The solution was then analyzed by atomic absorption spectrophotometry using Perkin Elmer A3300 (Figure 5B). This is a multielement spectrophotometer equipped with cationic lamps (*e.g.* Mn lamp), which are bought separately. The samples with metal are atomized in a flame and then absorb the specific wavelength emitted by the lamp. However, the elemental analysis by Atomic Absorption Spectrometry provides reliable and reproducible results when the concentration of metal in the samples is relatively high (higher than that met in natural conditions).
5. The obtained data were statistically analyzed using student's *t* tests and ANOVA (Excel) ($P < 0.05$) (Figure 5C).

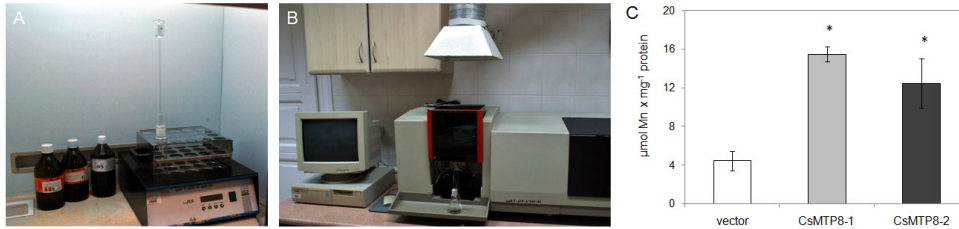


Figure 5. Details of the analysis of Mn accumulation in *Arabidopsis* plants. A. Dried plants were digested in closed glass vessels by heating in 10 ml of 65% HNO₃ in heating digester at 150 for 12 h. B. Atomic absorption spectrophotometer was used to estimate Mn content in the solution following digestion (mineralization). C. The example data are present as means ± standard deviations of three independent experiments with at least 15 plants each. Asterisks indicate significant differences between the line transformed with empty vector and two lines carrying *CsMTP8* (b) (P<0.05; ANOVA Student-t tests).

Recipes

1. Medium composition for growing plants on plates
 - a. For 1 L of 0.5 MS control medium
 - 2.2 g of Murashige & Skoog (MS) medium
 - Adjust pH to 5.7 with KOH
 - Add 5 g agar
 - Sterilized for 20 min at 121 °C/1 atm using an autoclave
 - b. For 1 plate of the medium MS supplemented with 2 mM MnSO₄
 - 1 ml of 100 mM MnSO₄ solution was added to 50 ml of medium
2. Medium composition for growing plants in liquid solution
 - a. For 1 L of control medium
 - Macroelements:

Ca(NO ₃) ₂ ·4H ₂ O	189 mg/L
MgSO ₄ ·7H ₂ O	270 mg/L
KH ₂ PO ₄	100 mg/L
K ₂ HPO ₄	10 mg/L
KNO ₃	200 mg/L

 - Microelements:

FeSO ₄ ·7H ₂ O	5.54 mg/L
MnSO ₄ ·5H ₂ O	0.59 mg/L
H ₃ BO ₃	0.56 mg/L
CuSO ₄ ·5H ₂ O	0.195 mg/L
ZnSO ₄ ·7H ₂ O	0.86 mg/L

(NH₄)₆Mo₇O₂₄·4H₂O 0.092 mg/L

Na₂EDTA·2H₂O 7.44 mg/L

- b. For 1 L of the medium supplemented with 50 μM MnSO₄
0.5 ml of 100 mM MnSO₄ solution was added to the medium
3. 10 mM Na₂EDTA
3.722 g disodium ethylenediaminetetraacetate-2H₂O (Na₂EDTA·2H₂O, Mw = 372.24) was added to 800 ml of double distilled H₂O and stirred vigorously
The pH was adjusted to 8.0 with NaOH and the volume of the solution was adjusted to 1 L with double distilled water
The solution was sterilized by autoclaving
4. 65% HNO₃
65% HNO₃ was purchased as a ready-to-use concentrated solution

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References

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