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Water status in *Mesembryanthemum crystallinum* under heavy metal stress

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ABSTRACT

Heavy metals (HMs) are known to have negative effects on plant water status; however, the mechanisms by which plants rearrange their water relations to adapt to such conditions are poorly understood. Using the model plant *Mesembryanthemum crystallinum*, we studied disturbances in water status and rapid plant defence responses induced by excess copper or zinc. After a day of HM stress, reductions in root sap exudation and water deficits in leaf tissues became evident. We also observed several primary adaptive events, including a rapid decrease in the transpiration rate and progressive declines in the leaf-cell sap osmotic potential. Longer HM treatments resulted in reductions of total and relative water contents as well as proline accumulation, an increase in water retention capacity and changes in aquaporin gene expression. After 3 h of HM exposure, leaf expression of the *McTIP2;2* gene, which encodes tonoplast aquaporin, was suppressed more than two-fold, thus representing one of the earliest responses to HM treatment. The expression of three additional aquaporin genes was also reduced starting at 9 h; this effect became more prominent upon longer HM exposure. These results indicate that HMs induce critical rearrangements in the water relations of *M. crystallinum* plants, based on the rapid suppression of transpiration flow and strong inhibition of root sap exudation. These effects then triggered an adaptive water-conserving strategy involving differential regulation of aquaporin gene expression in leaves and roots, further reductions in transpiration, and an accelerated switch to CAM photosynthesis.

1. Introduction

Increasing levels of environmental pollution underscore the need to improve our understanding of how plants adapt to toxic products of anthropogenic activities, including heavy metals (HMs). Plant strategies to survive under HM pollution have become the subject of many intensive studies that have been comprehensively reviewed (e.g., Hall, 2002; Clemens, 2006; Broadley et al., 2007; Khan and Singh, 2008).

Wild plants inhabiting soils naturally enriched in HMs evolved a constitutive tolerance to these elements. Plant-hyperaccumulators can grow in soils with extremely high HM concentrations and accumulate HMs in roots and shoots (Baker and Brooks, 1989; Salt and Kraemer, 1999; Yang et al., 2005). This ability to take up HMs through root cells and translocate them to shoots represents one of the most important strategies that plants use to adapt to high HM concentrations. However, this strategy might only be efficient in combination with certain basic cellular HM detoxification processes, including synthesis of substances involved in HM chelation, intracellular traffic and membrane sequesterisation (Kraemer et al., 1996; Rauser, 1999; Clemens, 2006; Yruela, 2009).

Plant HM tolerance is a polygenic trait (Macnair et al., 1999) likely controlled by multiple major genes and several minor genes. To understand the biological functions of these gene products, it is important to study HM tolerance in poorly studied plant species, with special attention given to the physiological and molecular mechanisms of their adaptation and survival under HM stress. In this respect, the ice plant (*Mesembryanthemum crystallinum* L., Aizoaceae) appears to be an ideal model because it is a wild plant with a relatively high potential HM tolerance whose adaptive mechanisms are poorly understood.

We previously analysed the effects of a wide range of copper and zinc concentrations on ice plants and determined their toxic and lethal concentrations, the extent to which they accumulate in shoots and the effects of HMs on various physiological processes (Kholodova et al., 2005). Based on these results, we characterised the ice plant as quite tolerant to zinc, capable of accumulating it at relatively high concentrations in shoots. Copper was much more toxic to the ice plant: it initiated ROS generation and induced oxidative stress. These data are not in complete agreement with the results of Thomas et al. (1998), who reported that ice plants can tolerate up to 8 mM $CuSO_4$; however, this conclusion only applied to short-term (3–5 days) exposures to the damaging factor.

Along with these data, we consistently observed that HMs disturbed the water status of ice plants. This effect was dose-dependent and manifested as plant wilting and a significant

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decrease in leaf water content; however, the plants could complete their life cycle and develop viable seeds while growing in medium containing as much as 100 μM $CuSO_4$ or 1 mM $ZnSO_4$ (Kholodova et al., 2005).

The negative effects of HMs on plant water status were also observed in several other studies, some of which presented direct measurements of basic water parameters during long-term HM treatments (e.g., Salah and Barrington, 2006; Llamas et al., 2008). However, little work has been done on the rapid changes in plant water relations that occur in response to HMs, and the initial events and mechanisms underlying these changes in the water status under HM stress have not been analysed.

The objectives of this work were to study: (1) rapid changes in the water parameters of *M. crystallinum* plants in response to HM treatments; (2) short-term adaptive rearrangements in plant water status; (3) plant capacity for induction and development of a water-conserving CAM-type photosynthesis strategy aimed at plant survival under HM stress; (4) expression of stress-related aquaporin genes involved in the maintenance of cell water status under extreme conditions.

2. Materials and methods

2.1. Plant material and growth conditions

M. crystallinum L. plants were grown in a 12-h photoperiod with a day/night temperature of 23–25/18–20 °C and illumination from Reflux HPS lamps (Reflux, Russia) 350 μmol/(m^2 s). Seeds were sown in trays containing perlite saturated with distilled water. At the age of three to four weeks, plants were transferred to 2-L glass vessels (three plants per vessel) in modified Johnson nutrient medium (Winter, 1973) with iron provided as $Fe(NO_3)_3$. Plants were grown at continuous aeration and with weekly replacement of nutrient medium. $CuSO_4$ (25 or 50 μM), $ZnSO_4$ (250 or 500 μM) or 400 mM NaCl (200 mM and 200 mM after 24 h to avoid toxic shock) were added to the nutrient medium when plants developed three to four pairs of primary leaves (5.5–6.5 weeks after sowing), which were used for analyses.

2.2. Total and relative water content (RWC)

Total leaf water content was determined by the gravimetric method after fixation at 90 °C for 30 min and subsequent drying to constant weight at 60 °C. To determine the water content of fully turgescent leaves, detached leaves were weighed, and petioles were placed in a glass beaker containing 0.25 mM $CaSO_4$ and kept in a darkened chamber with a water-saturated atmosphere at room temperature (20–22 °C) for 20 h. Water-saturated leaves were weighed, fixed, and dried as described above. RWC was calculated as the ratio of water content in the initial leaf to its content at complete saturation.

2.3. Rate of transpiration

The rate of transpiration was measured by a routine gravimetric method for 5 min immediately after leaf detachment (with the cut surface of the petiole sealed with plasticine). Leaf areas were measured with light-sensitive paper.

2.4. Leaf water retention

The water retention ability (WRA) of detached leaves (with sealed cut surfaces) was evaluated by incubating them for 48 h at 20–22 °C under safe light. Afterwards, weighed leaves were dried to a constant weight as described above. Water loss was determined as the difference between the weights of detached leaves before and after exposure. WRA was calculated as the fraction of the initial water content in the freshly detached leaf remaining after incubation.

2.5. Determination of osmotic potential

To determine the osmotic potential of freshly harvested or water-saturated leaves, samples were fixed in liquid nitrogen and stored at −20 °C. The cell sap was obtained from frozen and thawed leaves using a manual press. During leaf sample thawing, measures were taken to prevent water condensation from the air. Measurements were performed with an Osmomat 030 cryoscopic osmometer (Gonotac, Germany).

2.6. Estimation of free proline

The free proline content of leaf samples (200–400 mg) fixed in liquid nitrogen was estimated as described by Bates et al. (1973), using an acidic ninhydrin reagent.

2.7. CAM activity measurements

CAM activity was assessed by measuring the diurnal dynamics of proton content (Chu et al., 1990). Leaf material (0.2–0.5 g) was fixed in liquid nitrogen at 20:00 and 8:00; organic acids were extracted by boiling the sample for 10 min in 10 ml of distilled water with subsequent continuous shaking for no less than 4 h. The proton content was measured with a pH meter (Orion Research, USA) and titration to pH 7.00 ± 0.05 using small aliquots (10–100 μl) of 10–200 mM NaOH. CAM activity was expressed as $\Delta[H^+]$ between the ends of the dark and light periods.

2.8. Total RNA extraction and reverse transcriptase PCR analysis

To study the changes in aquaporin gene expression, total RNA was extracted using a protocol based on the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified with the Qiagen RNeasy MiniElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by UV absorption at 260 nm using a Genesys 10UV spectrophotometer (Thermo Electron Corporation, USA). Integrity and purity of the RNA samples were determined by agarose gel electrophoresis and OD 260/280 absorption ratios.

Reverse transcription was performed for every sample in three independent reactions using oligodT primers (Fermentas, Latvia) in accordance with the manufacturer's recommendations. As a control, RT-PCR was also carried out for the gene encoding *βAct*. Primers used in this study are listed in Supplemental Table S1. Aquaporin and actin sequences were taken from the www.tigr.org site (Institute for Genomic Research, USA). For cDNA synthesis, we mixed 5-μg samples of total RNA with 1 μl of oligo(dT)21VN (100 pmol/ml) and DEPC-treated water to a final volume of 12.5 μl. After incubation at 70 °C for 10 min and 3 min on ice, the reaction was started by adding 2 μl of 10 mM dNTP mix, 2 μl of 10× reverse transcriptase reaction buffer, 0.5 μl of RNase Inhib and 1 μl of RevertAid M-MuLV reverse transcriptase 200 units/μl. cDNA synthesis was run at 42 °C for 50 min and at 70 °C for 15 min.

The final reaction mixture was used for PCR following the manufacturer's protocol (Fermentas, Latvia). PCR parameters and the amount of cDNA were optimised in such a way that the amplification was linear with regard to the reaction products and the number of PCR cycles. PCR was run using a PTC-100TM Thermal Cycler Version 9.0 (MJ Research, USA). The reaction mixture contained 1 μl (100 pmol/ml) of each primer, 5.5 μl of 10× buffer for *Taq* DNA polymerase, 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 2 μl (0.25 μg) of cDNA, 1 μl (5 units) of *Taq* DNA polymerase,