Water relations responses of the pea (*Pisum sativum* L.) mutant SGECd^t to mercury

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Abstract. Mercury (Hg) is one of the most toxic heavy metals and has multiple impacts on plant growth and physiology, including disturbances of plant water status. The impact of Hg on water relations was assessed by exposing the unique Hg-sensitive pea (*Pisum sativum* L.) mutant SGECd^t and its wild-type (WT) line SGE in hydroponic culture. When the plants were grown in the presence of 1 or 2 μM $HgCl_2$ for 11 days, the SGECd^t mutant had lower whole plant transpiration rate and increased leaf temperature, indicating stomatal closure. Shoot removal of Hg-untreated plants resulted in greater root-pressure induced xylem sap flow in the SGECd^t mutant than WT plants. Treating these plants with 50 μM $HgCl_2$ (an inhibitor of aquaporins) for 1 h decreased xylem sap flow of both genotypes by about 5 times and eliminated differences between WT and mutant. Adding 1 mM dithiothreitol (the reducing thiol reagent used for opening aquaporins) to the nutrient solution of Hg-treated plants partially restored xylem sap flow in SGECd^t roots only, suggesting genotypic differences in aquaporin function. Thus root water uptake is important in mediating sensitivity of SGECd^t to toxic Hg.

1 Introduction

Heavy metals, such as cadmium (Cd) and mercury (Hg), are widespread soil pollutants inhibiting plant growth and nutrition [1]. These toxicants decrease xylem vessel size [2], root hydraulic conductivity [3-5], stomatal conductance [6] and inhibited activity of the molecular water channels aquaporins (AQPs) [7-8]. Moreover, Hg treatments are often used to inhibit AQP activity. However, some aquaporin genes can be upregulated by treatment with Hg [9-10] including the pea AQP *PsPIP-2* [11]. By lowering leaf and root water status, heavy metals can enhance biosynthesis of phytohormone abscisic acid which activates stomatal closure [12-14].

Plant mutants with altered tolerance to heavy metals can enhance our understanding of the mechanisms by which heavy metals affect plant water status. Among such mutants, Cd-sensitive mutants *cad1* and *cad2* of *Arabidopsis thaliana* deficient in phytochelatin (PC) synthase [15] and γ-glutamylcysteine synthetase [16], Cd-tolerant rice mutant *cadH-5* with efficient function of the ascorbate-glutathione cycle and antioxidant enzymes [17], and a low

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Cd accumulating *A. thaliana* mutant *cdr3-1D* with increased glutathione biosynthesis [18] should be mentioned. However, no mutants with altered tolerance to Hg have been described. In this respect, pea (*Pisum sativum* L.) line SGE and its unique mutant SGECd^t possessing simultaneously high Cd tolerance [19] and low Hg tolerance [20] provide a promising genetic model to study the effects of these metals on plant water relations. Previous experiments revealed increased root xylem sap flow rate of metal-untreated and Cd-treated SGECd^t plants, but Hg-treated mutant plants subjected to chronic exposure to these metals showed opposing responses [20].

The present report aimed to characterize in more detail the negative effects of Hg on water relations and subsequent stress responses of SGECd^t and wild-type (WT) plants to better understand physiological mechanisms mediating plant tolerance to this toxic metal.

2 Materials and methods

2.1 Hydroponic culture

Seeds of wild-type (WT) pea (*Pisum sativum* L.) line SGE and its mutant SGECd^t characterized by increased Cd tolerance [19] and decreased Hg tolerance [20] were surface sterilized and scarified by treatment with 98% H_2SO_4 for 30 min, rinsed carefully with tap water and germinated on filter paper in Petri dishes for three days at 25°C in the dark. Seedlings were transferred to plastic pots (two pots with 10 seeds per genotype and treatment) containing 1500 mL of nutrient solution (μM): KH_2PO_4, 400; KNO_3, 1200; $Ca(NO_3)_2$, 60; $MgSO_4$, 250; KCl, 300; $CaCl_2$, 60; KCl, 250; Fe-tartrate, 12; H_3BO_3, 2; $MnSO_4$, 1; $ZnSO_4$, 3; NaCl, 6; Na_2MoO_4, 0.06; $AlCl_3$, 1; $CoCl_2$, 0,06; $CuCl_2$, 0.06; KJ, 0.06; KBr, 0.06; $NiCl_2$, 0,06; pH = 5.5.

Plants were cultivated for 12 days in a naturally lit greenhouse (additional artificial lighting of 200 μmol m^{-2} s^{-1}, a 12 h photoperiod with minima/maxima temperatures of 18°C/23°C respectively). In experiments with long exposure to mercury (11 days), one day after planting (DAP) the nutrient solution was supplemented with 0.5, 1 or 2 μM $HgCl_2$. In experiments with short exposure to heavy metals (1 h), the plants were cultivated without metals for 11 days. On the 12-th day the nutrient solution was supplemented or not with 50 μM $HgCl_2$ (to inhibit AQPs). The plants were treated for 1 h and roots were briefly washed with deionized water for 20 s. Then plants from each treatment were divided into two sub-treatments by transferring to new pots with fresh nutrient solution supplemented or not with 1 mM dithiothreitol (DTT), the reducing thiol reagent used to open AQPs. In all experiments the nutrient solution was changed, and where necessary the supplements were added, at 5 and 9 DAP.

2.2 Anatomical and physiological measurements

Whole plant transpiration rate was measured gravimetrically by weighing the pots. Thermal images were taken and leaf temperature determined with an infrared camera (Terma CAM SC2000, FLIR Systems Inc, Boston, USA). At harvest, cross sections of roots (2-3 cm from cotyledons) were prepared, and stained with toluidine blue to measure xylem tissues using a light microscope AxioVertA1 (Carl Zeiss, USA) and software OPTIMAS 6.1 (Optimas Corporation, Houston, USA) as described previously [20]. To determine xylem sap flow from de-topped roots, shoots were cut 2 cm above the cotyledons and cotton-filled 0.5 mL Eppendorf tubes (of defined weight) placed on the cut stumps and wrapped with PARAFILM® to prevent evaporative losses of sap. The pots were covered with aluminum foil to avoid direct sunlight and sap was collected for 3 h. Then tubes were collected, weighed

and root sap flow rate (J_v) calculated per plant (total J_v) or per 1 mm^2 of xylem area (specific J_v).

2.3 Statistical analysis

Statistical analysis of the data was performed using the software STATISTICA version 10.0 (StatSoft Inc., USA). Confidence intervals and Fisher's LSD-test were used to evaluate differences between means.

3 Results and discussion

Using similar hydroponic culture and growth conditions, we demonstrated that the SGECd^t mutant is more sensitive to chronic treatment with 0.5 µM, 1 µM or 2 µM $HgCl_2$ than the wild type SGE [20]. Root and shoot growth inhibition was associated with decreased root sap flow rate, root xylem and phloem areas and stomatal conductance, suggesting that Hg negatively affected plant water relations [20]. Images of representative plants treated with 1 µM Hg supplements those results to visualize Hg-dependent differences in shoot (Fig. 1 A, B) and root (Fig.1 C, D) growth between genotypes. At 1 µM Hg, whole plant transpiration of the SGECd^t mutant was more inhibited than in the wild type SGE. At 2 µM Hg, this genotypic difference was approximately two-fold (Fig. 2A). Thus increasing Hg concentrations differentiated water relations of the two genotypes.

Thermal images of chronically Hg-treated plants revealed genotypic difference in leaf temperature (Fig. 1 E, F). In quantitative terms, a significant genotypic difference in leaf temperature was observed even at 0.5 µM Hg (Fig. 2B), suggesting that this is very sensitive parameter to determine negative Hg effects on plants. Under water limited conditions, plants decrease stomatal conductance to limit transpiration, thus increasing leaf temperature [21-22]. Although leaf temperature was widely applied to monitor stomatal conductance of laboratory [23] and field-grown plants [24-25], we are not aware of its application to assess the effects of heavy metals on plant water relations. However it was included in comparison study of tree species for phytoremediation potential in soils contaminated by herbicides [26]. We propose that leaf temperature could be useful in toxicological studies to determine genotypic differences in tolerance to toxic metals, as a rapid alternative and adjunct to traditional biomass-based measurements [27-28]. It should be mentioned that increased leaf temperature may not only reflect negative effects of stressors, but also itself disturb plant metabolism, e.g. changing enzyme activities [29].

The molecular water channels AQPs, a numerous and multi-form family of proteins, can regulate root hydraulic conductance and water transport in various plant species, particularly under abiotic stress conditions [30-31]. Millimolar Hg concentrations are often used to inhibit AQPs in plants [4, 7-8, 32], including pea [11] while DTT (a compound that reduces thiols) scavenges Hg ions and restores J_v [33-34]. Here, all plants were pre-cultivated for 12 days without Hg, with no genotypic differences in root and shoot biomass (data not shown). In such plants, J_v of Hg-untreated SGECd^t plants was significantly higher by 90% than WT plants, as previously observed [20]. Short (1 h) exposure to 50 µM $HgCl_2$ significantly decreased total (Fig. 3A) and specific (Fig. 3B) root exudation of both genotypes by 80%, eliminating any statistical difference between WT and mutant plants. Cadmium tolerance mechanisms in the SGECd^t mutant are induced only in the presence of toxic Cd concentrations during plant cultivation [35] and the present results indicate this also occurs in response to toxic Hg.

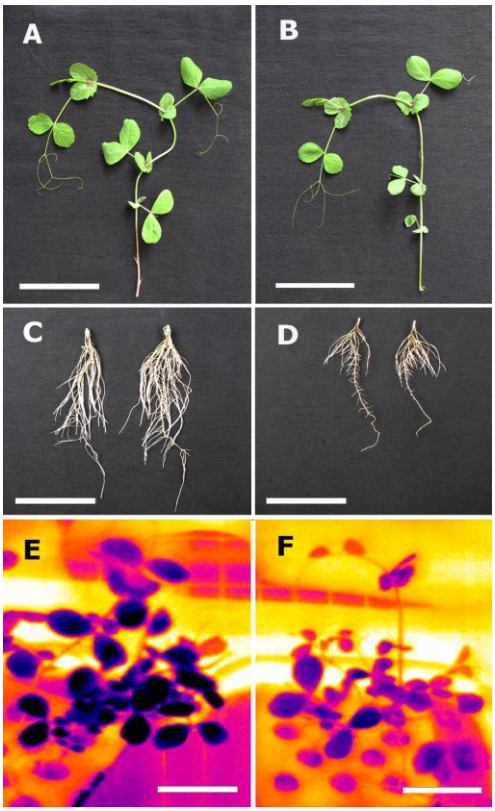

Fig. 1. Effect of mercury on growth and leaf temperature of SGE and SGECd^t plants grown for 11 days in hydroponics supplemented with 1 µM $HgCl_2$. A - SGE shoot, B - SGECd^t shoot, C - SGE roots, D - SGECd^t roots. *In situ* infrared images of SGE (E) and SGECd^t (F) shoots demonstrating increased SGECd^t leaf temperature expressed as more light color of leaves. Scale shows 5 cm.

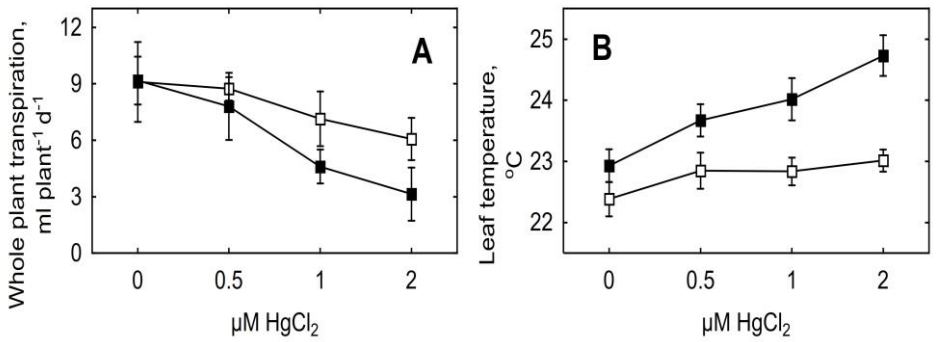

Fig. 2. Effect of mercury on whole plant transpiration (WPT) and leaf temperature of SGE and SGECd^t plants grown for 11 days in hydroponics supplemented with different $HgCl_2$ concentrations. A – whole plant transpiration; B - leaf temperature. Pea genotypes: □ – SGE, ■ – SGECd^t. Bars show confidence intervals (P = 0.05). Data are means of 2 experiments with 5 replications each.

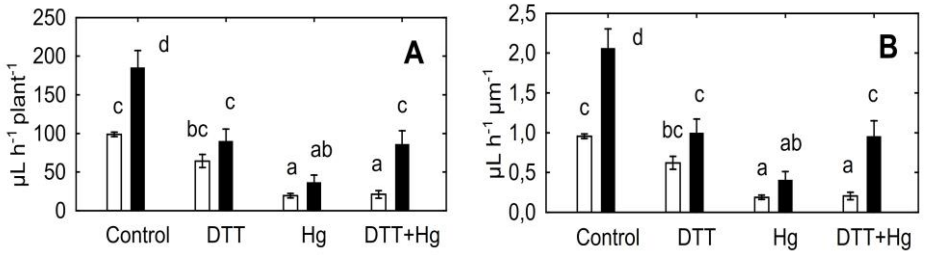

Fig. 3. Effect of dithiothreitol (DTT) on root sap flow rate of SGE and SGECd^t plants exposed for 1 h to mercury (Hg) and/or DTT in hydroponics. A – total root sap flow; B – specific root sap flow expressed per µm of xylem area. Pea genotypes: □ – SGE, ■ – SGECd^t. Treatments: Control – untreated control, Hg – 50 µM $HgCl_2$, DTT – 1 mM dithiothreitol, Hg+DTT – 50 µM $HgCl_2$ and 1 mM dithiothreitol. Different letters show significant differences between treatments (Fisher's LSD test; $P < 0.05$). Data are means of 2 experiments with 5 replicates each.

Surprisingly, treatment with 1 mM DTT alone decreased J_v of both pea genotypes, perhaps due to nonspecific and negative effects of this compound on root physiology [36]. In Hg-treated plants, DTT addition partially restored root sap flow only in the mutant plants (Fig. 3). Thus AQP regulation likely differs between SGECd^t mutant and WT plants, and AQPs may be involved in the increased J_v of SGECd^t mutant under unstressed conditions (Fig. 3), as well as decreased J_v under chronic Hg treatment [20]. Possibly Hg ions are more mobile and more readily scavenged by DTT in the SGECd^t mutant in alignment with its increased Hg sensitivity, although total root Hg concentration was approximately similar to WT [20]. On the other hand, DTT may have exerted some unknown effects thus partially restoring differences in water relations between the studied pea genotypes.

4 Conclusion

Taken together, the data confirm greater Hg sensitivity of SGECd^t mutant and demonstrate the importance of root water uptake in mediating this response. Genotypic difference in response of SGE and SGECd^t to toxic Hg concentrations is due to different regulation and/or function of AQPs. Leaf temperature was a sensitive variable to monitor the toxic effects of Hg on the studied pea genotypes. This suggests possibility for using it as a screening tool for differentiating genotypes in heavy metal tolerance.

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