MECHANISMS OF CHROMIUM TOLERANCE, UPTAKE AND ACCUMULATION IN PLANTS: A HAZARDOUS SUBSTANCE IN THE FOOD CHAIN

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Abstract- Anthropogenic activities have caused progressive environmental pollution and degradation. In particular, metal contamination has dramatically increased since the industrial revolution, causing severe damages to water sources and soil. To overcome these environmental hazards, phytoremediation is suggested as a sustainable approach for environmental rehabilitation, which aims at using plants to remove heavy metals from contaminated soil or water through their uptake by roots and transfer to the above-ground parts. However, the molecular and cellular mechanisms involved in transition metal uptake and accumulation are still not completely clear. Assessment of the possible role of sulfate transporters in chromium uptake and accumulation: results show evidence for chromate - sulfate competition, together with enhanced tolerance to chromium when root-localized high-affinity sulfate transporters are genetically silenced, and hypersensitivity when they were over-expressed in genetically transformed plants. Sulfate transporters are suggested to play an important role in chromium uptake from the soil environment into plant cells, and detrimental chromium effects may be caused by competition with sulfate at low chromium concentrations and by chromium toxicity at higher concentrations.

Keywords- Chromium, Heavy metals, Metal toxicity, Plant sulfur transport,

I. INTRODUCTION

Heavy metal, or transition metal, contamination by anthropogenic activity has various sources, amongst them urban, agricultural, mining, industrial and smelting processes. As a consequence of the increasing emission of excess concentrations of a large number of elements in the environment, public and governmental interest in trace element has emerged over recent decades. This has led to numerous research studies across a broad range of fields, including agricultural, environmental, geological, clinical and life sciences.

Soil is considered the main environmental component for trace elements released by human activities. The current status of these elements in the soil reflects their sources from parent material, but also from vast anthropogenic addition, even in relatively remote non-industrialized areas[1]. Metal contaminants can be transported to the soil through various pathways; by air, through deposition of dust, aerosol and gaseous forms of metal, by water, including runoff or flooding rivers followed by deposition of metal-containing soil and sewage or sludge emission, and by intentional placement, particularly in agriculture through commercial fertilizers, agrochemicals and irrigation.
Atmospheric deposition: this is the most extensive form of contamination, as contaminants may travel very long distances. Sources vary from electricity stations and industrial emissions to motor vehicle engines and domestic emissions, as well as fires and corrosion of metal structures. Large areas may be affected and contamination processes may continue for long time, although deposition rates may be lower compared to other transport mechanisms.

Flooding and sediment deposition: particles containing metals from various sources, predominantly sediments from metalliferous mines are suspended in river water. When the river floods, the suspended sediments will overflow onto land and deposit on alluvial soils. Nowadays, these sediment effluents are largely channeled into ponds and lagoons where water is let to sediment, though accidents happen occasionally, causing widespread soil and water contamination. Waste water and sewage sludge are another large source of metal contamination, as in many parts of the world it has been applied to land for decades, causing extremely high metal concentrations at the depositing sites. Introduction of regulations in many countries has achieved a certain reduction in heavy metal contamination from sewage dumping[2].

Intentional placement in agriculture: heavy metal input to soil is largely due to the use of inorganic fertilizers. Macronutrients such as nitrogen, phosphorus and potassium are the most commonly applied, but calcium, magnesium and sulfur are also used. Fertilizers, together with liming material and various agrochemicals, may all contain trace amounts of metals resulting in impurities. Another source is water used for irrigation, primarily gray water with little or no treatment.

Once metal contamination is introduced to soil it will undergo almost no chemical degradation at all and, unlike organic contaminants, will persist for a long time unless it leaches to ground water[3]. Although some trace elements, or metals, are essential plant micronutrients, excessive concentration as well as the presence of other non-essential heavy metals may

lead to their uptake and accumulation causing phytotoxicity. This non-selective uptake may become an environmental problem, when animals and human beings are nourished by such a plant, causing an inevitable bioconcentration of the element in their body. Other forms of exposure to metal contamination in soil include drinking water (after percolation of contaminants to ground water), and through the airways and skin.

Many heavy metals have well-documented effects on animal populations and human health. Some, such as lead, cadmium, mercury and arsenic have no essential function in the human body and are extremely toxic, even in trace amounts. Others, for example nickel, chromium and copper also have substantial effect on human health, while zinc is relatively less toxic, especially in low doses[4]. Amongst their broad toxic effects are serious brain and nervous system injuries, digestive system damage, carcinogenous activity and skin allergies[5].

Chromium occurs naturally in rocks, soil, plants and animals. Its atomic number is 24; it has an atomic mass of 51.9 g/mole and a density of 7.19 g/cm^3 at 20°C. It is the first element in group 6, together with molybdenum and tungsten, with which it shares chemical characteristics - a high melting point (1875°C for chromium) and the formation of compounds in different oxidation states. The most common chemical form for chromium is in the +3 oxidative state, which occurs naturally, and +6, which is generally produced anthropogenically by fuel combustion, waste incineration and industrial processes such as metal industries. Both forms are bound strongly in soil by cation exchange, becoming more mobile and bio-available in acidic conditions. Many chromium compounds have intense colors, giving the element its name - chroma (χρῶμα), color in Greek.

Trivalent chromium is required in animals and humans as a trace element for sugar metabolism as a constituent ingredient of Glucose Tolerant Factor, although its necessity is somewhat controversial, owing to the lack of direct evidence of chromium deficiency in humans[6]. Hexavalent chromium, on the other hand, is extremely toxic and mutagenic when inhaled. The World Health Organization recommends a maximal total chromium concentration of 0.05 mg/liter[7], that has been adopted worldwide and by the Israeli regulators.

Occurrence and contamination: Chromium concentration is the highest in ultramafic rocks reaching a concentration of 2300 mg/kg, while other geological sources are gabbro and basalt with 250 mg/kg, 100 mg/kg in shale, a maximum of 700 mg/kg in black or oil shale, 35 mg/kg in the upper crust, 20 mg/kg in coal, 10 mg/kg in granite and granodiorite and 5 mg/kg in limestone. Median chromium concentration in earth topsoil is 42 mg/kg, with a wide variation when comparing different regions. It reaches a maximum of 5910 mg/kg in a sample site in north California and as low as less than 0.1 mg/kg in the Netherlands[2]. The disparities reflect differences in geochemical composition, but also in the degree of soil contamination. Ultramafic rocks are notable because of their relatively high concentration of chromium and nickel, together with a low Ca:Mg ratio and low phosphorus and potassium. High chromium concentrations, as found in California for example, are due to the abundance of this parent material. Although high chromium concentration may be found in many sites, only small quantities are bioavailable, while most of it is strongly bound to soil particles[8].

Chromium does not occur naturally in elemental form, but only in compounds, and is mined mainly as chromite, $FeCr_2O_4$, and ferrochromium, FeCr. The leading country of production is South Africa, followed by Kazakhstan, India and China. The global production of chromium has grown progressively since the industrial revolution, with a dramatic increase since the middle of the 20th century, and a continuous rise over the last two decades (Fig.1). Chromium is second only to lead in abundance of heavy metal found in contaminated sites, followed by arsenic, zinc, cadmium, copper, mercury and nickel[4]. A substantial number of industrial sources contribute to chromium contamination, among them electrical components, steel work, metal smelting, tanning industry, printing and graphic, pigments and paints and textiles[2].

Chromium (VI) is a carcinogen when inhaled (predominantly respiratory cancer) though oral exposure was not shown to increase tumor incidence. IARC, the International Agency for Research on Cancer, has therefore classified chromium (VI) compounds in Group 1 (human carcinogen), while chromium (III), with no carcinogenicity activity, in Group three[9]. The oxyanion chromate interferes with the metabolism of structurally related ions in the living cell. Chromium (VI) toxicity is related to oxidative stress both in eukaryotes and prokaryotes, negatively effecting membrane integrity and electron transport chain. DNA damage has been reported when cells were exposed to chromium (VI)[10]. Chronic exposure to high levels can damage liver, kidney, nerve tissue and may lead to death. Chromium (VI) is also a skin irritant, causing allergic contact dermatitis[11]. Here we describe the investigation of plant sulfate transporters in the response and accumulation of chromium in a model plant with implication on chromium accumulation in the food chain through plants.

II. DETAILS EXPERIMENTAL

2.1. Plants grown on solidified medium: Artificial growth media used for most experiments was based on Nitsch medium including nutrients and vitamins (Duchefa). Chromium was added from stock solution to reach final concentrations of 5 to 40 mg Cr(VI)/L, and pH was adjusted to 5.8 using NaOH. Plant Agar (Duchefa) was added to a final concentration of 0.8% prior to autoclaving. Antibiotics were added after autoclaving and cooling of the media.

For sulfate-free media, nutrients were added to reach the following final concentrations: 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM NaH_2PO_4, 0.1 mM FeNaEDTA, 1 mM $MgCl_2$, 50 µM H_3BO_3, 50 µM $MnCl_2$, 15 µM $ZnCl_2$, 3 µM $NaMoO_4$, 2.5 µM KI, 0.05 µM $CuCl_2$, 0.045 µM $CoCl_2$, 5-10 mM Glc. Sulfate was added as K_2SO_4 to reach final concentration of 1 mM. Djenkolate, $C_7H_{14}N_2O_4S_2$ (Tokyo Chemical Industry Co.), was added from a 50 mM stock dissolved in NaOH 0.1 M, to reach final concentration of 100 µM. Agarose (Lonza) was added to solidify the media instead of plant agar, to avoid sulfate traces.For growth under other potentially phytotoxic-ions, the following final concentrations were used: 100, 400, 600 µM selenate (added from a 50 mM Na_2SeO_4 stock solution), 40, 150 µM cadmium (added from a 1 mM CdN_2O_6 stock solution) and 750, 2000 µM zinc (added from a 100 mM $ZnSO_4$ stock solution). Every genotype was sown on 4 different plates in every experiment.

2.2 Seed surface sterilization: ~100 *A. thaliana* seeds were placed in a 1.7 ml tube. The tube was then left open inside a desiccation jar next to a beaker containing 100 ml sodium hypochlorite. 5 ml of HCl (32%) were added to the bleach beaker, and the jar quickly sealed. Seeds were kept in the jar for 2 hr. After sterilization, seeds were sown and vernalized at 4°C for 24h.

2.3 Molecular biology and DNA manipulations:
Plant genomic DNA extraction
One to two leaves (˜100 mg) were placed in a 2 ml Eppendorf tube, and put immediately into liquid nitrogen. Leaves were crushed using a Qiagen Retsch TissueLyser, and were homogenized with 400 µl DNA extraction buffer. The tubes were then vortexed and centrifuged for 10 min at 13,200 rpm. The supernatant was extracted with 300 µl isopropanol and incubated 10-15 min at room temperature followed by 15 min centrifugation. The pellet was washed with 400 µl 70% ethanol and centrifuged 10 min at 13,200 rpm. DNA pellet was air-dried and resuspended in 50 µl DDW, containing 1 µl of 10 µg/µl RNAse A. DNA extraction buffer contains the following final concentrations: 200 mM Tris-HCl pH 7.5, 25 mM EDTA, 250 mM NaCl, 0.5% SDS. PCR Reaction conditions were as follows: 0.2 mM dNTPs, 200-500 nM of each primer, 1 X Taq reaction buffer, 1.5-2.5 mM MgCl2, 0.05 unit/µl of Taq DNA polymerase (expressed and purified in out lab) and 1-500 ng DNA template in a final volume of 20 µl. The reaction conditions were: 3 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 55°C and 1-2 min at 72°C, followed by an additional step of 7 min at 72°C.Taq reaction buffer (x10) contains the following final concentrations: 100 mM Tris-HCl, pH 8.8, 500 mM KCl, 25 mM MgCl2, 0.8% IGEPAL. RNA extraction: Roots of different genotypes of *A. thaliana* were collected, placed into a 2 ml Eppendorf tube, and put immediately into liquid nitrogen, followed by -80°C storage. Samples were homogenized using a tissuelyzer. Procedure was carried out using the QIAGEN RNeasy Mini Kit according to manufacturer's protocol. Reverse-Transcriptase PCR:
A two-step RT-PCR has been conducted, using Masterscript™ RT-PCR System (5 Prime) according to manufacturer's protocol. Plasmids:
pBTEX:SULTR1;2: SULTR1;2 CDS was amplified from *A. thaliana* cDNA using the gene-specific primers XmaI:sultr1;2_CDS_fw and sultr1;2_CDS:SalI_rv. The 2Kb PCR product was purified and concentrated, then digested with XmaI and SalI and ligated into the binary vector pBTEX. Positive pBTEX:SULTR1;2 plasmid was isolated, sequenced and transformed into *A. tumefaciens*, followed by floral-dip stable transformation of *A. thaliana*.
pSAT-C:SULTR1;2: SULTR1;2 CDS was amplified from pBTEX vector harboring SULTR1;2 using the gene-specific primers pSAT-C::XhoI_sultr1;2 and pSAT1-C::sultr1;2_XmaI. The 2Kb PCR product was purified and concentrated, then digested with XhoI and XmaI and ligated into pSAT1-venus-C1 vector. Positive pSAT-C1:SULTR1;2 plasmid was isolated and digested with AscI, then ligated to the binary vector pPZP. Positive pPZP:venus-SULTR1;2 plasmid was isolated, sequenced and transformed into *A. tumefaciens*, followed by floral-dip stable transformation of *A. thaliana.*
PSAT-N:SULTR1;2: SULTR1;2 CDS was amplified from pBTEX vector harboring SULTR1;2 using the gene-specific primers pSAT-N::NcoI_sultr1;2 and pSAT-N::sultr1;2_XmaI. The 2Kb PCR product was purified and concentrated, then digested with NcoI and XmaI and ligated into pSAT6-EYFP-N1 vector. Positive pSAT-N1:SULTR1;2 plasmid was isolated, sequenced and digested with PI-PspI, then ligated to the binary vector pPZP. Positive pPZP: SULTR1;2-EYFP plasmid was isolated and transformed into *A. tumefaciens*, followed by floral-dip stable transformation of *A. thaliana* and transient transformation of *N. benthamina.* Molecular biology procedures were performed according to manufacturer's instructions. PCR amplification towards cloning performed was conducted using

Phusion® high-fidelity DNA polymerase (New England BioLab).DNA purification kit for PCR product of Zymo research. Ligations: Takara's Mighty Mix (Cat 6023) was used. Restriction enzymes used were from NEB and Takara.Plasmid isolation by HiYield™ Plasmid Mini Kit (RBC Bioscience).

III. RESULTS AND DISCUSSION

3.1. Sulfate transporter mutants

Ten *A. thaliana* mutant lines of 6 different genes encoding sulfate transporters were ordered from the ABRC stock center (Table 2). PCR reactions were performed on genomic DNA to confirm homozygous T-DNA integration using gene-specific primers. All homozygous mutant lines were subjected to preliminary screens for chromium (VI) response and examined for germination rates and root elongation under chromium (VI) exposure, using agar plates with concentrations between 10 to 40 mg/L chromium. Preliminary results indicated mutants *sultr1;2* and *sultr3;5* to have a higher germination rate on chromium-containing medium, and therefore a higher tolerance to chromium (VI) compared to wild type. In these mutants the activity of the transporters SULTR1;2 and SULTR3;5, located at the root epidermis and pericycle, respectively, is impaired and sulfate uptake and xylem loading is disrupted[13]. Regarding *sultr4;1* (SULTR4;1 is located at the root tonoplast), the mutant presented lower tolerance, or higher sensitivity to chromium (VI). The results suggested that SULTR family members play a role in chromium homeostasis in *A. thaliana*.

Table 1. A list of *A. thaliana* mutant lines of 6 genes encoding sulfate transporters

Transporter	Locus No.	Tissue	ABRC Mutant
SULTR1;2	AT1G78000	Root epidermis and cortex	SALK_122974
			SALK_133651C
SULTR1;1	AT4G08620	Root epidermis and cortex	SALK_093256C
SULTR 3;5	AT5G19600	Root pericycle	SALK_128559
SULTR 2;1	AT5G10180	Root pericycle and Shoot xylem parenchyma	SALK_109907C
			SALK_111923
SULTR 4;1	AT5G13550	Root tonoplast	SALK_054520C
			SALK_103873C
SULTR 4;2	AT3G12520	Root tonoplast	SALK_103827C
			SALK_116166

3.2. The high-affinity sulfate transporters SULTR1;1 and SULTR1;2

The two root-localized high-affinity transporters SULTR1;2 and SULTR1 were investigated in greater detail[14]. These transporters are the only ones to be identified to uptake sulfate by the roots, even though other non-specific uptake pathways exist, therefore are good candidates to play a major role in chromium (VI) uptake. SALK_093256C is a SULTR1;1 knockout mutant. The T-DNA insertion is located inside the coding sequence. SALK_122974 and SALK_133651C are two mutant lines in which the T-DNA insertions are located in proximity but outside of SULTR1;2 coding sequence. SALK_122974 (named *sultr1;2_1*) has an insertion located about 450 bases up-stream of the start codon of *SULTR1;2*, at the gene's putative promoter. The T-DNA insertion of the second allele, SALK_133651C (named *sultr1;2_2*), is located about 500 bases downstream of the gene stop codon (Fig. 2). The insertion site locations were determined using PCR with specific primers.

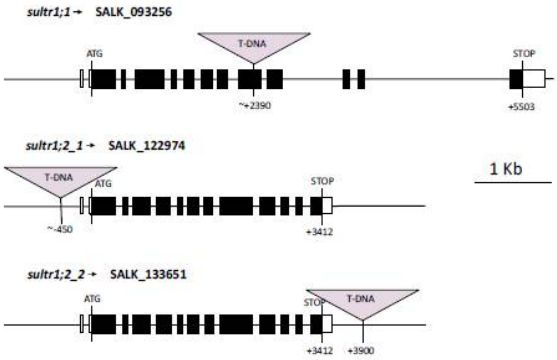


Fig. 2. Graphic illustration of T-DNAs location within *sultr1;1* and two *sultr1;2* alleles. Black bars indicate exons and lines introns, white bars indicate untranslated regions. ATG stands for the initiation codon.

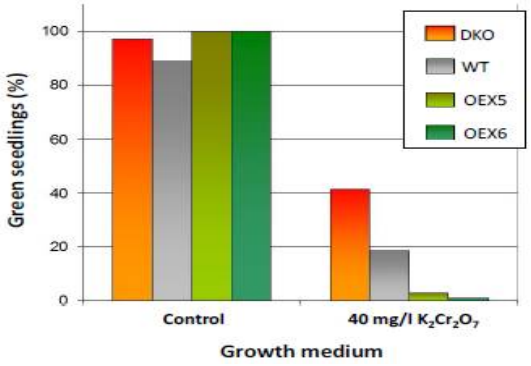


Fig. 3. Survival rates of seedlings of three *Arabidopsis thaliana* (Col.) line types. *sultr1;1:sultr1;2* double knockout mutants (DKO), two independent SULTR1;2 overexpressor lines (OEX) and wild type were germinated on agar medium containing 40 mg/L Cr (VI) and no chromium as control. Values are percentage of seedlings germinated and survived after 12 days on the medium (n=36). The differences in survival rates between the genetically modified lines and the wild type on 40 mg/L Cr (VI) are statistically significant (*Pv*<0.05, chi test). No

statistically significant differences were found for control conditions.

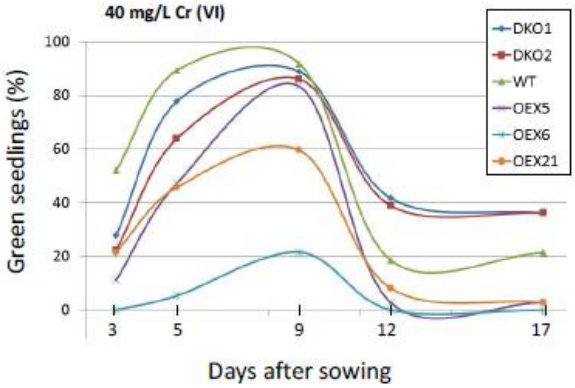


Fig. 4. Germination and survival rates of seedlings of three *Arabidopsis thaliana* (col.) line types. Two alleles of *sultr1;1-sultr1;2* double knockout mutants (DKO), three independent SULTR1;2 overexpressor lines (OEX) and wild type were germinated on agar medium containing 40 mg/L Chromium (VI).

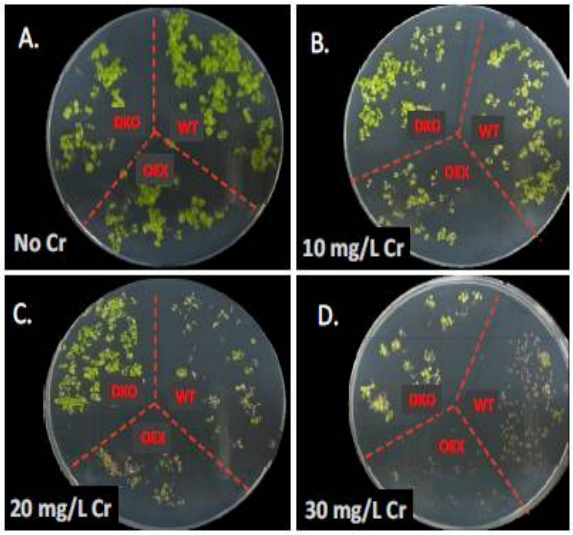


Fig. 5. Germination of three *Arabidopsis thaliana* lines, wild type (WT, top right), *sultr1;1-sultr1;2* double knockout (DKO, top left) and SULTR1;2 overexpressor (OEX, bottom) on solid substrate (agar) containing no chromium as control (A), and 10 mg/l (B), 20 mg/l (C) 30 mg/l (D) chromium (VI).

CONCLUSIONS

Relative tolerance to chromium (VI) exposure, measured as high germination and survival rates of the double-knockout mutant *sultr1;1-sultr1;2* may result from low chromium uptake as a result of the absent of full-functioning sulfate transporters. On the other hand, hypersensitivity to chromium (VI) is presented by the SULTR1;2 overexpressor lines, likely to be caused by elevated efflux of chromate ions into the roots. Thus sulfate transporters likely mediate the uptake of chromium into plants and facilitate the occurrence of this hazardous substance in the food chain.

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