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Short communication

# Exposure to Cr(VI) induces organ dependent MSI in two loci related with photophosphorylation and with glutamine metabolism

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ABSTRACT

Chromium (Cr), as a mutagenic agent in plants, has received less attention than other metal pollutants. To understand if Cr induces microsatellite instability (MSI), *Pisum sativum* seedlings were exposed for 28 days to different concentrations of Cr(VI) up to 2000 mg $L^{-1}$, and the genetic instability of ten microsatellites (SSRs) was analyzed. In plants exposed to Cr(VI) up to 1000 mg $L^{-1}$, MSI was never observed. However, roots exposed to 2000 mg $L^{-1}$ displayed MSI in two of the loci analyzed, corresponding to a mutation rate of 8.3%. SSR2 (inserted in the locus for plastid photosystem I 24 kDa light harvesting protein) and SSR6 (inserted in the locus for *P. sativum* glutamine synthetase) from Cr(VI)-treated roots presented alleles with, respectively, less 6 bp and more 3 bp than the corresponding controls. This report demonstrates that: (a) SSRs technique is sensitive to detect Cr-induced mutagenicity in plants, being Cr-induced-MSI dose and organ dependent (roots are more sensitive); (b) two Cr-sensitive loci are related with thylakoid photophosphorylation and with glutamine synthetase, respectively; (c) despite MSI is induced by Cr(VI), it only occurs in plants exposed to concentrations higher than 1000 mg $L^{-1}$ (values rarely found in real scenarios). Considering these data, we also discuss the known functional changes induced by Cr(VI) in photosynthesis and in glutamine synthetase activity.



## Introduction

Genotoxicity, and in particular mutagenicity, is a highly dramatic form of toxicity that may be generated by metals of contaminated soils. In general, metal genotoxicity is originated through the indirect action of these pollutants, either by the generation of reactive oxygen species and associated DNA damage, or by the inhibition of the DNA repair systems, causing genetic instability and mutation accumulation (Monteiro et al., 2012). Concurrently, some metals can directly interact with DNA and cause further damages/mutations (Peterson-Roth et al., 2005).

Chromium is a toxic and carcinogenic metal, the most toxic valence being Cr(VI). The environmental attenuation of Cr(VI) may be feasible as several natural reductants can transform Cr(VI) to Cr(III). However, the phytoxicity of Cr(VI)-contaminated soils and water has been clearly demonstrated (e.g., Shanker et al., 2005; Goupil et al., 2009).

In animals and microorganism two classes of Cr-DNA phosphate adducts may exist: the majority (about 90%) being non-mutagenic monofunctional Cr-phosphate complexes, while the minority are mutagenic microchelates, which involve a phosphate group and the $N^7$ position of G (Salnikow and Zhitkovich, 2008). In yeasts, Cr(VI)-induced base substitution mutations accounted for at least 83.9% of the mutations observed, while deletions and insertions only corresponded to 16.1% (O'Brien et al., 2009). Concerning the Cr-induced mutagenicity in plants, Labra et al. (2003) demonstrated that *Arabidopsis thaliana* plants treated with Cr(VI) presented more polymorphic AFLP (amplified fragment length polymorphism) bands than control plants. However, no further studies on Cr mutagenicity in plants using molecular markers were developed. Recently, Rodriguez et al. (2011) demonstrated by flow cytometry that Cr(VI) induced DNA degradation, clastogenicity and cell cycle arrest at the $G_2$/M checkpoint. This checkpoint's activation provides the necessary time for cells to repair damaged DNA prior to mitosis (O'Connell and Cimprich, 2005).

Microsatellites (SSRs) are one of the most commonly used molecular markers mainly due to their abundance through eukaryotic genomes, random occurrence and high degree of polymorphism (Burstin et al., 2001). SSRs have therefore been used to study Cd induced microsatellite instability (MSI), for example in mice and in human cell lines (e.g. Oliveira et al., 2012). In plants, SSRs were mostly used in taxonomy, genetic mapping or developmental biology (e.g., Depeiges et al., 2005; Golubov et al., 2010). However, MSI application to survey metal mutagenicity in plants is restricted to rare studies as those of Kovalchuk et al. (2000) who analysed chernobyl ionizing radiation mutagenicity in plants and,

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**Table 1**
Description of the microsatellites that amplified and that were used to assess MSI. Locus, EMBL-Bank accession number (http://www.ebi.ac.uk/ena/data/view/), forward and reverse primers sequence and motif were taken from Burstin et al. (2001). $T_m$ – annealing temperature used.

| SSR | Locus | Accession no. | Forward primer | Reverse primer | Motif | $T_m$ (°C) |
|---|---|---|---|---|---|---|
| 1 | PEAATPSYND | M94558 | 5′-CTCCAGCCCAATAGTCGAAG-3′ | 5′-TCACAACCGAAGTCACAACC-3′ | $(AC)_6$ | 61.5 |
| 2 | PEACPLHPPS | L19651 | 5′-GTGGCTGATCCTGTCAACAA-3′ | 5′-CAACAACCAAGAGCAAAGAAAA-3′ | $(AT)_6$ | 61.5 |
| 3 | PSRBCS3C | X04334 | 5′-CCCAGTGAAGAAGGTCAACA-3′ | 5′-CAATGGTGGCAAATAGGAAA-3′ | $(AT)_6$ | 57.5 |
| 4 | PSARGDECA | Z37540 | 5′-CTGTTCCTCTTTCAAGCACTCC-3′ | 5′-GGGAAAGCAAAGCATGCGGATC-3′ | $(TC)_7$ | 61.5 |
| 5 | PEARHOGTPP | L19093 | 5′-ACGCTTCAACGGCAAAAT-3′ | 5′-AGGACCCCAATCACTCTCAC-3′ | $(TC)_5$ | 57.5 |
| 6 | PSGSR1 | X04763 | 5′-TGAAACCACCATTCTCTGGA-3′ | 5′-AAGACCCCACTTGAAAATTACTTC-3′ | $(ATT)_5$ | 55.9 |
| 7 | PSAJ3318 | AJ223318 | 5′-CAGTGGTGACAGCAGGGCCAAG-3′ | 5′-CCTACATGGTGTACGTAGACAC-3′ | $(CAT)_6$ | 61.5 |
| 8 | PSBT2AGEN | X96764 | 5′GCAGCAGAGCTTGTCTTTGAG-3′ | 5′-GGAATCAGAAACAGCCTTGGG-3′ | $(CCT)_5$ | 57.5 |

more recently, our studies on Cd mutagenicity in lettuce (Monteiro et al., 2007, 2009). In these works, SSRs were recognised as reliable molecular markers in ecotoxicological assays.

The objective of the present work was to evaluate if the supply of Cr(VI) to plants induces MSI. For that we applied our standard SSRs protocol to assess MSI in plants (Lopes et al., 2006; Monteiro et al., 2007, 2009). As model species, we used a pea cultivar (*Pisum sativum* var. *macrocarpon* cv. 'Corne de Bélier') that survives to Cr(VI) doses as high as 2000 mg $L^{-1}$, and whose physiological responses to Cr(VI) were characterized elsewhere (Rodriguez et al., 2011). Plants were exposed to doses up to 2000 mg $L^{-1}$. Although this high concentration is above the recommended maximum concentration in European Union, it is observed in extreme environmental situations. For example, Khasim et al. (1989) described discharged waters showing values as high as 1240 mg $L^{-1}$. Also electroplating industrial effluents with 5000 mg/L Cr(VI) ions were reported by Kavita and Keharia (2012).

## Materials and methods

### *Plant conditions and Cr(VI) treatment*

Pea seeds (*Pisum sativum* L. cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48 h and then placed in pots containing a peat:perlite mixture (4:1) a combination commonly used in commercial greenhouse production of crops. Seedlings were grown during 28 days at 24 ± 1 °C, under light intensity of 200 μmol $m^{-2}$ $s^{-1}$ and a photoperiod of 16/8 h (light/dark). For Cr(VI) treatments, different groups of plants were watered twice per week with 100 mL of a 1:10 Hoagland's solution (Sigma–Aldrich, USA; pH = 5.8) containing different concentrations of $K_2Cr_2O_7$ (≥99.5% purity, Sigma–Aldrich, USA) that provided the following concentrations of Cr(VI): 0 (control), 20, 200, 1000 and 2000 mg $L^{-1}$ as described by Rodriguez et al. (2011). The real levels of total Cr of these solutions were determined by inductively coupled plasma spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France) and were, respectively, of 0, 19.95, 201.88, 998.98 and 2004.62 mg $L^{-1}$. The soil pH was measured before (pH = 5.8 ± 3) and after the experiment (at this time the amplitude of pH changes compared to the initial values were below 0.5). For each Cr(VI) concentration, groups of 25 plants were used.

After 28 days of exposure, plant organs were collected and sampled for the subsequent analyses described below. Roots were cleaned from adhered substrate and then were rinsed thoroughly in distilled water to remove adsorbed Cr (Rodriguez et al., 2011).

### *Total chromium analysis*

The amount of total Cr accumulated by control and Cr-treated organs (at least 3 plants per condition, with 2 independent assays) was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, JY70Plus, France). Leaves and roots were dried to constant weight at 60 °C, and incinerated at 520 °C for 14 h (Azevedo et al., 2005). Ashes were digested by adding HCl and heating. After this, samples were filtered by vacuum suction and final volume adjusted. Samples were analyzed by ICP-AES (Silva et al., 2010). Statistical significance of treatments was assessed by One-Way ANOVA, using SigmaStat 3.5 for WINDOWS (SPSS Inc., Chicago, USA).

### *Microsatellite analysis*

Total DNA was extracted from roots and leaves (100 mg each) of control and Cr(VI)-treated plants (3 individuals per condition from 2 independent experiments), using the DNeasy® Plant Mini Kit (QIAGEN, Germany) as instructed by the manufacturer. Ten SSR were chosen from the ones reported by Burstin et al. (2001), to represent different repetitive motifs, size and locations in the sequence and number of repeats. The primers were synthesized by Invitrogen (UK). From the chosen ten SSRs, two could never be amplified. The 8 SSRs that amplified were used to evaluate Cr(VI)-induced MSI. The primers, repetitive motif and annealing temperatures are given in Table 1. The remaining PCR conditions were as instructed by the PCR's kit manufacturer (TAQ PCR core Kit, Qiagen): each assay contained 30 ng of template DNA, in 20 pM of each primer, 0.2 mM dNTP, 1 × Taq buffer containing 1.5 mM $MgCl_2$, 1 U Taq polymerase in a total volume of 25 μL. PCR was performed in a MyiQ2 (Biorad, CA, USA) and included 1 step of 3 min at 94 °C followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at the required $T_m$ (Table 1) and 45 s elongation at 72 °C. The final step was a 5 min elongation at 72 °C. PCR products were electrophoresed in 2% agarose gels stained with ethidium bromide and running in 1 × TBE buffer. The bands were visualized with a UV transilluminator (G:Box, Syngene, UK). The software (GeneSnap) was used for image acquisition and analysis (Lopes et al., 2006). PCR products were evaluated by Capillary Electrophoresis (CE) on an ABI Prism 310® Genetic Analyser (Applied Biosystems, USA). Each sample was prepared according to the modified protocol from Life Technologies Corporation: 1 μL of PCR product was mixed with 25 μL deionized formamide (HI-Di™ Formamide, Applied Biosystems). Also, 1.0 μL of internal size-standard labelled with ROX™ (GeneScan™ 500 ROX™ Size Standard, Applied Biosystems) was added to all the samples to be used as a reference with known size. Each PCR analysis was repeated twice. The mutation rate (%) was calculated as: [(number of organ samples with MSI/3) × (number of SSR with MSI/8)]*100.

## Results and discussion

The total Cr contents in plants are presented in Table 2, all the values being significantly different from control ($P < 0.005$). Internal Cr levels were maximal in plants exposed to 2000 mg $L^{-1}$. In these plants leaves had half the amount of Cr levels observed in roots being 27.5 μg $gdw^{-1}$ and 51.5 μg $gdw^{-1}$, respectively (Table 2). The internal accumulation of Cr showed the following correlations with the external dosage of Cr(VI): $y = 5.23x - 1.0779$ ($R^2 = 0.8317$) for