Interactions between Chromium and Sulfur Metabolism in *Brassica juncea*

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The effects of chromate on sulfate uptake and assimilation were investigated in the accumulator *Brassica juncea* (L.) Czern. Seven-day-old plants were grown for 2 d under the following combination of sulfate and chromate concentration: (i) no sulfate and no chromate (−S, −Cr), (ii) no sulfate and 0.2 mmol L\(^{-1}\) chromate (−S +Cr), (iii) 1 mmol L\(^{-1}\) sulfate and no chromate (+S, −Cr), or (iv) 1 mmol L\(^{-1}\) sulfate and 0.2 mmol L\(^{-1}\) chromate (+S +Cr). Despite the toxic effects exerted by chromate as indicated by altered level of reducing sugars and proteins in leaves, the growth of *B. juncea* was only weakly reduced by chromate, and no variation in chlorophyll \(a\) and \(b\) was measured, regardless of S availability. Chromium (Cr) was stored more in roots than in leaves, and the maximum Cr accumulation was measured in −S +Cr plants. The significant decrease of the sulfate uptake rates observed in Cr-treated plants was accompanied by a repression of the root low-affinity sulfate transporter (BjST1), suggesting that the transport of chromate in *B. juncea* may involve sulfate carriers. Once absorbed, chromate induced genes involved in sulfate assimilation (ATP-sulfurylase: apsR3; APS-reductase: apr2; Glutathione synthetase: gsh2) and accumulation of cysteine and glutathione, which may suggest that these reduced S compounds play a role in Cr tolerance. Together, our findings indicate that when phytoremediation technologies are used to recover Cr-contaminated areas, the concentration of sulfate in the plant growth medium must be considered because it may influence the ability of plants to accumulate and tolerate Cr.

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**The properties that make a plant species suitable for phytoremediation include heavy metal tolerance, accumulation, and biomass production. These properties depend on such factors as metal uptake and translocation inside plants, intracellular metal sequestration, and stress-resistance mechanisms (Pilon-Smiths and Pilon, 2002). The heavy-metal accumulator *Brassica juncea* is a high biomass crop that represents a good candidate for application in phytoremediation technologies (Dushenkov et al., 1995; Salt et al., 1995, 1998; Pilon-Smiths and Pilon, 2002). In this species, heavy metal accumulation and the parallel synthesis of phytochelatins (PCs) have been investigated in depth (Speiser et al., 1992; Schäfer et al., 1998; Heiss et al., 1999). Moreover, in recent years, the use of genetic engineering approaches has indicated that cadmium (Cd) accumulation in *B. juncea* may be increased by the overexpression of genes involved in glutathione and PC production (Zhu et al., 1999a, 1999b).

The present study was conducted to investigate the physiological and molecular mechanisms involved in chromium (Cr) tolerance and accumulation in *B. juncea*. This knowledge may improve the use of this plant species for the remediation of Cr-contaminated sites by classical breeding or with the use of genetic engineering. The transition metal Cr is an important environmental pollutant. Chromium mining, smelting, and its use in industries such as tanning and electroplating lead to Cr discharge into aquatic bodies and the atmosphere (Nichols et al., 2000). Chromium is a primary noxious substance and is considered to pose a significant potential risk to human health because of its mutagenic and carcinogenic properties (Chaney et al., 1997; USEPA, 2000). In recent years, Cr pollution has become a serious problem worldwide, and as a consequence the remediation of Cr-contaminated areas has received considerable interest (Chandra et al., 1997; Zayed and Terry, 2003). Although phytoremediation could be an attractive alternative to most conventional (and often prohibitively expensive) methods of heavy metal remediation, few studies have been performed on the molecular and physiological basis of plant Cr tolerance and accumulation.

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**Abbreviations:** Cys, cysteine; GSH, glutathione; HPLC, high-performance liquid chromatography; PC, phytochelatin; RT-PCR, reverse transcriptase–polymerase chain reaction.*
Chromium is widespread in nature and is most frequently found in the trivalent (+3) and hexavalent (+6) oxidation states (Zayed et al., 1998). These two forms can interchange depending on several physical, biological, and chemical processes that occur in waters and soils (Kotas and Stasicka, 2000), and differ in toxicity (Wetterhahn and Hamilton, 1989; Katz and Salem, 1994). Hexavalent chromium is highly toxic; it causes cellular and DNA damage due to its powerful oxidizing properties. Trivalent chromium is less soluble and less toxic, but at high doses it is able to inhibit various enzyme systems or react with organic molecules (Barceló and Poschenrieder, 1997).

Plant growth and development are generally injured by exposure to heavy metals (Hagemeier, 2004). High levels of Cr are toxic for plants, limiting their growth. A number of toxic effects have been reported to be induced by Cr, including elicitation of polyamine synthesis followed by a decrease in growth (Jacobson et al., 1992), interveinal chlorosis of young leaves and tissue necrosis (Sharma et al., 1995), altered water content of leaves (Barceló et al., 1986), root system damage (Terry, 1981; Vázquez et al., 1987; Shanker et al., 2005), impaired uptake of various mineral nutrients (Dube et al., 2003; Gardea-Torresdey et al., 2005), and oxidative damage to cell membranes due to the formation of reactive oxygen species (Aijar et al., 1991; Stohs and Bagchi, 1995; Pandey et al., 2005).

Depending on the oxidation state of Cr, there are different mechanisms through which the metal can enter cells (Zaccheo et al., 1985). Although Cr(III) is thought to be passively taken up by plants, the entry of Cr(VI) compounds (chromates and dichromates) into the cytosol is mediated by an active process requiring metabolic energy provided by ATP hydrolysis (Skeffington et al., 1976). It has been suggested that chromate influx and accumulation by plants may involve sulfate transporters (Shewry and Peterson, 1974; Skeffington et al., 1976; Kleiman and Cogliatti, 1997). These studies were originally restricted to kinetic experiments aimed at verifying the role of sulfate as a strong inhibitor of chromate uptake on the basis of their high chemical similarity. Sulfate was found to inhibit chromate uptake in barley seedlings (Shewry and Peterson, 1974) and in wheat (Kleiman and Cogliatti, 1997). Furthermore, Kleiman and Cogliatti (1997) observed an increase of chromate influx in sulfate-depleted wheat plants, which they attributed to the absence of sulfate competition.

More recent transgenic studies provided evidence that suggests that chromate is absorbed via sulfate transporters in plants. The overexpression of a yeast transcriptional activator MSN1 was found to promote the accumulation of Cr and S and increased the expression of the Nicotiana tabacum sulfate transporter NtST1. In addition, the expression of NtST1 in Saccharomyces cerevisiae enhanced the ability to take up Cr and S (Kim et al., 2006). Moreover, expression of a plant high-affinity sulfate transporter (SHST1) enhanced Cr accumulation in Indian mustard (Lindblom et al., 2006).

Once absorbed by plants, Cr(VI) is generally retained in roots after being converted to the less toxic trivalent form (Zayed et al., 1998; Smith et al., 1989). The concentration of Cr recorded in roots is typically 10- to 100-fold higher than in shoots (Zayed et al., 1998). Only when chromate supply to plants is high and exceeds the ability of root cells to reduce it to Cr(III) and to retain it in the vacuoles does Cr accumulation in the aerial plant tissues become significant (Barceló et al., 1986; Vázquez et al., 1987).

Despite the overall tendency to keep Cr stored in the root, plants show quantitative differences in their ability to translocate Cr. Plants that accumulate Cr in their shoot are potentially useful for environmental cleanup of Cr. Among high biomass crops, B. juncea (Indian mustard) has been widely used in phytoextraction. This species is able to take up and accumulate in its aboveground tissues appreciable quantities of heavy metals like cadmium, copper, nickel, zinc, lead, hexavalent chromium, and selenium (Dushenkov et al., 1995; Bañuelos et al., 2005; Le Duc and Terry, 2005).

Apart from the role of sulfate transporters in Cr uptake, S metabolism may affect Cr tolerance and accumulation due to the capacity of certain reduced S compounds to bind and detoxify metals and metalloids (Freeman et al., 2004; Pilon-Smits et al., 1999; Zhu et al., 1999a, 1999b). To further investigate the potential role of plant S metabolism in Cr accumulation and tolerance, the interactions between sulfur (S) nutrition and Cr tolerance and accumulation were considered in this study.

Brassica juncea plants were supplemented with different chromate and sulfate concentrations, and plant growth and Cr concentration in different plant tissues were measured. Because relatively little is known about the phytotoxicity of Cr for this plant species, the present study analyzed the effects of Cr stress on a number of physiological parameters, including root growth, biomass production, and tissue levels of pigments and soluble sugars. The effects exerted by Cr(VI) treatments on sulfate influx and S accumulation were also analyzed. Moreover, the gene expression of a putative low-affinity sulfate transporter (BjST1) was examined via reverse transcriptase–polymerase chain reaction (RT-PCR), and the amounts of the thiols glutathione (GSH) and cysteine (Cys) were determined to investigate the role of these reduced S compounds in Cr detoxification. Related to this, the transcript levels corresponding to genes encoding components of the sulfate assimilation pathway (ATP-sulfurylase, APS-reductase) and GSH synthesis (glutathione synthetase) were determined in the roots of B. juncea plants via RT-PCR.

**Materials and Methods**

**Plant Material**

Seeds of B. juncea (L.) Czern. (Cv. PI 426314) were surface sterilized by rinsing in 70% (v/v) ethanol for 30 to 60 s and then in 5% (v/v) sodium hypochlorite (NaClO) for 30 min while rocking on a platform and washed in distilled water for 5 × 10 min. The seeds were allowed to germinate for 2 d in the dark at 20°C on filter papers saturated with double-distilled water. Germinated seedlings were transferred for 7 d to 35-L plastic tanks containing a thoroughly aerated Hoagland modified nutrient solution with the following composition (μmol L⁻¹): KH₂PO₄ 40, Ca(NO₃)₂ 200, KNO₃ 200, MgSO₄ 1000, FeNaEDTA 10, B 4.6, Cl 1.1, Mn 0.9, Zn 0.09, Mo 0.01. The nutrient solution was renewed every 2 d. The seedlings were grown in a chamber with a 13 h light/11 h dark
cycle, air temperature of 20/15°C, relative humidity of 70/85%, and at a photon flux density of 280 mol m⁻² s⁻¹.

To study interactions between S metabolism and Cr, *Brassica* seedlings were divided into four groups and placed in 2.5-L beakers containing the nutrient solution described previously, supplemented with the following final concentrations of sulfate and chromate: no sulfate (−S), no sulfate and 0.2 mmol L⁻¹ chromate (−S +Cr), 1 mmol L⁻¹ sulfate (+S), 1 mmol L⁻¹ sulfate, and 0.2 mmol L⁻¹ chromate (+S +Cr). Cr(VI) was furnished as potassium chromate (K₂CrO₄). In the solution without sulfate, 1 mmol L⁻¹ MgCl₂ replaced MgSO₄.

*B. juncea* plants were harvested after 2 d, carefully washed with EDTA and distilled water to remove any Cr bound to the root surface, and dried with blotting paper. Part of the plant material was immediately frozen with liquid nitrogen and kept at −80°C to undergo further molecular and physiological analyses. For fresh and dry weight measurements, 10 plants per treatment were divided into roots and shoots and weighed separately. The samples were placed in a drying oven for 2 d at 70°C and allowed to cool for 2 h inside a closed bell jar before measuring the dry weight. The experiment was replicated three times.

To determine the tolerance of *B. juncea* seedlings to Cr, the root length of individual seedlings was measured after 2 d of growth on media with or without Cr at the described concentration. For each measurement, three independent experiments were performed, and each time the root length of 10 plants was measured for each treatment.

**Determination of Pigment Content**

Chlorophyll *a* and *b* levels were determined by the method of Welburn and Lichtenthaler (1984). Fresh foliar tissue (300 mg) was ground in liquid nitrogen and extracted with 15 mL ethanol (96% v/v). The samples were kept in the dark for 2 d at 4°C, and the extracts were filtered and subsequently analyzed via a spectrophotometer (UV/VIS Lambda 1; PerkinElmer, Norwalk, CT) at 665 and 649 nm. The Chla and Chlb concentrations were calculated using the Welburn and Lichtenthaler (1984) formula and expressed in grams of pigment per kilogram of leaf fresh weight. Two measurements were performed for each plant, and six plants of three independent experiments were tested for each treatment.

**Quantification of Soluble Sugars**

Foliar tissues (100 mg) were dried for 48 h at 80°C, ground in liquid nitrogen, and extracted with 2.5 mL 0.1 N H₂SO₄. Samples were incubated in a heat block for 40 min at 60°C and centrifuged at 6000 × g for 10 min at 4°C. The supernatants were analyzed by high-performance liquid chromatography (HPLC) (PerkinElmer 410) after being filtered. The soluble sugars were separated using a Biorad Aminex 87 C column (Biorad, Hercules, CA) (300 × 7.8 mm) using H₂O as eluent at a flow of 0.6 mL min⁻¹. Sugar concentration was expressed in g Kg⁻¹ dry weight and measured two times each for three replicates per experimental treatment. Three independent experiments were performed.

**Elemental Analysis**

Foliar and root tissues harvested from three running growth experiments were dried for 48 h at 80°C and digested with 5 mL of nitric acid 99% (v/v) in a microwave mineralizer (Milestone Ethos model 1600; Milestone, Shelton, CT). Total Cr determination in three replicates of each sample was realized using inductively coupled plasma atomic emission spectroscopy (Spectro CirosCCD; SPECTRO Analytical Instruments, Kleve, Germany), using appropriate standards. The values obtained were expressed in milligrams element per kg dry weight.

**Sulfate Content**

Fresh foliar and root tissues (500 mg) were ground in liquid nitrogen and extracted with 10 mL of distilled water. The samples were incubated for 2 h in a heating block at 85°C. The extracts obtained were filtered and analyzed by HPLC using a Dionex IonPac AS11 (Dionex, Sunnyvale, CA) 4-mm column coupled to guard column AG 14. The column was eluted for 18 min with 3.5 mmol L⁻¹ Na₂CO₃/1 mmol L⁻¹ NaHCO₃ in H₂O at a flow rate of 0.9 mL min⁻¹ and at 1400 PSI pressure. Sulfate content was expressed in mg kg⁻¹ fresh weight. Each determination was performed three times on samples obtained from three independent experiments.

**Sulfate Influx**

Sulfate influx experiments were performed as described by Quaggiotti et al. (2003), and the radioisotope ³⁵S⁻ (SO₄⁻) was furnished by PerkinElmer Life Sciences (Boston, MA). Groups of three Cr(VI)-exposed, sulfur-starved, and sulfur-supplied plants were transferred for 10 min to vessels containing complete nutrient solution buffered with 15 mmol L⁻¹ Tris–2[N-Morpholino]ethanesulfonic acid (pH 5.6) and 1 mmol L⁻¹ MgSO₄ labeled with ³⁵SO₄⁻ (5 MBq mmol⁻¹). The temperature of the solutions was maintained at 23°C, and a 400 W lamp with a photon flux density of 280 µmol m⁻² s⁻¹ provided an adequate light supply. After the uptake period, the plants were removed and placed with their roots in ice-cold unlabeled solution for 2 min to remove sulfate that had passively entered the apoplastic root space. Shoots and roots were harvested separately, blotted dry, weighed, and digested for 24 h with 10 mL of 0.1 N HCl at room temperature. Extract (1 mL) was added to 4 mL of liquid scintillation fluid (Hionic-Fluor 6013319; Packard Bioscience, The Netherlands), and the radioactivity was determined using a liquid scintillation counter (Packard Instruments, Downers Grove, IL). The experiment was replicated three times.

**Determination of Thiol Content**

For isolation of thiols according to Wirtz et al. (2004), frozen foliar and root tissues of *Brassica* plants were ground in liquid nitrogen and incubated in extraction buffer (100 mmol L⁻¹ phosphate buffer [pH 7.1], 50% methanol, and 5 mmol L⁻¹ dithiothreitol) for 10 min at 60°C while shaking. Homogenates were centrifuged twice at 15,400 × g for 5 min at room temperature, and the supernatant fluid was used for further analysis. Reduction of oxidized...
thiols in the extracts was performed at room temperature for 60 min in a total volume of 0.27 mL containing 134 mmol L\(^{-1}\) Tris (pH 8.3) and 1 mmol L\(^{-1}\) dithiothreitol. Thiols were derivatized for 15 min by adding monobromobimane (Calbiochem, La Jolla, CA) to a final concentration of 3 mmol L\(^{-1}\). Resulting monobromobimane derivatives were stabilized by adding 5% (v/v) acetic acid, and thiols were detected fluorometrically (Fluorometer RF 551; Shimadzu, Milton Keynes, UK) at 480 nm by excitation at 380 nm after separation by reverse-phase HPLC using a Waters HPLC System (Waters Multisolvnet Delivery system, Autosampler 717 plus; Waters, Milford, MA) connected to a Nova-Pak C18 4.6 × 250 mm column (pore size 4 μm). Glutathione and cysteine were separated by applying an isotropic flow (1.3 mL min\(^{-1}\)) of buffer containing 100 mmol L\(^{-1}\) potassium acetate and 9% methanol for 12.5 min. The column matrix was washed with 100% methanol for 3 min and re-equilibrated for 8.5 min using the same buffer.

Data acquisition and processing was performed with Millennium\(^{32}\) software (Waters). The measurement of thiols was performed on three replicates for each treatment.

**Expression Analysis via Semi-quantitative RT-PCR**

Total RNA was extracted from the roots of *B. juncea* seedlings. After harvest, the plant samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. RNA isolation was performed using the Nucleon Phytopure kit (Amersham-Pharmacia, UK) following the protocol provided by the manufacturer. The RNA amount and quality were analyzed spectrophotometrically by measuring the absorption at 230, 260, and 280 nm. Electrophoresis analysis was performed in a 1% (w/v) agarose gel to verify the absence of nucleic acid degradation. Forty micrograms of total RNA was treated with 10 U of Dnase RQ1 (Promega, Milano, Italy) in a heat block at 37°C for 30 min. At the end of the Dnase reaction, the samples were extracted with phenolchloroform (3:1), and the RNA was precipitated as described by Sambrook et al. (1989). The pellets obtained were washed in 1 mL of 75% (v/v) ethanol and resuspended in 20 μL of sterile Rnase-free water, and the RNA amount was estimated as described previously. After Dnase treatment, 5 μg of RNA of each sample was used to synthesize the first-strand cDNAs, by means of 200 U of Moloney Murine Leukemia Virus reverse transcriptase (Promega, Milano, Italy) and oligo(dT) as primers, in 20-μL reactions. The reaction conditions were 37°C for 60 min and 70°C for 5 min. Cooling to 4°C for 5 min stopped the RT reaction.

Reverse transcriptase–polymerase chain reaction experiments with specific primers were performed to evaluate the expression level of the *B. juncea* genes reported in Table 1. For all PCR reactions, 1 μL of the cDNA obtained was used in 20-μL reactions, using 0.025 U μL\(^{-1}\) of Taq-polymerase (Amersham-Pharmacia-Biotech, Piscataway, NJ), and a set of different numbers of cycles ranging from 18 to 30 was tested to determine the optimal number of cycles corresponding to the exponential phase in the amplification for each gene. In this phase, increasing numbers of PCR cycles resulted in a higher amount of PCR product, indicating that the reactions were not in the stationary phase and the reaction components were not limiting. Each PCR cycle consisted of 3 min initial denaturation at 95°C, 30 s denaturation at 95°C, 30 s annealing, 30 s extension at 72°C, and 7 min final extension at 72°C. The annealing phase temperature was modified depending on the gene transcript.

The constitutively expressed *B. juncea* actin gene (*β-actin2*) (Heiss et al., 2003) was used as internal control to normalize the obtained gene expression results. RT-PCR analysis was performed using the Gen Amp PCR system 9700 (PE Biosystems, Branchburg, NJ), and PCR products were separated by electrophoresis in a 1 to 1.5% agarose gel stained with ethidium bromide. The DNA fragments were visualized under UV light and quantified through the ImageJ program (ImageJ 1.23j; Wayne Rasband, National Institutes of Health, Bethesda, MD). To confirm the expression analysis results, PCR reactions were performed on cDNAs obtained from two different RNA extractions performed on roots of seedlings of two independent experiments and repeated at least four times for each cDNA. Polymerase chain reaction products obtained from the gene expression analysis were further sequenced to verify the specificity of amplification of each gene. DNA was extracted from the agarose gel and eluted by the QIAquick Gel Extraction-Kit Protocol kit (QIAGEN, Milano, Italy). The eluted DNAs were electrophoresed in agarose gel together with a molecular marker to be quantified. Gene sequencing was performed according to Sanger et al. (1977) at the CRIBI at Padua University, using the ABI PRISM original Rhodamine Terminator kit (PerkinElmer Biosystems) and specific and universal primers. Blastx and Blastn (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) programs served to compare the gene sequences.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ223495, AJ223499, AJ001207, Y10984.

**Statistical Analysis**

Analysis of variance was performed followed by pair-wise post-hoc analyses to determine which means differed significantly. Statistically significant differences (\(P < 0.05\)) are reported in the text and shown in the figures.

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Table 1. Oligonucleotide primer name and sequence for primers of *Brassica juncea* used in reverse transcriptase–polymerase chain reactions.

<table>
<thead>
<tr>
<th>Genet</th>
<th>Forward primer (5′ → 3′)</th>
<th>Reverse primer (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin2</td>
<td>TGTTCAACACACACAGGAGG</td>
<td>CACCTGTGCCGGGGTAACTCG</td>
</tr>
<tr>
<td>BjSt1</td>
<td>TACCTCACAATGGAACAGAG</td>
<td>GGCAAACTATCATTATTCTG</td>
</tr>
<tr>
<td>atps6</td>
<td>CGAGACGGGTTGGTACACATGCA</td>
<td>TTGATAGGCATTTTGTACCTTC</td>
</tr>
<tr>
<td>aprs2</td>
<td>ACATGGAAGCCTCAGCTGAGG</td>
<td>CGTCCTTGTAATGGTGCCTGTGGA</td>
</tr>
<tr>
<td>gsh2</td>
<td>CGAGACGGGTTGGTACACATGCA</td>
<td>TTGATAGGCATTTTGTACCTTC</td>
</tr>
</tbody>
</table>

† aprs2, APS reductase; atps6, ATP sulfurylase; BjSt1, low-affinity sulfate transporter; gsh2, glutathione synthethase.
Results

Effects of Chromium on Plant Growth in Relation to Sulfur Status

To examine the possible interactions between sulfate and chromate, *B. juncea* plants were grown for 2 d in complete nutrient solution containing four different combinations of sulfate and chromate concentrations: (i) no sulfate and no chromate (−S), (ii) no sulfate and 0.2 mmol L\(^{-1}\) chromate (−S +Cr), (iii) 1 mmol L\(^{-1}\) sulfate and no chromate (+S), or (iv) 1 mmol L\(^{-1}\) sulfate and 0.2 mmol L\(^{-1}\) chromate (+S +Cr). The chromate treatments did not result in chlorosis or necrosis. In +S plants chromate had a negative effect on all parameters of plant growth, but under S starvation Cr only slightly affected root growth (Table 2). Sulfur starvation negatively affected shoot weight and root length in the absence of Cr but not in its presence (Table 2). Thus, the two stresses, Cr toxicity and S deficiency, seemed to have a similar negative effect on growth without additive effect. The dry matter content (ratio of dry weight to fresh weight) was similar in +S and −S plants and did not change under chromate treatment (Table 2).

Effects of Chromium on Chlorophyll, Protein Content, and Soluble Sugars

To further evaluate the effects of chromate on plant metabolism in relation to S status, the *B. juncea* plants grown under the four treatments (−S, −S +Cr, +S, +S +Cr) were analyzed for their levels of chlorophyll, total protein, and soluble sugars (Fig. 1). Regardless of Cr supply, *Chl a* and *Chl b* levels in S-supplied plants were about twofold higher than in S-starved plants (Fig. 1A). In contrast, chromate treatment led to changes in the accumulation of proteins and soluble sugars (glucose and fructose). The total protein content in leaves was reduced by 25 to 30% in plants grown with −S +Cr or +S +Cr, compared with the respective −S and +S controls (Fig. 1B). Sulfur deficiency and Cr toxicity seemed to have an additive negative effect on protein content because −S +Cr plants contained the lowest protein amount of all treatments. In the root, no significant differences were observed between any of the treatments (Fig. 1B). The leaf glucose levels increased approximately twofold in *B. juncea* plants grown in the presence of chromate, compared with the respective −S and +S controls, and, regardless of Cr treatment, S deficiency reduced leaf glucose levels by twofold (Fig. 1C). Sulfur deficiency and Cr toxicity thus seemed to have opposite effects on leaf glucose concentration. These effects seemed to be additive because +S +Cr plants contained the highest glucose levels among all treatments. Leaf fructose level was also augmented by Cr only in S-starved plants (Fig. 1D), and S status did not seem to affect fructose concentration.

Effect of Sulfate and Chromate Availability on Tissue Chromium, Sulfate, and Sulfur Levels

Because previous reports suggest a competition between sulfate and chromate for the same plasma membrane transporters...
while entering the root cells, the amount of Cr (Fig. 2), sulfate, and total sulfur (Fig. 3) in foliar and root tissues was measured. Chromium was more accumulated in roots than in leaves for −S and +S plants (10- to 30-fold), and the maximum Cr accumulation was observed under S deprivation (1.6-fold higher concentration than +S; \( P < 0.05 \)) (Fig. 2A, 2B). S-repleted plants exhibited a higher Cr translocation from roots to the aerial part. For comparison, the shoot-to-root ratio of Cr under −S conditions was 0.03, whereas under +S conditions it was 0.09.

With respect to sulfate accumulation, B. juncea plants from all treatments contained more sulfate in foliar tissues than in roots (2- to 2.5-fold) regardless of sulfate and chromate availability (Fig. 3A). After exposure to chromate, the accumulation of sulfate was significantly reduced in leaves of +S plants but was unaffected in −S plant leaves; Cr treatment did not significantly affect root sulfate levels. The level of total sulfur was also higher in the aerial tissues than in roots and was significantly diminished by chromate only in roots of S-sufficient plants (Fig. 3B).

**Sulfate Uptake Capacity and BjST1 Gene Expression**

Sulfate uptake rates were measured in relation to Cr and S supply to investigate how sulfate uptake systems in B. juncea are regulated by the combination of these elements. When radiolabeled SO\(_4^{2-}\) (at 1 mmol L\(^{-1}\) external sulfate concentration) was supplied to plants over a 10-min pulse period, the rates of sulfate uptake in −S plants were approximately two-fold higher than in +S plants under no Cr and +Cr conditions (Fig. 4A). The supplement of chromate in the presence or absence of S decreased the sulfate uptake rates by about 30% compared with the respective −S and +S controls. Thus, sul-
and Glutathione Levels

Fig. 5. Effect of chromium exposure on cysteine (Cys) (A) and total glutathione (GSH) (B) in Brassica leaves and roots. Plants were grown for 7 d in Hoagland modified complete nutrient solution and treated for 2 d with no sulfate (−S), no sulfate and chromate 0.2 mmol L⁻¹ (−S +Cr), 1 mmol L⁻¹ sulfate (+S), or 1 mmol L⁻¹ sulfate and 0.2 mmol L⁻¹ chromate (+S +Cr). Thiols are expressed as μmol Kg⁻¹ fresh weight. Data acquisition and processing was performed with Millennium³² software (Waters). The measurement of thiols was realized in three replicates (±SE) of three running experiments. Values marked with different letters (lowercase for leaves and uppercase for roots) are significantly different (P < 0.05).

To further investigate the influence of chromate on sulfate transport in B. juncea, the expression pattern of the putative low-affinity sulfate transporter BjST1 was analyzed in roots. Overall, BjST1 transcript abundance correlated with sulfate uptake rates (Fig. 4A, 4B). The mRNA level of BjST1 was reduced by chromate in −S and +S plants, and in the absence of Cr, S starvation induced BjST1 transcript accumulation, which is in agreement with other studies (Takahashi et al., 1997; Quaggiotti et al., 2003; Hopkins et al., 2004). However, in the presence of Cr, BjST1 was not upregulated under S starvation.

The Effects of Chromium and Sulfur Supply on Cysteine and Glutathione Levels

Because reduced S compounds are known to play a key role in plant heavy metal detoxification and may regulate sulfate uptake, the content of Cys and total glutathione GSH was determined in the plants grown under the four different Cr and S levels. Both Cys and GSH were accumulated more in plants grown under adequate S supply in comparison with S-deprived plants (Fig. 5A, 5B). The effect of chromate on Cys levels was similar in root and leaves but differed for −S and +S plants. The levels of Cys were the same with or without Cr for S-starved plants, whereas in S-supplied plants more Cys was present in plants exposed to chromate (Fig. 5A). Thus, it seems that chromate caused Cys accumulation in roots and leaves but only when sufficient S was available.

Although GSH levels were generally higher in leaves than in roots of −S plants, the S-supplied plants showed the highest levels of GSH in their roots, correlating with the content of Cys (Fig. 5B). In −S plants, chromate treatment had a small positive effect on leaf GSH levels and did not affect root GSH levels. Chromate had a larger stimulatory effect on GSH levels in the roots of +S plants (Fig. 5B).

Effects of Chromate on Gene Expression of Components of the Sulfate Assimilation Pathway and GSH Biosynthesis

To further explore the biochemical mechanisms causing the positive effects of chromate treatment on Cys production, the gene expression was analyzed of several components of the sulfate assimilatory pathway. ATP sulfurylase (atp6) and APS reductase (apsr2) are involved in sulfate activation and reduction (Heiss et al., 1999), whereas glutathione synthetase (gsh2) is responsible for the biosynthesis of GSH (Schäfer et al., 1998). The gene expression analyses were performed via semi-quantitative RT-PCR using roots of B. juncea plants from the four treatments.

As expected based on earlier studies (Lappartient and Touaraine, 1996; Hopkins et al., 2004), the transcript corresponding to the atp6 gene was more accumulated in −S plants than in S-supplied plants (Fig. 6). The effect of chromium on atp6 expression was striking: under −S and +S conditions Cr treatment resulted in a pronounced increase in atp6 transcript levels. A similar pattern of gene expression was observed for gsh2 (Fig. 6). The transcript level of apsr2 also showed induction by Cr but no induction by −S conditions.
Discussion

This study provides evidence of interactions between plant S metabolism and Cr. Chromate and sulfate seemed to compete for uptake because plant Cr levels were higher under −S than +S conditions. In addition, chromate negatively affected sulfate uptake rates, perhaps due to the observed negative effect on the expression of the low-affinity sulfate transporter BjST1, although a competition for the active binding site of the sulfate transporter cannot be excluded. Thus, chromate may act as an inhibitor of sulfate uptake by root cells. Chromate exposure increased the transcript levels of the S-related genes atp6, apr2, and gsh2, which are involved in Cys and GSH production. Indeed, GSH and Cys levels were increased to some extent in Cr-supplied plants. These results may indicate that S metabolism plays a role in Cr tolerance and accumulation in B. juncea, as was shown for other metals and metalloids like cadmium and arsenic (Zhu et al., 1999a, 1999b; Pickering et al., 2000).

The limited effect of 0.2 mmol L−1 chromate on plant growth indicated that B. juncea well tolerated 2 d of chromate treatment at this concentration, in contrast with the inhibition of root length up to 90% in B. oleracea after 2 d of Cr supply at 100 μM observed by Sanità di Toppi et al. (2002). Therefore, these two species of Brassica seem to show substantial variation in Cr tolerance. Such large variation in Cr tolerance between related taxa has also been reported by Corradi et al. (1991). Although the ratio of dry weight to fresh weight did not change appreciably on chromate exposure, the tendency of this parameter to increase was associated with the reduction of the shoot and root fresh biomass. The decrease in fresh weight, which is indicative of reduced water content, may be due to the high Cr concentration in plant tissues, as reported by other authors (Barceló et al., 1986; Nichols et al., 2000; Pandey et al., 2005). The inhibition of B. juncea growth in the presence of Cr may be also related to the observed lower synthesis of total foliar proteins. Indeed, a toxic tissue Cr concentration may bring about quantitative and qualitative changes in plant protein production, which are often associated with stunted plant growth (Labra et al., 2006).

Free hexose sugars such as glucose and fructose are generally not very abundant in photosynthetic cells (Salisbury and Ross, 1991). The supply of chromate enhanced the accumulation of these sugars in foliar tissues. A similar effect was reported by Sharma et al. (1995) in wheat. Similar to Cr, other heavy metals, including cobalt, nickel, and zinc, have been found to induce this effect when supplied to plants at toxic concentrations (Agarwala et al., 1977). The observed increase in levels of reducing sugars may be due to interference of Cr with carbohydrate metabolism or with photoassimilate export from leaves by inhibiting phloem loading (Sharma et al., 1995). Evidence for the former comes from investigations by Labra et al. (2006), where induction of proteins involved in sugar metabolism occurred in maize plants in response to chromate stress. The buildup of sugars and starch may inhibit photosynthesis at some point (Salisbury and Ross, 1991). Investigations performed by Araya et al. (2006) indicate that the negative effect of carbohydrate accumulation on photosynthesis is significant in the source leaves but not in the young sink leaves and is mainly attributable to a decline in Rubisco content. In the present study, the accumulation of Cr and the concomitant accumulation of hexoses did not seem to affect the levels of photosynthetic pigments, as judged from Chl a and b measurements. These findings are in agreement with the observations by Sharma et al. (2003) in maize plants treated with chromate concentrations of 0.25 and 0.5 mmol L−1, where Chl a slightly decreased from the no Cr condition, and Chl b remained unchanged.

High levels of Cr were detected in roots. Brassica juncea accumulated more Cr when chromate was offered in the absence of sulfate. This suggests inhibition of chromate uptake and accumulation by sulfate when both anions are present in the growth medium, likely due to competition for the active binding site of the same sulfate transporters. Interaction between sulfate and chromate uptake is also supported by the results obtained from the sulfate uptake experiment, which showed a markedly diminished ability of plants exposed to chromate to take up sulfate from the nutrient solution.

Concomitant with the reduction in sulfate uptake rates, a significant decrease of transcript accumulation for the low-affinity sulfate transporter BjST1 was observed in plants grown in the presence of Cr under −S and +S conditions. Therefore, other than the probable competition with sulfate for the binding to the same carriers, chromate had an additional way to interfere with sulfate absorption (i.e., the inhibition of low-affinity sulfate transporters transcription).

Earlier studies reported a downregulation of BjST1 associated with the activation of the first steps of sulfate reduction and the concomitant enhanced accumulation of thiols in B. juncea in response to cadmium stress (Heiss et al., 1999). Similar to these results, in Brassica plants exposed to chromate, the observed downregulation of BjST1 was concomitant with an induction of three genes encoding components of the sulfate assimilation pathway and GSH biosynthesis. The expression of atp6, apr2, and gsh2 seemed to be regulated in a coordinated way: all were induced on chromate treatment in −S and +S plants. Similar coordinated changes in transcript amounts of BjST1, ATP-sulfurylase, and APS-reductase, together with variations in contents of O-acetylserine(thiol)lyase and thiols observed in B. juncea plants in response to Cd, were hypothesized by Heiss et al. (1999) to represent an essential part of the cellular response in countering Cd toxicity. Consequently, the higher sulfate uptake at root level and the enhanced sulfate assimilation were thought by the authors to be necessary for providing metal-chelating S compounds, such as GSH and phytochelatins (PCs).

A similar explanation could in part be extended to Cr stress. We found the levels of Cys and GSH considerably increased in roots of Cr-treated S-supplied plants, whereas the amounts of these compounds remained almost unchanged during S starvation. These results were consistent with the observed differences in sulfate content, which was lower in plants grown under S starvation than in +S condition, thus limiting the possibility to form cysteine and GSH. Total S being equal between +S +Cr−-treated and +S control plants,
the reduction in sulfate concentration observed in leaves of +S +Cr plants and the lower accumulation of sulfate in roots than in foliar tissues may be explained by the increased request for sulfate for local assimilation in Cys and GSH. Although Heiss et al. (1999) found a depression of GSH levels in plants supplied with Cd, which was parallel with the increased synthesis of PCs, in our study GSH was accumulated more in Cr-treated plants, in agreement with observations by Sanità di Toppi et al. (2002) in B. oleracea.

Therefore, our findings suggest a coordinated transcriptional regulation of the sulfur assimilation enzymes in response to an augmented demand for cysteine and GSH, as already hypothesized for Cd (Heiss et al., 1999). However, the higher accumulation of GSH observed in B. juncea in response to Cr stress is unlikely to be required for PCs synthesis. This notion is supported by previous studies that report no synthesis of PCs in B. oleracea plants in concomitance with the increased content of GSH (Sanità di Toppi et al., 2002) and no formation of PC-Cr complexes in transgenic tobacco plants with enhanced tolerance to Cr (Kim et al., 2005).

Rather than being a substrate for PCs biosynthesis during Cr stress, GSH may play a major role in the reduction of Cr(VI) to the less toxic trivalent form. In such a reaction, the transfer of three electrons is required, and only few biological compounds in cells function as efficient reductants for Cr(VI), including GSH and ascorbate (Kaim and Schwedersky, 1994). The oxidation of GSH and Cys by Cr(VI) has been previously reported by McAuley and Olatunji (1977a, 1977b). Furthermore, during Cr(VI) to Cr(III) reduction, reactive oxygen species can be produced, and in this view the ascorbate/glutathione cycle might represent a fundamental mechanism regulating the cellular oxidative balance (Noctor and Foyer, 1998).

Conclusions

Chromate uptake in B. juncea may involve sulfate transporters because chromate competed with sulfate for uptake. Apart from this competition effect, chromate also had a direct negative effect on sulfate uptake at transcriptional level. Once inside the plant, chromate induced genes involved in sulfate assimilation and GSH biosynthesis and led to the accumulation of Cys and GSH, suggesting these reduced S compounds play a role in Cr tolerance. These findings have some practical implications. First, because B. juncea accumulated Cr to substantial levels in roots and had higher chromium translocation than many previously investigated vegetable crops (Zayed et al., 1998), this species may be a good candidate for Cr phytoextraction via phytoremediation or rhizofiltration. Furthermore, in view of the interactions between chromate and sulfate, the concentration in the growth media must be taken into consideration when B. juncea plants are going to be used for Cr phytoremediation. Finally, the involvement of S transporters and S assimilatory enzymes in Cr uptake and accumulation suggest that the manipulation of the genes encoding these proteins may be a promising strategy to improve Cr tolerance and accumulation of B. juncea.

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References

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