SHORT COMMUNICATION

Expression of stress-related genes in tomato plants exposed to arsenic and chromium in nutrient solution

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Summary

The molecular responses of hydroponically cultivated tomato plants to As(V) or Cr(VI) were assessed by transcript accumulation analysis of genes coding for products potentially involved in heavy metal tolerance. A quantitative real-time PCR experiment was performed with *Hsp90-1*, *MT2*- and *GR1*-like protein genes using RNA isolated from tomato roots or shoots treated for 24 h with As(V) or Cr(VI) at concentrations ranging from 80 to 640 μM. Both transient metallic treatments induced *Hsp90-1* transcript accumulation in tomato plants. *MT2*- and *GR1*-like transcripts accumulated in tomato roots treated with As(V) but were only slightly affected by Cr(VI) treatment. Tomatoes showed phenotypic symptoms to heavy metal toxicity when plants were exposed to Cr(VI) but not As(V). Plant lethality was observed at 1280 μM Cr(VI), indicating that tomatoes were more tolerant to As than Cr stress under the experimental conditions used here.

Introduction

Arsenic (As) and chromium (Cr) are highly toxic elements naturally present in a number of minerals

and released into the environment by industrial and agricultural activities (He et al., 2005). As and Cr exist in various forms that differ in biological properties and degrees of toxicity. Both elements predominate in inorganic forms, i.e. arsenate As(V) and arsenite As(III), Cr(VI) and Cr(III). In their different oxidation states, these elements are mobile and stable; they can be absorbed and accumulated by plants, not necessarily causing phenotypic symptoms of toxicity (Patra et al., 2004; Shanker et al., 2005). Any basal metal tolerance found in most plant species including crops could lead to metal concentrations potentially health-threatening for consumers (McLaughlin et al., 1999).

Current knowledge on the ubiquitous basic metal tolerance indicates that plants share several common mechanisms (Clemens, 2001, 2006; Hall, 2002) preventing the damaging effects of the metallic stress instead of developing proteins that could resist the heavy metal effects. These mechanisms involve reduced metal uptake, oxidative defense, metal chelation, repair of stress-damaged proteins and vacuolar compartmentalization.

In the present work, the early molecular response of hydroponically cultivated tomato plants to As(V) or Cr(VI) was examined by transcript accumulation analysis of three stress-related genes: (i) *Hsp* (heat shock proteins) gene, an environmental toxicology stress marker (Feder and Hofmann, 1999), (ii) *MT* (metallothionein) gene, coding for a metal-binding protein (DalCorso et al., 2008) (iii) *GR* (glutathion reductase) gene, a marker of enzymatic ROS scavenging mechanism (Schützendübel and Polle, 2002; Apel and Hirt, 2004).

Our results suggest that tomato plants might develop different strategies to cope with As(V) and Cr(VI) toxicity by manipulating the expression level of stress-related genes. This molecular analysis, combined with the observation of toxicity symptoms, allowed us to illustrate the potential tolerance of tomato plants to As(V).

Material and methods

Plant material and growth conditions

Tomato (*Lycopersicon esculentum* cv. VFN-8) was grown in vermiculite under greenhouse conditions. After 15 days of growth, seedlings were transferred onto a hydroponic support containing macronutrient solution in mM: 3.9 $Ca(NO_3)_2$, 6.5 KNO_3, 2 $MgSO_4$, 0.9 KH_2PO_4 plus micronutrients in μM: 90 Fe-EDTA, 2.7 $MnSO_4$, 0.8 $ZnSO_4$, 4.5 H_3BO_3, 4 $CuSO_4$ and 2.0 $Mo_7O_{24}(NH4)_6$ at pH 6. After 7 d of acclimatization with a light/dark photoperiod of 16:8 h at 25 °C, $AsNa_2O_4$ or $K_2Cr_2O_7$ was added to the hydroponic solution for 24 h at a concentration from 0 (control), 80, 160, 320, 640 and 1280 μM of As(V) or Cr(VI).

RNA isolation and first strand cDNA synthesis

RNA extraction was performed using Tri-reagent (Euromedex) and the Euroscript Reverse Transcriptase (Eurogentec) was used for cDNA synthesis, both according to manufacturer's instructions. RNA integrity was verified on a 1% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent.

Primer design

Primers used for the amplification of target cDNAs were designated according to tomato genes available in the databank (http://www.ncbi.nlm.nih.gov/). Both forward and reverse primers must frame a relatively short sequence (approximately 150 bp) suitable for qPCR, designed with a GC percentage of around 60% and a Tm of between 58 and 60 °C. Primers *Hsp90-1*, *MT-*, *GR-* and *Actin*-like protein genes were designated on sequences of tomato genes:

LeHsp90-1 Fw: 5′-GAGAATCATGAAGGCACAAGC-TCTC,
LeHsp90-1 Rev: 5′-CTTATCAGCATCAGCTCTCTTC-CTG,
LeMT Fw: 5′GCTGTGGATCTAGCTGCAAGTGCG,
LeMT Rev: 5′-AAGGGTTGCACTTGCAGTCAGATCC,
LeGR Fw: 5′-TCCCATCGGCTCTGAAGTTAGTGGG,
LeGR Rev: 5′-TCTTTGCATCCTCCAGTTCTGGCCC,
LeActin Fw: 5′-GGGATGGAGAAGTTTGGTGGTGG,
LeActin Rev: 5′-CTTCGACCAAGGGATGGTGTAGC.

Real-time quantitative PCR

The identity of each RT-PCR product was confirmed by direct sequencing: PCR products were resolved on a 1.2% (w/v) agarose gel for size verification, purified with a DNA gel band purification kit (GFX™ PCR, Amersham Pharmacia Biotech) according to manufacturer's protocol, and then sequenced using Genome Express services. Sequence homology searches were carried out using the BLAST search facility available through NCBI (http://www.ncbi.nlm.nih.gov/).

Table 1.   Homologies of qPCR amplified fragments to sequences in the databases.

PCR fragments	Length[a] (bp)	Accession number	Homology[b]	BLAST score
Hsp90-1	85	EU884311	*Lycopersicon* esculentum molecular chaperone Hsp90-1 mRNA (AY368906.1)	6e−34
MT2	169	EU884310	*Lycopersicon esculentum MT2*-like protein gene (L77966.1)	4e−47
GR1	86	FJ265823	*Brassica rapa* glutathione reductase (BcGR1) mRNA (AF008441.2)	5e−20
Actin1e-52	120	EU884309	*Solanum lycopersicum Actin* isoform B mRNA (BM956640.1)	1e−52

[a]Sequence provided by MWG/OPERON.
[b]GenBank accession numbers of sequences homologous to qPCR fragments are in parentheses.

The quantitative assessment of mRNA levels was performed using the iCycler iQv3 (BIO-RAD). Real-time quantitative PCR, based on the fluorescence emitted by the amplification products in the presence of SYBR Green, facilitates quantification of the target transcript accumulation relative to the *Actin* transcripts taken as reference. The reactions were prepared using the qPCR kit Mastermix for SYBR Green (Eurogentec) according to the manufacturer's protocol. The cDNA concentration used produced a CT (threshold cycle) between 15 and 30 cycles. The abundance of targeted gene transcripts was normalized to *Actin* mRNA and set relative to control plants (no heavy metal exposure) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Results and discussion

A range of concentrations of the two elements was used in our experimental conditions to mimic their potential effect in polluted soil and assess the response of the plant at the molecular level. High metal concentrations can be found in agricultural soils, as anthropogenic processes such as intensive use of fertilizers, organic manures and industrial wastewaters for plant irrigation are commonly practiced. Historically, As-containing pesticides such as plant defoliants and herbicides have resulted in As topsoil accumulation at $124\,mg\,kg^{-1}$ (Smith et al., 1998). Repeated use of organic materials such as biosolids or composts containing $40–2800\,mg\,kg^{-1}$ of Cr has increased heavy metal bioavailability (Zayed and Terry, 2003; He et al., 2005). In our experiments, the tomato plants were cultivated hydroponically. This is an advantageous system because it could manipulate the exogeneously supplied bioavailable metal and correlate the metal concentration with the plant response or symptom. Compared to plant soil culture, it avoids the various rhizosphere physical and biological parameters limiting element bioavailability (Hinsinger et al., 2006).

Transcript levels were assessed by quantitative real-time PCR using RNAs isolated from roots and shoots of hydroponically cultivated tomato plants. The primer pairs designed for *Hsp90-1*, *MT2-* and *GR1*-like protein genes facilitated the amplification of a single isoform (data not shown). Table 1 depicts the size and the homologies of PCR amplified fragments to sequences in databases.

Figure 1 shows transcript accumulation of *Hsp90-1*, *MT2-* and *GR1*-like protein genes in tomato plants treated with different concentrations of As(V) or Cr(VI) for 24 h.

Both metals induced higher *Hsp90-1* gene expression. Nevertheless, As(V) induction was marked in roots and Cr(VI) induction in shoots. These results indicate that both elements were absorbed by tomato plants in 24 h to trigger the molecular response. The hexavalent Cr is a stable form of Cr. Its mobilization and uptake involves sulfate carriers (Cervantes et al., 2001). As(V) transport pathway involves phosphate transporter active mechanisms (Meharg and Hartley-Whitaker, 2002).

Hsp transcripts are useful biomarkers because their induction is much more sensitive to stress than traditional indices such as growth inhibition. Hsps act as molecular chaperones in normal protein folding and assembly, and may also play important physiological roles in the creation of supramolecular structures or the restriction of coagulation of polypeptides. Protection and repair of protein folding under stress conditions has been also reported (Del Razo et al., 2001). In our experiments, both metals induced the expression of the abundant and highly conserved molecular chaperone *Hsp90-1* protein gene. These data suggest that tomato plants sense the potent metallic stress and respond to As and Cr stress by activating mechanisms to alleviate the protein damage and preserve cellular homeostasis.

Exposure of tomato plants to As(V) led to dramatic changes in the abundance of stress-related transcripts,
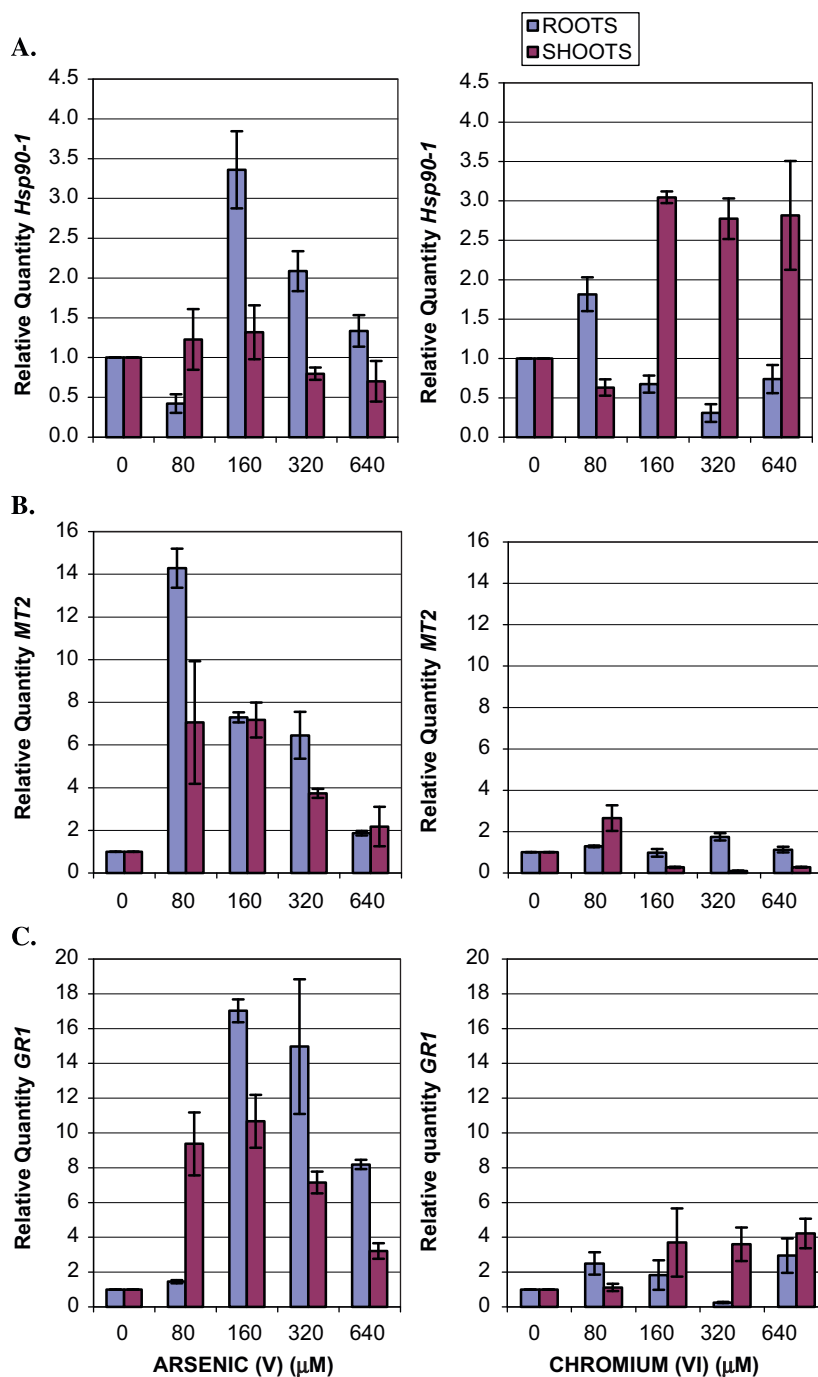
Figure 1. Stress-related transcript accumulation after As(V) and Cr(VI) treatments of hydroponically cultivated tomato plants for 24 h. The amount of transcript encoding *Hsp90-1*(A), *MT2-* (B) and *GR1*-like (C) genes was quantified by RT-qPCR and normalized to the amount of the housekeeping *Actin* transcript. Values are expressed relative to the control (no heavy metal treatment) value. Bars represent mean values $\pm$ SD from three CT values of two independent experiments.

MT2 and *GR1* (Figure 1). The highest response of the *MT2*-like gene was at 80 μM (14 and 7 times to the control in roots and shoots, respectively). The highest levels of *GR1*-like protein transcript accumulation were detected at 160 μM (17 and 11-fold increases in the roots and shoots, respectively). The As(V) treat-ment, in general, induced greater transcript accumu-lation in roots compared to shoots. A bell-shaped curve of transcript accumulation for the three genes was apparent in roots, showing that higher metalloid concentrations may result in destabilization of cellular homeostasis.

In Cr(VI) hydroponically treated plants, the molecular response of *MT2*- and *GR1*-like genes led to lower levels of transcript accumulation compared to the As(V) treatment. However, the bell-shaped curve of transcript accumulation observed in As(V)-treated plants was not apparent when hydroponic cultures were supplied with Cr(VI).

A marked differential molecular response was evidenced between As(V)- and Cr(VI)-treated tomato plants. In our experiments, As(V) treatment induced *MT2*-like protein transcript accumulation mostly in roots of the tomato plants. The Cr(VI) treatment, on the contrary, did not change drastically the *MT2*-like protein transcript levels in roots or shoots. These data suggest that As(V) efficiently induced *MT2*-related tolerance mechanisms in tomato plants, but Cr(VI) stress did not. The well-characterized cysteine-rich polypeptides, MTs, can bind to heavy metals in the cytoplasm and sequester them into the vacuoles or out of the cells. Most heavy metals induce the synthesis of MTs, preventing the disruption of homeostasis due to environmental stress (DalCorso et al., 2008). This detoxification mechanism is particularly efficient in metallophyte plant tolerant varieties with high transcription rate of MTs (Hall, 2002). In tomato, type 2 metallothionein-like genes have been isolated previously (Whitelaw et al., 1997), and showed relatively specific expression in roots under heavy metal stress (Giritch et al., 1998). The massive accumulation of *MT2*-like protein transcripts in roots under As(V) stress might contribute to high As(V) concentration-tolerance of tomato plants.

The antioxidant enzyme, GR, has shown also differential responses under As(V) and Cr(VI) stress. The ascorbate–glutathione cycle key enzyme can protect the cell against oxidative damage, maintaining a high GSH/GSSG ratio (Schützendübel and Polle, 2002; Foyer and Noctor, 2005). Because As(V) induced the *GR1*-like protein gene, the antioxidant efficiency in tomato plants could be considered potentially high under As stress. It is conceivable that, in tomato plants, the highly generative ROS metal Cr(VI) (Panda and Choudhury, 2005) either did not stimulate non-enzymatic antioxidant metabolism involving glutathion within 24 h, or Cr(VI) may not influence GR synthesis. Alternatively, other non-GR mechanisms within the cell could prevent the toxic effects exerted by Cr.

As(V) induced the strongest molecular response in roots. Previous work has reported tomato as a plant model tolerant to As pollution, and suggested that the potential mechanism of tolerance developed by tomato plants could involve the limited upward As transport to the shoots (Carbonell-Barrachina et al., 1997). Upon As treatment, tomato plants accumulated As primarily in roots, and only relatively low quantities were translocated to shoots (Burló et al., 1999). Our report provides insight into the potential tolerance processes of tomato root tissues. Because *Hsp90-1*, *MT2*- and *GR1*-like protein transcripts all accumulated under As stress, a combinatorial type of tolerance mechanism related to protein damage repair, metal chelation and antioxidative metabolism could be effectively activated in tomato plants to provide protection against As toxicity. No visual symptoms of vegetative injury (chlorosis, wilting or necrosis) were observed in As-treated tomato plants throughout our experiments, and all plants tolerated even higher As concentrations (1280 μM) (Figure 2). As the *Hsp90-1*, *MT2*- and *GR1*-like transcripts accumulated higher in roots than in shoots, the As detoxification mechanisms in tomato plants might be effective enough *in situ* to minimize the impact of high As concentration of the hydroponic culture.

For Cr, the profile of the *Hsp90-1* transcript accumulation suggested that tomato plants respond efficiently to the heavy metal treatment. The induction was more marked in shoots, indicating that the upward Cr transport to the shoots was effective. However, the low accumulation levels of *MT2*- and *GR1*-like transcripts in root and shoots suggests that tomato plants might not develop molecular mechanisms allowing protection to Cr toxicity via the MT2 and GR pathways. The low but noticeable accumulation of *GR1*-like transcripts indicates that highly generative ROS metal Cr(VI) moderately stimulates the *GR1*-like gene. Because, in most plants, there are a number of GR isoforms, it is anticipated that transcript analysis of most GR isoforms would provide new insights in Cr detoxification via the GR pathway. Nevertheless, phenotypic symptoms of tomatoes supported this notion of tomato sensitivity to Cr(VI) (Figure 2). Cr (VI) treatment induced withered leaves and shoot necrotic areas leading to plant bending when high metal concentration was added to the hydroponic cultures (640 μM Cr(VI)). The highest concentration (1280 μM) was drastic with unrecovering withered plants (Figure 2). These lethal phenotypic symptoms could be the result of high ROS production induced by this redox metal, which might lead to cellular membrane alterations (Panda and Choudhury, 2005).

The authors agree with and adopt the definition of tolerance to heavy metal in plants as the ability to survive in a soil that is toxic to other plants, and manifested by an interaction between a genotype and its environment (Macnair et al., 2000; Yang et al., 2005). In this paper, we analyzed the molecular

Figure 2. Images of hydroponically cultivated tomato plants treated for 24 h in nutrient solutions supplemented with As(V) (A) or Cr(VI) (B) in concentration ranging from 0 to 1280 μM. Tomato plants were not affected by As(V) treatment. High concentrations of Cr(VI) induced stunting. Arrows point the brownish and necrotic shoot leading to plant bending.

response on tomato plants submitted hydroponically to unnatural concentrations of Cr and As. The no-metallophyte tomato plants behaved as a Cr-sensitive and As-tolerant since the threshold of metal phyto-toxicity could be reached with Cr but not with As in the range of metal concentration used.

Acknowledgments

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