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Expression analysis of three stress-related genes in response to excess of boron in *Solanum lycopersicum* cv Poncho Negro

Análisis de expresión de tres genes relacionados a estrés en respuesta a un exceso de boro en Solanum lycopersicum *cv Poncho Negro*

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ABSTRACT

Boron (B) is an essential micronutrient for higher plants, but is very toxic at higher concentrations. This study evaluated the transcriptional activity of three stress-related genes under conditions of excess B in *Solanum lycopersicum* cv Poncho Negro, a cultivar originating from the Lluta Valley (Arica, Chile), where it grows in environments with very high concentrations of B. Poncho Negro tomato seedlings were grown under hydroponic conditions and stressed with B (20 ppm); root and leaf samples were collected at 3 and 96 h after stress. The activity of three stress-related genes, *Hsp90*, *MT* and *GR*, was analyzed by quantitative reverse transcription PCR. A marked differential molecular response was observed between leaves and roots. The three genes were overexpressed in leaves in response to B, while in roots *Hsp90* decreased, *MT* increased and *GR1* did not change its activity. Activation of these genes may indicate participation of genes involved in stress oxidative processes as an early response to B stress and may represent a complementary mechanism to boron transport.

Key words: Boron tolerance, gene expression, Poncho Negro tomato.

RESUMEN

El boro (B) es un micronutriente esencial para la mayoría de las plantas, pero es muy tóxico a altas concentraciones. En éste trabajo se evaluó la actividad transcripcional de tres genes relacionados al estrés, en condiciones de exceso de B, utilizando el tomate Poncho Negro, un cultivar originario del valle de Lluta (Arica, Chile), el cual crece y se desarrolla en ambientes con concentraciones muy altas B. Plántulas de tomate Poncho Negro, crecidas en hidroponía, fueron estresadas con un exceso de 30 ppm de B y muestras de raíz y hoja fueron colectadas a las 3 y 96 h. Se analizó la actividad de tres genes relacionados al estrés Hsp90*,* MT *y* GR*, mediante de PCR en tiempo real. Una marcada diferencia en la respuesta molecular fue observada entre hojas y raíces. En hojas, los tres genes fueron sobre-expresados en respuesta al exceso B. En raíces, la actividad de* Hsp90 *disminuye,* MT *aumenta y* GR1 *no cambia su actividad bajo un estrés inducido por un exceso de B. La activación de estos genes puede indicar la participación de genes involucrados en procesos estrés oxidativo como una respuesta temprana a un estrés por B y puede representar un mecanismo complementario al transporte de B.*

Palabras claves*: Tolerancia a boro, expresión de genes, tomate Poncho Negro.*

Introduction

Boron (B) is an essential micronutrient required for plant growth and development; its availability in soil and irrigation water is an important determinant of agricultural production. Boron is present in soil solution in several forms; it is accepted that plants take up B from soil in the form of boric acid (Tanaka and Fujiwara 2008). B has the narrowest range of all plant nutrient elements between deficient and toxic soil concentrations. Both B deficiency and toxicity severely limit quality and crop production worldwide. South Australia, Iraq, Turkey, California and northern of Chile are some regions or countries with B toxicity problems in agricultural lands (Tanaka and Fujiwara 2008).

Typical B toxicity symptoms occur in the margin of mature leaves, and these portions become

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chlorotic or necrotic. Boron tends to accumulate in old leaves, especially at the margins (Tanaka and Fujiwara 2008). The physiological effects of B toxicity in plants include reduced root cell division, decreased shoot and root growth (Nable *et al.* 1990), decrease in leaf chlorophyll, inhibition of photosynthesis, lower stomatal conductance, deposition of lignin and suberin, reduced proton extrusion from roots, increased membrane leakiness, peroxidation of lipids and altered activity of anti-oxidation pathways (Reid 2007).

Depending on B availability, boric acid uptake and transport can be carried out by three different molecular mechanisms: i) passive diffusion across the lipid bilayer due to its uncharged property; ii) facilitated transport by the major intrinsic protein (MIP) channel and iii) an energy-dependent high-affinity transport system induced in response to low B supply, which is mediated via BOR transporters (Camacho-Cristóbal *et al.* 2008; Tanaka and Fujiwara, 2008). In addition to these transport mechanisms, the activation of other protection mechanisms that cope with toxic levels of B have been reported (Tombulogu *et al.* 2012). In tomato, some stress-related genes are expressed differentially in the presence of high B concentration: *heat shock protein90-1* (*HSP90-1*), a marker of environmental stress (Feder and Hofmann 1999); *metallothionein2* (*MT2*), which encodes a protein that binds to heavy metals (DalCorso *et al.* 2008) and *glutathione reductase1* (*GR1*), which encodes an enzyme that participates in the reduction of reactive oxygen species (ROS) (Schützendübel and Polle 2002), suggesting a role in the regulation of cellular homeostasis under high B level (Tombulogu *et al.* 2012).

Solanum lycopersicum cv. Poncho Negro is a local variety developed by the farmers of the Lluta Valley (Arica, Chile). The soils of the Lluta Valley have high levels of B and salinity, among other elements, which strongly limit crop production. Concentrations of B in the soil and irrigation water fluctuate between 4.0-20.0 and 12.4-16.0 ppm, respectively (Torres and Acevedo 2008). Under these conditions this cultivar grows successfully, suggesting that Poncho Negro tomato has an inherent tolerance to high levels of B. The objective of this study is to evaluate the activity of *HSP*90-1, *MT2* and *GR*1 genes in Poncho Negro tomato under stress induced by an excess of B.

Materials and methods

Plant growth and stress application

Tomato (*Solanum lycopersicum* cv. Poncho Negro) seeds were germinated in perlite under greenhouse conditions for 3 weeks. These seedlings were transferred to hydroponic culture with Hoagland's solution, which was renewed every 3 days. After 10 days of acclimatization, seedlings were exposed to 20 ppm of B for 3 and 96 h. Root and shoot samples from three plants were pooled, frozen in liquid nitrogen and immediately stored at -80 ºC until RNA isolation.

Isolation of RNA and synthesis of first strand cDNA

Total RNA from shoot and root tissues was isolated using the Trizol ® reagent (Invitrogen) according to procedures specified by the manufacturer. The extracted RNA was resuspended in 20 µl of RNase-free water and stored at -80 ºC. Integrity was verified by electrophoresis in 1% agarose gels. RNA was purified with the RNeasy ® MinElute™ Cleanup Kit (QIAGEN) according to procedures specified by the manufacturer. Then 2 µg of RNA were treated with DNase I in a final volume of 10 µl and incubated at 37 ºC for 1 hour. The reaction was stopped by adding 1 µl of EDTA (25 mM) at 65 ºC for 5 minutes.

The synthesis of the first strand of cDNA was performed as follows: to 2 µg of RNA treated with DNase I (Invitrogen) 3 µl oligo dT (10 pmol / µl) and 3 µl dNTP's (2.5 mM) were added in a final volume of 17 µl, which was incubated at 65 ºC for 5 minutes. Then 8 µl of 5x First Strand Buffer, 2 µl 0.1 M DTT, 1 µl RNasaOut (Invitrogen) and 1 ml SuperScript™ III RT (Invitrogen) were added in a final volume of 40 µl. Reverse transcription was performed in a thermocycler model NYX TECHNIK Amplitronyx™ 6, using the following program: 15 ºC for 10 minutes, 25 ºC for 16 min, 42 ºC for 60 min, 70 ºC for 10 min and 4 ºC final hold. The tubes were stored at -20 ºC until further use.

Design of primers

The primers used were obtained according to Tombuloglu *et al.* (2012). The expected sizes for

genes *Actin-7*, *Hsp90-1*, *MT2* and *GR1* were 166, 130, 207 and 183 bp, respectively.

Quantitative Reverse Transcription PCR (RT-qPCR) analysis

For quantification analysis, the genes of interest were amplified using the Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific) and real-time PCR was performed on an Eco Real-Time System (Illumina). Tomato *Actin* gene was used as housekeeping gene for normalization (Tombuloglu *et al.* 2012). The reaction conditions used were as follows: 95 ºC for 10 min, 40 cycles of 95 ºC for 15 sec, 60 ºC for 15 sec, 72 ºC for 15 sec, and an analysis of dissociation or melting of 55 to 95 ºC with a temperature increase of 0.3 ºC sec^{-1}.

The efficiency of the PCR reaction in real time was calculated for each of the transcripts used with 6 serial dilutions of cDNA, with an initial concentration of 32 ng and a dilution factor of 1:2. The efficiency was determined with the following formula: Efficiency (E) = $10^{(-1/slope)}$ -1.

Quantification and data analysis

Considering an efficiency of about 100% in all the reactions, the quantification method that was used to measure the relative changes in gene expression was $2^{-\Delta\Delta Ct}$, based on Livak and Schmittgen (2001). The RT-qPCR data were analyzed using a t-test between the average and 1, which was used as a reference value for no change in relative gene expression.

Results and discussion

Solanum lycopersicum var. lycopersicum is classified as a B-sensitive crop because it is able to grow in a range of 1-2 mgL^{-1} B in irrigation water (Mcfarland *et al.* 2014). *S. lycopersicum* cv. Poncho Negro, a Chilean germoplasm developed by local farmers of the Lluta valley, is adapted to soil and irrigation water whose B level reaches 4.0-20.0 and 12.4-16.0 ppm, respectively (Torres and Acevedo 2008) and could be considered as a B-tolerant germplasm (Bastías *et al.* 2011; Diaz *et al.* 2011). This is according to Bañuelos *et al.* (1999), who suggested that northern Chilean germplasms are better adapted to high B levels.

We examined the activity of *Hsp90-1*, *MT2* and *GR1* genes in roots and leaves of Poncho Negro tomato under stress induced by 20 ppm B; the expression patterns of these genes were analyzed by qRT-PCR. To verify the identity of the analyzed genes, PCR products for each gene were visualized on 2% agarose gels and were of the expected size range. Sequence analysis of the PCR products showed 100% identity with the expected gene product using NCBI BLAST analysis (Table I).

We measured expression at 3 and 96 h after B stress, because 3 h is enough time to trigger to the early stress response and after 72 h plants begin a period of acclimation to an abiotic stress, which implies that the early response genes decrease their activity and late response genes are expressed (Wang *et al.* 2003). Poncho Negro leaves did not show any visible symptoms after 96 h of B stress compared to control plants; there were no significant changes in plant height, and characteristic symptoms of B toxicity were not observed (data not shown).

The transcriptional activity of each gene was normalized with reference gene *Actin* and compared to the control (not stressed).

Hsp90 is one of the most abundant and highly conserved molecular chaperone proteins and is essential for viability in eukaryotes (Breiman 2014). Most of the client proteins of Hsp90 control multiple signal transduction pathways involved in hormone signaling, cell cycle control, differentiation and developmental processes and stress defense

Table I. Homologies of RT-qPCR amplified sequences.

Amplified Sequence	Length (bp)	Homology	E-value
Actin-7	166	*Solanum lycopersicum* actin-7-like (LOC101264601), mRNA.	2e-80
HSP90-1	130	*Solanum lycopersicum* molecular chaperone Hsp90-1 (Hsp90-1), mRNA.	1e-60
MT2	207	*Solanum lycopersicum* metallothionein-like protein (LOC778298), mRNA.	2e-10
GR1	183	*Solanum lycopersicum* cytosol glutathione reductase, transcript variant 1 (LOC100301935), mRNA.	7e-90

(Taipale *et al*. 2010). In *Dunaliella salina*, a dshsp90 was up-regulated by heat and salt stress (Wang *et al*. 2012). In rice, rHsp90 increased its activity in the presence of several stresses including salt, desiccation and high temperature. Transgenic tobacco seedlings over-expressing this *rHsp90* showed tolerance to high salt concentrations (Liu *et al*. 2006). Its activity is also induced by metals such as cadmium, chromium and arsenic (Goupil *et al*. 2009), confirming its important roles in multiple environmental stresses.

A marked differential molecular response was observed between leaves and roots after B stress. In our experiments, Poncho Negro tomato stressed with 20 ppm B showed an increase of *Hsp90-1* transcript accumulation in leaves of almost three times within 3 h after stress; this activity was maintained for at least 96 h. However, in the root this activity decreased significant over time (Figure 1A and 1D). Similar results were observed in tomato cv Oturak exposed to B ranging from 80 to 5120 µm for 24 h (Tombuloglu *et al*. 2012).

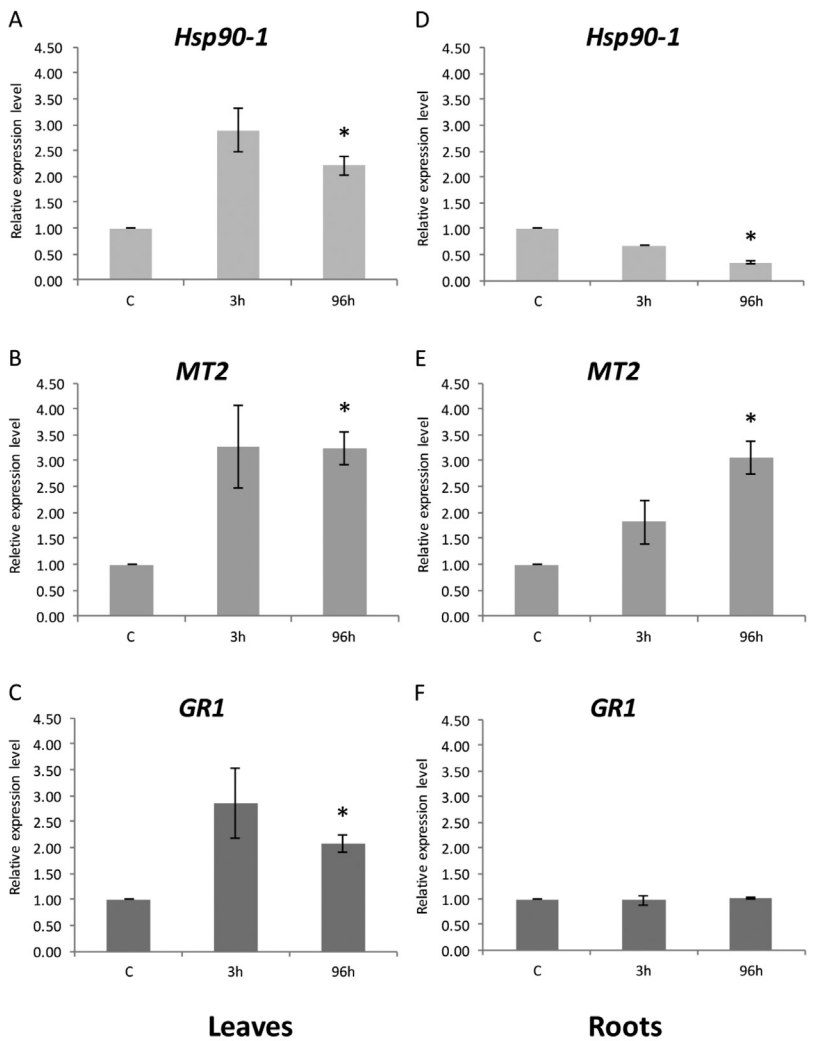

Figure 1. **Relative expression of tomato stress genes in response to B toxicity.** We measured the activity of the *Hsp90-1*, *MT2* and *GR1* genes by RT-qPCR in leaf and root samples of Poncho Negro tomato stressed with 20 ppm B. Samples were collected at 3 and 96 h after stress. The *Actin* gene was used for normalization of gene expression. The values are presented as average and standard error (SE) of three biological replicates and three technical replicates. The asterisks represent significant differences in transcript accumulation at 3 and 96 h after stress compared to the control (C). (*) P<0.05.

Our results indicate that the induction of *Hsp90-1* in leaves can induce a mechanism that prevents protein damage and cell homeostasis destabilization exerted by B. Other non-*Hsp90-1* mechanisms could probably carry out these functions in roots.

Metallothioneins (MTs) are cysteine-rich proteins of low molecular weight with many attributed functions; their most important role is in tolerance to the metals Zn, Cu and Cd (Lv *et al.* 2012), providing protection against oxidative stress (Hall 2002). The mechanism for metal tolerance in plants is most likely the result of the ability of MTs to maintain the homeostasis of essential metal ions, detoxifying heavy metals or scavenging reactive oxygen species (ROS) (Guo *et al.* 2008). MTs also play important roles in alleviation of salinity and drought stress (Zhou *et al.* 2012). In B-treated Poncho Negro tomato, the *MT2* gene in leaves significantly tripled its activity at 3 h compared to the control, and this expression pattern was maintained at 96 h (Figure 1B). In roots, *MT2* gene activity increased two-fold at 3 h after B stress, and this expression pattern continued to increase, reaching over three-fold at 96 h (Figure 1E). Similar results were observed in tomato plants, in which *MT2* accumulation increased in shoot and root over 80 μM B, and then declined above 320 μM (Tombuloglu *et al.* 2012). Our results suggest that Poncho Negro tomato detects B-stress and activates mechanisms that alleviate the cell damage induced and preserve cell homeostasis in roots and leaves. The presence of a high concentration of arsenic (As) also activates *MT2* expression in roots and leaves of tomato (Goupil *et al.* 2009). The presence of high concentrations of As in irrigation water (0.1 - 0.6 mg/lt) and soil (197.5 - 254.7 μg/g) (DGA, 2004) in the Lluta Valley allow us to suggest that Poncho Negro tomato may also be able to tolerate As stress.

Glutathione reductase (GR) is one of the important antioxidant enzymes in plants. This enzyme catalyzes the reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH) with the accompanying oxidation of NADPH. GR plays an essential role in the defense system against ROS, replenishing the reduced glutathione pool; this activity is crucial for plant tolerance to a variety of stresses (Gill and Tuteja 2010). It has also been reported that GR activity increases in the presence of cadmium, arsenic and chromium stress (Gill and Tuteja 2010; Tombuloglu *et al.* 2012). In our system, the *GR1* expression in leaves doubled its activity within 3 h in relation to the control and this behavior was maintained up to 96 h after stress (Figure 1C). However, in roots its activity was not altered in presence of an excess of B up to 96 h (Figure 1F). The activity of GR is primarily associated with chloroplasts; probably for this reason we did not detect any change in its expression in the root, or a separate mechanism may be responsible of the B tolerance in this tissue. The activation of the *GR1* gene in leaves suggests its participation in cell damage protection under B-induced oxidative stress.

Conclusion

The transcriptional activity of three stress-related genes *Hsp90-1*, *MT2* and *GR1* in Poncho Negro tomato is altered in the presence of high B concentration. Activation of these genes may indicate participation of oxidative stress genes in the early response to B stress and may represent a complementary mechanism to the boron transport mechanisms, maintaining the mechanisms that regulate cellular homeostasis in the presence of excess of boron. Poncho Negro tomato could be a good system to study how crops have managed to adapt under extreme conditions.

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