

Grafting response to excess boron and expression analysis of genes coding boron transporters in tomato

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ABSTRACT

Boron (B) is essential for plant growth, however its excess in soil and/or in irrigation water can severely compromise plant growth and yield. The goal of this work was to determine whether grafting onto ‘Arnold’, a commercial interspecific hybrid (*Solanum lycopersicum* x *S. habrochaites*) rootstock, which in a previous study was found to be tolerant to salt stress, could improve tomato (*S. lycopersicum* L. ‘Ikram’) tolerance to excess B, and whether this effect is associated with an exclusion mechanism. Non-grafted, self-grafted and grafted plants were hydroponically grown in a greenhouse with B concentration in the nutrient solution of 0.27 (control), 5, 10 and 15 mg L⁻¹). A transcription analysis was carried out on *SINIP5* and *SIBOR1* genes, which encode putative B transporters. Grafting ‘Ikram’ onto ‘Arnold’ rootstock reduced B concentration in leaf tissue of plants exposed to B concentrations of 10–15 mg L⁻¹. At high B levels, *SINIP5* was down-regulated in all grafting combinations, while *SIBOR1* was down-regulated only in the roots of plants grafted onto ‘Arnold’. We conclude that grafting the susceptible tomato cultivar ‘Ikram’ onto the commercial rootstock ‘Arnold’ improved tolerance to excess B by reducing expression of genes encoding for B transporters at the root level, thus partially reducing the root uptake of B and its accumulation in the shoot.

Keywords: boron transporters; rootstock; *SIBOR1*; *SINIP5*; *Solanum lycopersicum* x *Solanum habrochaites*; vegetable grafting.

INTRODUCTION

Boron (B) is an essential micronutrient for plants, as it is an important constituent of cell walls (Matoh 1997). Boron is also thought to play a role in membrane function by complex formation with glycoproteins (Goldbach & Wimmer 2007), and indirectly in the metabolism of many substances such as nucleic acids, proteins, carbohydrates, plant-growth regulators and phenols (Shaaban 2010). On the other hand, plant growth and crop yield may be severely limited by excess B (Nable et al. 1997). Exposure to excess B causes alteration of the cell wall structure, and reduction of root cell division, leaf chlorophyll content and CO₂ fixation (Nable et al. 1997; Guidi et al. 2011). Boron contamination of both soil and groundwater is a major issue in many arid and semi-arid regions of the world, including coastal areas of the Mediterranean Basin (Nable et al. 1997). Soil and groundwater B contamination occurs primarily through natural processes such as seawater intrusion along coastal areas, volcanic and geothermal activity, or weathering of B-rich rocks (Vengosh et al. 2004). Boron contamination may also arise from anthropogenic activities: industrial wastes, sewage and sludge run-off, over-fertilisation, irrigation with desalinated water or recycled municipal wastewater, often characterised as having high B concentrations (Venturi et al. 2015).

Remediation of B-toxic soils is impractical in most cases. In the last decades, much effort has been made to identify and select plant genetic resources tolerant to B toxicity, and to investigate the major mechanisms of tolerance to excess B. However, conventional breeding programmes have failed to provide high-yielding cultivars tolerant to excess B (Reid 2010). Plant tolerance to B is generally associated with the ability of the plant to limit B uptake and accumulation in leaf tissues (Nable et al. 1997). Boron-tolerant plants can regulate the accumulation of B in roots cells by limiting its uptake or enhancing its efflux (Hayes & Reid 2004; Sutton et al. 2007); this suggests a pivotal role of roots in B tolerance. However, the molecular mechanisms responsible for B tolerance are still not entirely clear. Several studies have revealed that B uptake by roots from the soil solution is promoted by both a channel-mediated diffusion and the presence of energy-dependent active transporters (Takano et al. 2008). In *Arabidopsis thaliana*, for instance, two types of B transporter were identified, NIP5;1 and BOR1, both of which play an important role in B transport under conditions of B deficiency. The protein NIP5;1 belongs to the aquaporin family and primarily functions as a boric acid channel for B uptake under B limiting conditions (Takano et al. 2006). In *A. thaliana*, BOR1 contributes to the xylem loading of B and its activity increases under B limitation and rapidly decreases when plants are exposed to high B levels (Takano et al. 2008).

Among vegetable crops, tomato is considered one of the most tolerant species to excess B, as it can tolerate exposure to B concentrations up to 4–6 mg L⁻¹ (Alpaslan & Gunes 2001).

Therefore, tomato may constitute a good crop candidate to maintain productive B-rich soils.

Given the importance of the root system in the mechanism of B tolerance, grafting could represent an alternative and more rapid approach to conventional breeding and selection of B-tolerant cultivars in herbaceous plants. Grafting commercial cultivars of vegetable species onto vigorous rootstocks can improve crop productivity under stress conditions, including excess B (Schwarz et al. 2010; Di Gioia et al. 2013). For instance, Edelstein et al. (2007) found that melon plants grafted onto *Cucurbita maxima* Duchesne x *Cucurbita moschata* Duchesne rootstock ‘TZ-148’ accumulated less B in the shoot and provided higher fruit yield than non-grafted melons exposed to similar B levels (0.1–10.4 mg L⁻¹) in the irrigation water. The lower accumulation of B and the higher fruit yield observed in grafted plants was attributed to a higher selectivity and lower absorption of B by the rootstock as compared to the non-grafted plants.

As B-tolerant genotypes seem to also have a higher tolerance to other types of salt stress (Edelstein et al. 2016), in the present study, we hypothesised that grafting tomato onto vigorous interspecific hybrids (*S. lycopersicum* x *S. habrochaites*), which have been demonstrated to be tolerant to salinity stress (Di Gioia et al. 2013), might raise tomato tolerance to excess B. Therefore, we investigated how grafted, self-grafted and nongrafted tomato plants, grown in hydroponic culture, respond to excess B (up to 15 mg L⁻¹) in terms of growth, B uptake and its partitioning in plant tissues. The expression and molecular regulation in roots, leaf blade and petiole tissues of two genes coding for a tomato boric acid channel (*SINIP5*) and a boric acid/borate exporter (*SIBORI*) were also analysed.

MATERIAL AND METHODS

Plant material and growth conditions

The experiment was conducted between September and October 2012 at Mola di Bari, Italy (41°03' N, 17°40' E; 24 m a.s.l.) in a polymethacrylate greenhouse. Tomato cultivar ‘Ikram’ (Syngenta Seeds, Greensboro, NC, USA), which in previous studies proved to be more susceptible than others to B excess (Princi et al. 2016), was chosen as the scion. Seedlings of ‘Ikram’ were grown in peat-filled plastic trays under greenhouse conditions. Fourteen days after sowing, when the plants had two true leaves, they were either self-grafted or grafted onto ‘Arnold’, an inter-specific hybrid (*S. lycopersicum* x *S. habrochaites*) rootstock (Syngenta Seeds). Grafting was performed using the splice-tube method (Di Gioia et al. 2010). The seeds of the rootstock were sown 5 days before those of the scion. Fourteen days after grafting, non-grafted, self-grafted and grafted plants were

transplanted into 4.5-l pots containing perlite (Agrilit n. 3, Perlite Italiana, Corsico, Milano, Italy), which were placed in the greenhouse with a density of 3.4 plantsm².

Plants were drip-irrigated with a nutrient solution, prepared with tap water and 10.7 mM NO₃, 1.6 mM H₂PO₄, 6.1 mM K, 1.9 mM Mg, 3.0 mM Ca, 2.9 mM SO₄, 20 uM Fe, 5 uM Mn, 2 uM Zn, 0.5 uM Cu and 0.1 uM Mo, resulting in an electrical conductivity (EC) of 1.8 dS m⁻¹. Boron was added at different concentrations ranging from 25 to 1388 uM, using boric acid. When needed, the pH of the nutrient solution was adjusted to 5.6 with sulphuric acid. Irrigation frequency and volume were adjusted to maintain a drainage percentage between 40% and 80% in order to minimise the variation of EC, pH and nutrient concentration in the substrate. During the experiment, daily air temperature in the greenhouse averaged 24.6 °C, ranging from 12.0 °C to 44.8 °C. Daily relative humidity was on average 81% and ranged between 60% and 96%. The daily light integral ranged from 3.0 mol m⁻² day⁻¹ to 15.9 mol m⁻² day⁻¹ and averaged 10.5 mol m⁻² day⁻¹.

Experimental design

The following B concentrations were tested: 25 uM (0.27 mg L⁻¹), 463 uM (5 mg L⁻¹), 925 uM (10 mg L⁻¹) and 1388 uM (15 mg L⁻¹). Control plants were grown at 0.27 mg L⁻¹ B, which is an optimal concentration for most species grown in hydroponic culture (Sonneveld & Voogt 2009). Boron levels were differentiated 1 week after transplanting. Treatments were arranged in a split-plot experimental design with three replicates, with the B levels arranged in the main plots and grafting combinations in the sub-plots. Each experimental unit consisted of seven plants. The treatments lasted 4 weeks.

Plant determinations

At the end of the experiment, 28 days after treatment initiation, one plant was sampled from each experimental unit for growth measurements and chemical analyses. Sampled plants were separated into roots, stems and leaves to determine their biomass (dry weight, DW). Bottom (older) leaves (i.e. the first ten leaves) were separated from the top (younger) leaves. Plant samples were dried in a ventilated oven at 65 °C until reaching a constant weight. Leaf area was determined with a digital planimeter (LI-COR 3100 Leaf Area Meter; LI-COR, Lincoln, NE, USA). Leaf B concentration was measured on 0.5 g dry samples. Samples were digested with 2.5 mL nitric acid (HNO₃) and 1 mL perchloric acid (HClO₄) at 230 °C for 2 h, and determined colorimetrically using the azomethine-H method (Wolf 1974) from absorbance measurement at 420 nm taken using a T60

UV-Visible spectrometer (PG Instruments, Leicester, UK). Root samples were oven-dried overnight at 100 °C, weighed and finely ground with a mortar and pestle. Dried samples of 0.1 g were digested in solutions containing trace-metal grade concentrated HNO₃ and H₂O₂, and digested in a microwave digestion system, Milestone MLS 1200 MEGA (FKV, Sorisole, BG, Italy). After cooling, deionised water was added and the solution was filtered through a Whatman filter paper 40 into a 25 mL volumetric flask. The obtained volume of filtrate was brought to 25 mL using deionised water. Root B concentration was determined by ICP-MS (Brown et al. 1992). Tissue B concentrations were expressed as mgkg⁻¹ of DW. On the same day, leaf blades, petioles and roots of another plant per experimental unit were sampled with sterilised scissors and were stored at 80 °C for gene expression analysis.

Extraction of RNA and cDNA synthesis

Total RNA of all tissue samples and treatments was extracted using Trizol (Life Technologies, Carlsbad, CA, USA) reagent according to the manufacturer's instructions. Extracted RNA was dissolved in RNase-free water and stored at 80 °C. RNA concentration was calculated using the Qubit fluorometric system (Life Technologies). The first strand of cDNA was synthesised using 2 lg total RNA, oligo (dT) primer and TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's standard protocol. The reverse transcriptase was heat-inactivated at 95 °C for 5 min.

Isolation of NIP and BOR genes from *S. lycopersicum*

To obtain cDNAs coding putative tomato B transporters, degenerate primers were designed, based on highly conserved regions of other plant NIP and BOR genes, and were used to amplify cDNA fragments using PCR. The 5' and 3' terminal regions of partial cDNAs were obtained using the SMARTer Race 5'/3' kit (Clontech, Takara Bio, Mountain View, CA, USA) following the manufacturer's instructions. All products were cloned into pCR4-TOPO vector (Life Technologies) according to manufacturer's instructions and both strands were sequenced. Full-length cDNAs were obtained that had high homology to *A. thaliana* genes and named SINIP1-1-like, *SINIP5*, *SINIP6*, *SIBOR1* and *SIBOR2*.

Sequence alignment and phylogenetic analysis

To study the phylogenetic relationship of tomato BORs with Arabidopsis, wheat and rice B transporter homologues, full length amino acid sequences were aligned with ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), utilising a protein weight matrix BLOSUM (BLOCKS SUBstitution Matrix). A phylogenetic tree, based on BOR family members from *A. thaliana*, rice, wheat and *S. lycopersicum* was constructed through MEGA7 (Molecular Evolutionary Genetics Analysis, version 7.0; Kumar et al. 2016) using the maximum parsimony (MP) method. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. The analysis involved 12 amino acid sequences. All position containing gaps and missing data were eliminated. Bootstrap support for individual branches of the tree was obtained using 100 bootstrap replicates.

Gene expression

The PCR primers for qPCR were designed using Primer Express 3.0 (Applied Biosystem; Table 1). Real-time PCR was performed with SYBR green fluorescence detection in a real-time PCR thermal cycler (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems). The PCR mix was prepared using 100 ng cDNA, 12.5 ul Power SYBR Green RT-PCR Master mix (Applied Biosystems), 1.0 uM forward and reverse primers, in a total volume of 25 ul. The cycling conditions were: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analysis was performed after PCR to evaluate the presence of non-specific PCR products and primer dimers. The real-time PCR data were plotted as the ΔR_n fluorescence signal versus the cycle number. The ABI PRISM 7900HT Sequence Detection System software calculated the ΔR_n using the equation:

$$\Delta R_n = (R_{n+}) - (R_{n-}) \quad [1]$$

where R_{n+} is the fluorescence signal of the product at any given time and R_{n-} is the fluorescence signal of the baseline emission during cycles 6–13. An arbitrary threshold was set at the midpoint of the log ΔR_n versus cycle number at which the ΔR_n crosses the threshold (CT = cycle threshold). The efficiency (E) of the target amplification was evaluated for each primer pair and the corresponding value was used to calculate the fold changes (FC) with the following formula:

$$FC = (1 + E)^{-\Delta\Delta CT} \quad [2]$$

where:

$$\Delta\Delta CT = \text{Treatment } (CT_{\text{target gene}} - CT_{\text{ref.gene}}) - \text{Control } (CT_{\text{target gene}} - CT_{\text{ref.gene}}) \quad [3]$$

The FC values were separately computed for each grafting treatment and the CT data were expressed as the average of three experimental replicates.

In this work, eight consistently expressed genes were used as potential reference genes. They encode for actin (*ACT*; TC194780), b-tubulin (*b-TUB*; Q205342), elongation factor 1a (*EF1*; TC65250), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; TC198136), phosphoglycerate kinase (PGK; TC203809), ribosomal protein L2 (*RPL2*; X64562), ubiquitin (*UBI*; TC193502) and a catalytic subunit of protein phosphatase 2 (*PP2A*; AY325817). The coefficients of variation (CV = ratio between the SD and the mean) based on qRT-PCR data were: $CV_{actin} = 0.025$; $CV_{EF} = 0.058$; $CV_{GAPDH} = 0.259$; $CV_{PGK} = 0.107$; $CV_{PP2A} = 0.049$; $CV_{RPL} = 0.065$; $CV_{tubulin} = 0.041$; $CV_{ubiquitin} = 0.115$. Therefore, among the eight putative reference genes, only *actin*, *PP2A* and *tubulin* had CVs < 0.05. In the present work, *actin* was selected as reference gene since it had the lowest CV.

Statistical analysis

Plant growth, root and leaf B concentration data were subjected to two-way ANOVA. Mean values were separated by Duncan's multiple range test ($P = 0.05$). Statistical analysis was performed using the GLM procedure in SAS version 9.4 (SAS Institute, Cary, NC, USA). Expression data were considered biologically relevant when a difference between the control and one of the treated samples was >2 or <0.5 FC and statistically relevant when the $P < 0.05$. The P-value was calculated using the parametric paired t-test and adjusted with the Benjamini-Hochberg FDR (false discovery rate) method.

RESULTS

Plant growth

Leaf area and shoot DW were influenced by both B level and grafting combination, and no interaction was observed between the two factors (Fig. 1). Plants grown with 5, 10 and 15 mg L⁻¹ B showed a reduction in leaf area of 9.3%, 29.5% and 30.6%, respectively, as compared to plants grown with 0.27 mg L⁻¹ B (control). Shoot DW decreased on average 21% in plants grown at 10 and 15 mg L⁻¹ B as compared to the control. Shoot DW was not different in plants grown with 0.27 and 5 mg L⁻¹ B, between plants grown at 5 and 10 mg L⁻¹ B, and between those grown at 10 and 15 mg L⁻¹ B. Plants grafted onto 'Arnold' had higher leaf area and shoot DW than nongrafted and self-grafted plants.

Tissues B concentration

Root B concentration was not influenced by grafting combination, while it was affected by the level of B applied in the nutrient solution (Fig. 2). Root B concentration did not differ in plants exposed to 0.27, 5 and 10 mg L⁻¹ B in the nutrient solution, and was on average 28.1 mg kg⁻¹ DW. Whereas roots of plants grown at the higher B level (15 mg L⁻¹) had a significantly higher concentration of B, on average 42.8 mg kg⁻¹ DW across grafting combinations. Apical leaves accumulated less B than bottom leaves, on average, younger leaves accumulated 45.9% less B than older leaves, regardless of grafting combination and B concentration in the nutrient solution (Fig. 2). Leaf B concentration of bottom (older) and apical (younger) leaves increased with increasing B concentration and was significantly lower in plants grafted onto ‘Arnold’ than in non-grafted and self-grafted plants (Fig. 2). A significant interaction was observed between B level and grafting combination. Plants grafted onto ‘Arnold’ accumulated much less B in older and younger leaves than the other plants at the highest B levels (10 and 15 mg L⁻¹), while similar B concentrations were found in all grafting combinations with 0.27 and 5 mg L⁻¹ B in the nutrient solution (Fig. 2).

Cloning and sequence analysis of tomato NIP and BOR genes

Degenerate oligonucleotide primers designed from highly conserved regions of plant B transporters were used to obtain tomato full-length cDNAs encoding putative B transporters. This led to isolation of three cDNAs coding for putative aquaporins, SINIP1-1-like, *SINIP5* and *SINIP6* and two cDNAs for putative BOR transporters, *SIBOR2* and *SIBOR1*. All resulting amino acid sequences were compared with plant databases through the NCBI database (<http://www.ncbi.nlm.nih.gov>) and Solgenomics network (<http://solgenomics.net>).

Protein–protein BlastP and multiple alignment analysis showed that the deduced amino acid sequences had high similarity with NIP and BOR sequences from other plant species, suggesting they belong to the NIP and BOR families.

In particular, *SIBOR1* amino acidic sequence showed a similarity of 79% with AtBOR2 and 75% with AtBOR1, *SIBOR2* showed 77% similarity with AtBOR2, 72% with AtBOR1 and only 61% with AtBOR4. The phylogenetic analysis with wheat, rice and Arabidopsis sequences showed two main clades, and *SIBOR2* was grouped with AtBOR4 and OsBOR4, while *SIBOR1* was grouped with three wheat genes TaBOR.1.1, TaBOR1.2, TaBOR1.3 and rice OsBOR1 (Fig. 3).

Gene expression

Among the five genes analysed in root, leaf blade and petiole tissues of plants non-grafted, self-grafted and grafted onto ‘Arnold’ grown at different B levels, only *SIBOR1* and *SINIP5* showed a significant variation of expression in response to B treatments. In all grafting combinations, at different B levels, *SIBOR2*, *SINIP1-1-like* and *SINIP6* were not differentially expressed in roots, leaves or shoots (data not shown).

SINIP5

Tomato *SINIP5* was significantly down-regulated by B treatments. The roots of plants grafted onto ‘Arnold’ or self-grafted showed low levels of *SINIP5* transcripts when exposed to 5, 10 and 15 mg L⁻¹ B (Fig. 4). In non-grafted plants the down-regulation was not significant. In petioles of plants grown at 10 and 15 mg L⁻¹ B, *SINIP5* was down-regulated regardless of the grafting combination. In the petioles of non-grafted plants, *SINIP5* was also down-regulated at 5 mg L⁻¹ B. In leaf blades, *SINIP5* was down-regulated in all grafting combinations when exposed to excess B, except for plants grafted onto ‘Arnold’ at 5 mg L⁻¹ B. Moreover, the expression level of *SINIP5* in roots was higher than in petioles (FC = 2.7 ± 0.2) and in leaves (FC = 3.1 ± 0.3).

SIBOR1

In the roots of plants grafted onto ‘Arnold’, *SIBOR1* was downregulated with 5 mg L⁻¹ B and its expression decreased with increasing B level (Fig. 5). In contrast, *SIBOR1* was not differentially expressed in non-grafted and self-grafted plants grown at 5 mg L⁻¹ B. In non-grafted plants, the expression of *SIBOR1* decreased only at the highest B level. In petioles, *SIBOR1* was more than two-fold down regulated in plants grafted onto ‘Arnold’, non-grafted and self-grafted grown at 15 mg L⁻¹ B. Moreover, *SIBOR1* was more than two-fold down-regulated in non-grafted plants grown at 5 and 10 mg L⁻¹ B (Fig. 5). In leaf blades the *SIBOR1* transcript decreased only in plants grafted onto ‘Arnold’ grown at 10 and 15 mg L⁻¹ B (Fig. 5). As observed for *SINIP5*, *SIBOR1* also showed a higher level of expression in roots than in petioles and leaves (FC = 2.5 ± 0.2 and FC = 3.4 ± 0.3, respectively).

DISCUSSION

Grafting tomato plants on ‘Arnold’ rootstock stimulated dry matter accumulation and leaf expansion, regardless of the level of B supplied, and enhanced tolerance to excess B by reducing leaf B accumulation in the presence of 10 and 15 mg L⁻¹ B (Figs 1 and 2). Similar results were

obtained on melon grafted on pumpkin rootstock ‘TZ-148’ (Edelstein et al. 2007, 2011). The lower growth performance of self-grafted plants as compared to plants grafted onto ‘Arnold’ suggests also that grafting itself does not improve tomato plant growth and tolerance to excess B, but the rootstock plays an important role.

The similar dry biomass observed in non-grafted and grafted tomato shoots exposed to 0.27 and 5 mg L⁻¹B (Fig. 1) confirmed that tomato itself is characterised by a high tolerance to B toxicity (Nable et al. 1997). Tomato shoot dry biomass was not significantly affected at 5 mg L⁻¹B, while a strong reduction of shoot DW and leaf area was observed at 10 and 15 mg L⁻¹ B. The reduction in dry matter accumulation was likely the result of lower leaf expansion (Fig. 1) and less photosynthesis (Edelstein et al. 2011; Guidi et al. 2011). No significant differences were found between plants grown at 10 and 15 mg L⁻¹ B and this was likely due to the relatively short period (28 days) of exposure to toxic B levels.

The lower B concentration observed in both bottom and top leaves of plants grafted onto ‘Arnold’ compared to the other grafting combination when grown at 10 and 15 mg L⁻¹ B, cannot be explained by a simple dilution effect. It suggests instead that ‘Arnold’ roots could enhance the tolerance to excess B, probably through an exclusion mechanism, in agreement with previous findings in other species (e.g. wheat and barley, Nable et al. 1997; *A. thaliana*, Tanaka & Fujiwara 2008; melon, Edelstein et al. 2011). Nevertheless, based on analysis of the root B concentration, no differences were observed between any of the grafting combinations tested (Fig. 2). Although an exclusion of B at root level cannot be dismissed, B exclusion was not influenced by the grafting combination, and thus could not explain the lower B concentrations observed in lower and apical leaves of plants grafted onto ‘Arnold’. Such results suggest that the ability of grafted plants to accumulate less B in the leaves may rather be associated with a reduced transfer of B from root to shoot or to B recirculation within the plant. Di Gioia et al. (2013) demonstrated that tomato plants grafted onto ‘Arnold’ were able to tolerate salinity stress better than non-grafted plants as a result of regulation of Na⁺ partitioning within the shoot. Plants grafted onto ‘Arnold’ accumulated more Na⁺ in the older leaves, thus reducing exposure of the younger and most actively growing leaves to toxic levels of this ion. Di Gioia et al. (2013) hypothesised that plants grafted onto ‘Arnold’ have a greater ability to recirculate Na⁺ via the phloem and accumulate it in less metabolically active plant tissues such as the older leaves. Some species can redistribute B within the plant under conditions of B deficiency (Tanaka & Fujiwara 2008), while passive transport via the xylem seems to be predominant when plants are exposed to excess B (Tanaka & Fujiwara 2008). The higher B concentration consistently observed in the bottom leaves as compared to the top leaves is likely due to the difference in

cumulative transpiration among leaves of different ages, supporting the hypothesis that, under conditions of excess B, transport in the shoot occurs mainly via the xylem. Similar results were found on tomato (Guidi et al. 2011) and on other species in which B is phloem-immobile, such as strawberry (Brown et al. 1999) and basil (Pardossi et al. 2015). These findings suggest that the phloem mobility of B is very limited in tomato. Based on these results, the higher B tolerance of plants grafted onto ‘Arnold’ is most likely due to an exclusion mechanism at the root level, combined with restricted translocation from root-to-shoot, rather than B translocation within the shoot, as observed for Na⁺. An exclusion mechanism at the root level was described for *A. thaliana* grown under conditions of excess B, and it was demonstrated that channel proteins (NIP5) and active efflux pumps (BOR1) play an active role in B absorption and translocation within the plant (Takano et al. 2008).

In this study, *SIBOR1* and *SIBOR2* were identified as *Solanum* genes coding B efflux transporters. The phylogenetic analysis showed that the *SIBOR1* is more similar to rice OsBOR1 and three wheat BOR1s (TaBOR1.1, TaBOR1.2, TaBOR1.3; Fig. 3) than to AtBOR1. These differences in gene phylogenetic relationships are likely to be a reflection of different B transport systems, requirements and regulation in different plants. *SIBOR2* was not regulated by B status in any grafting combinations (data not shown), and its expression pattern was not consistent with the up-regulation of *AtBOR4*, coding for efflux type B transporters involved in B exclusion at root level, as observed by Miwa et al. (2014). The putative *SINIP5* and *SIBOR1* were differentially regulated in roots, petioles and leaf blades of grafted, self-grafted or non-grafted tomato plants (Figs 4 and 5). *SINIP5* expression was very sensitive to B concentration (Fig. 4). The consistent *SINIP5* down-regulation observed at high B levels in roots, petioles and leaf blades of all of the grafting combinations tested leads to the hypothesis that this channel could play a key role in tomato response to B excess. Nevertheless, these results could not explain the lower B accumulation of plants grafted onto ‘Arnold’ as compared to those self-grafted and non-grafted. Moreover, it is not possible to speculate whether the lower expression of *SINIP5* is a component of the molecular response of the tomato plant to excess B, or *SINIP5* is down-regulated simply as a result of the high B cellular concentrations.

Similarly, in barley (*Hordeum vulgare* L.), differential expression was measured in aquaporin transcripts upon excess B treatment. The reduced expression of HvNIP2;1 was determinant in limiting B uptake, and thus increased barley tolerance to excess B, along with the increased expression of HvBot1 to remove B from roots and sensitive tissues (Schnurbusch et al. 2010). In more detail, HvNIP1;1 was highly up-regulated in barley roots and, in contrast, down-regulated in

the leaf blades, indicating that plasma membrane and vacuoles play a role in the B cellular concentration. In addition to HvNIP2;1, expression of HvNIP2;2 was reduced, contributing to tolerance to excess B (Tombuloglu et al. 2016). The differential expression level of aquaporins in roots and leaf blades may be involved in B uptake and partitioning within the plant tissues. The slightly different expression of *SINIP5* observed in non-grafted and self-grafted plants in roots in the presence of 5 and 10 mg L⁻¹ B did not result in a different concentration of B in the roots and/or leaves (Figs 2 and 4). The higher level of down-regulation of *SINIP5* observed in roots of self-grafted plants compared to non-grafted plants grown at 5 and 10 mg L⁻¹ B, may be attributed to an effect of the grafting per se, as the same level of down-regulation was observed in plants grafted onto

‘Arnold’.

In the case of the efflux-type B transporter *SIBOR1*, plants grafted onto ‘Arnold’ consistently decreased the expression of *SIBOR1* with increasing B levels in roots, leaf blades and petioles. While in non-grafted and self-grafted plants, *SIBOR1* was down-regulated only in the petioles. In the case of non-grafted plants, *SIBOR1* was down-regulated in the roots, but only when exposed to 15 mg L⁻¹ B. Such results suggest that the interspecific hybrid rootstock ‘Arnold’ increases tomato tolerance to excess B, at least in part, by limiting translocation of B from the root to the shoot through the xylem, and by decreasing *SIBOR1* expression in the roots already in the presence of 5 mg L⁻¹ B. Such results are consistent with the lower accumulation of B observed in bottom and apical leaves of plants grafted onto ‘Arnold’ and suggest that the tomato *SIBOR1* gene is regulated at the transcriptional level. The down-regulation of *SIBOR1* observed in roots of non-grafted plants grown with 15 mg L⁻¹ B did not result in a lower concentration of B in the leaves, as observed in plants grafted onto ‘Arnold’ (Figs 2 and 5). This was not consistent with the level of expression observed in roots of self-grafted plants. This may be explained by the ability of ‘Arnold’ to limit B accumulation already in presence of 5 mg L⁻¹ B, while in non-grafted plants *SIBOR1* was down-regulated only at the highest B level.

Leaunghitikanachana et al. (2013) working on wheat (*Triticum aestivum* L.), identified three genes (*TaBOR1.1*, *TaBOR1.2*, *TaBOR1.3*) encoding functional efflux-type B transporters, the mRNA accumulation of *TaBOR1* was regulated by B status, and the patterns were different from previously characterised genes *AtBOR1* (Takano et al. 2008) and *OsBOR1* (Nakagawa et al. 2007). In wheat *TaBOR1.1* and *TaBOR1.3* transcript accumulation were reduced under excess B (Leaunghitikanachana et al. 2013) as observed for tomato *SIBOR1* in this study. Conversely, in *A. thaliana*, *AtBOR1* transcript in both root and shoot was not affected by B supply. In the presence

of high B levels *AtBOR1* expression was negligible, and had post-transcriptional regulation, where *AtBOR1* proteins are incorporated into endosomes and transported back to the vacuole for degradation (Takano et al. 2006). The B-regulated mechanism of *SIBOR1* expression may contribute to reducing the accumulation of B in the shoot, thus reducing B toxicity. Another efflux-type borate transporter, *AtBOR4*, a *BOR1* paralogue, is not regulated at the post-translational level (Miwa et al. 2007) and has been reported to confer B tolerance, when expressed in transgenic rice plants (Miwa et al. 2007; Kajikawa et al. 2011). In barley landrace Sahara 3771, *Bot1*, a *BOR1* orthologue, has been identified as the gene responsible for B tolerance (Reid 2007). *TaBOR2* and *HvBOR2* were up-regulated in roots of tolerant cultivars and a positive correlation was found between the level of expression and the degree of tolerance (Reid 2007). These and previous findings suggest that *BOR* genes have different roles and modes of regulation in different plant species. However, different tolerance levels in different genotypes seem to be highly related to differences in transporter activity, partic- transporters to confirm their role in the mechanism of B tolerularly at root level. Therefore, the grafting technique, with the ance in tomato plants. selection of tolerant rootstocks, may be a good strategy to overcome stress associated with excess B in tomato.

In conclusion, in the presence of excess B, tomato plants cv ‘Ikram’ grafted onto ‘Arnold’, a commercial interspecific hybrid rootstock, accumulated less B than ‘Ikram’ non-grafted or self-grafted plants, in both lower and apical leaves. Under conditions of excess B, the rootstock ‘Arnold’ reduces B translocation from the roots to the shoot by decreasing expression of genes coding for the B transporters *SINIP5* and *SIBOR1*. These results provide preliminary information concerning the possible mechanism of tolerance to excess B in tomato, suggesting a key role played by B transporters that have not yet been identified and characterised. Further studies are required to analyse the mechanism of transport in the presence of excess B, and to characterise the function of these B transporters to confirm their role in the mechanism of B tolerance in tomato plants.

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REFERENCES

- Alpaslan M., Gunes A. (2001) Interactive effects of boron and salinity stress on the growth, membrane permeability and mineral composition of tomato and cucumber plants. *Plant and Soil*, 236, 123–128.
- Brown P.H., Picchioni G., Jenkins M., Hu H. (1992) Use of ICP-MS and ^{10}B to trace the movement of boron in plants and soils. *Common Soil Science and Plant Analysis*, 23, 2781–2807.
- Brown P.H., Hu H., Roberts W.G. (1999) Redefining boron toxicity symptoms in some ornamentals. *Slosson Report*, 95–98, 1–7.
- Di Gioia F., Serio F., Buttaro D., Ayala O., Santamaria P. (2010) Influence of rootstock on vegetative growth, fruit yield and quality in ‘Cuore di Bue’, an heirloom tomato. *Journal of Horticultural Science and Biotechnology*, 85, 477–482.
- Di Gioia F., Signore A., Serio F., Santamaria P. (2013) Grafting improves tomato salinity tolerance through sodium partitioning within the shoot. *HortScience*, 48, 855–862.
- Edelstein M., Ben-Hur M., Plaut Z. (2007) Grafted melons irrigated with fresh or effluent water tolerate excess boron. *Journal of the American Society for Horticultural Science*, 132, 484–491.
- Edelstein M., Plaut Z., Ben-Hur M. (2011) Mechanism responsible for restricted boron concentration in plant shoots grafted on pumpkins rootstocks. *Israel Journal of Plant Science*, 59, 207–215.
- Edelstein M., Cohen R., Baumkoler F., Ben-Hur M. (2016) Using grafted vegetables to increase tolerance to salt and toxic elements. *Israel Journal of Plant Science*, 63, 1–18.
- Goldbach H.E., Wimmer M. (2007) Boron in plants and animals: is there a role beyond cell-wall structure? *Journal of Plant Nutrition and Soil Science*, 170, 39–48.
- Guidi L., Degl’Innocenti E., Carmassi G., Massa D., Pardossi A. (2011) Effects of boron on leaf chlorophyll fluorescence of greenhouse tomato grown with saline water. *Environmental and Experimental Botany*, 7, 57–63.
- Hayes J.E., Reid R.J. (2004) Boron tolerance in barley is mediated by efflux of boron from the roots. *Plant Physiology*, 136, 3376–3382.

- Kajikawa M., Fujibe T., Uraguchi S., Miwa K., Fujiwara T. (2011) Expression of the Arabidopsis Borate Efflux Transporter Gene, AtBOR4, in rice affects the xylem loading of boron tolerance to excess boron. *Bioscience, Biotechnology and Biochemistry*, 75, 2421–2423.
- Kumar S., Stecher G., Tamura K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870–1874.
- Leaunghitikanachana S., Fujibe T., Tanaka M., Wang S., Sotta N., Takano J., Fujiwara T. (2013) Differential expression of three BOR1 genes corresponding to different genomes in response to boron conditions in hexaploid wheat (*Triticum aestivum* L.). *Plant and Cell Physiology*, 54, 1056–1063.
- Matoh T. (1997) Boron in plant cell walls. *Plant and Soil*, 193, 59–70.
- Miwa K., Takano J., Omori H., Seki M., Shinozaki K., Fujiwara T. (2007) Plants tolerant of high boron levels. *Science*, 318, 1417.
- Miwa K., Aibara I., Fujiwara T. (2014) Arabidopsis thaliana BOR4 is upregulated under high boron conditions and confers tolerance to high boron. *Soil Science and Plant Nutrition*, 60, 349–355.
- Nable R.O., Banuelos G.S., Paull J.G. (1997) Boron toxicity. *Plant and Soil*, 193, 181–198.
- Nakagawa Y., Hanaoka H., Kobayashi M., Miyoshi K., Miwa K., Fujiwara T. (2007) Cell-type specificity of the expression of OsBOR1, a rice efflux boron transporter gene, is regulated in response to boron availability for efficient boron uptake and xylem loading. *The Plant Cell*, 19, 2624–2635.
- Pardossi A., Romani M., Carmassi G., Guidi L., Landi M., Incrocci L., Maggini R., Puccinelli M., Vacca W., Ziliani M. (2015) Boron accumulation and tolerance in sweet basil (*Ocimum basilicum* L.) with green or purple leaves. *Plant and Soil*, 395, 375–389.
- Princi M.P., Lupini A., Longo C., Miller A.J., Sunseri F., Abenavoli M.R. (2016) Long- and short-term effects of boron excess to root form and function in two tomato genotypes. *Plant Physiology and Biochemistry*, 109, 9–19.
- Reid R. (2007) Identification of Boron transporter genes likely to be responsible for tolerance to Boron toxicity in wheat and barley. *Plant and Cell Physiology*, 48, 1673–1678.

- Reid R. (2010) Can we really increase yields by making crop plants tolerant to boron toxicity? *Plant Science*, 178, 9–11.
- Schnurbusch T., Hayes J., Hrmova M., Baumann U., Ramesh S.A., Tyerman S.D., Langridge P., Sutton T. (2010) Boron toxicity tolerance in barley through reduced expression of the multifunctional aquaporin HvNIP2;1. *Plant Physiology*, 153, 1706–1715.
- Schwarz D., Rouphael Y., Colla G., Venema J.H. (2010) Grafting as a tool to improve tolerance of vegetables to abiotic stresses: thermal stress, water stress and organic pollutants. *Scientia Horticulturae*, 127, 162–171.
- Shaaban M.M. (2010) Role of boron in plant nutrition and human health. *American Journal of Plant Physiology*, 5, 224–240.
- Sonneveld C., Voogt W. (2009) *Plant nutrition of greenhouse crops*. Springer, New York, USA.
- Sutton T., Baumann U., Hayes J., Collins N.C., Shi B.J., Schnurbusch T., Hay A., Mayo G., Pallotta M., Tester M., Langridge P. (2007) Boron-toxicity tolerance in barley arising from efflux transporter amplification. *Science*, 318, 1446–1449.
- Takano J., Wada M., Ludewig U., Schaaf G., Wiren N.V., Fujiwara T. (2006) The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *The Plant Cell*, 18, 1498–1509.
- Takano J., Miwa K., Fujiwara T. (2008) Boron transport mechanisms: collaboration of channels and transporters. *Trends in Plant Science*, 13, 451–457.
- Tanaka M., Fujiwara T. (2008) Physiological roles and transport mechanisms of boron: perspectives from plants. *Pflügers Archiv - European Journal of Physiology*, 456, 671–677.
- Tombuloglu H., Ozcan I., Tombuloglu G., Sakcali S., Unver T. (2016) Aquaporin in boron tolerance: identification, characterization, and expression analysis. *Plant Molecular Biology Reporter*, 34, 374–386.
- Vengosh A., Weithal E., Kloppmann W. (2004) Natural boron contamination in Mediterranean groundwater. *Geotimes*, 49, 20–25.
- Venturi S., Vaselli O., Tassi F., Nisi B., Pennisi M., Cabassi J., Rossato L. (2015) Geochemical and isotopic evidence for a severe anthropogenic boron contamination: a case study from Castelluccio (Arezzo, central Italy). *Applied Geochemistry*, 63, 146–157.

Wolf B. (1974) Improvement in the Azomethine-H method for determination of boron. *Communications in Soil Science and Plant Analysis*, 5, 39–44.

Table 1. Primers used for NIPs and BORs gene expression studies. Target genes and their relative GenBank IDs are reported.

Primer sequences (5' to 3')	Target gene	GenBank ID
AGAACAGCGCGTCAGTAACTTG TGGCCATGTAAGCGAAATATCC	Probable boron transporter 1 (BOR1)	XM_004229320
CCTTCCGTGGGCGTGTT TCCATCCCCCACGAATTTTA	Probable boron transporter2 (BOR2)	XM_004241450
GTTCATTGATGCTCCTGATATTGG TGATAATAGAGCCATTGGAGGACTTG	NIP1;1 like aquaporin	NM_001287775
CCCTTCATGTCTGGTGGAGTTAC CAGTGACAACGAAAAGGAGATTGA	Aquaporin, Major intrinsic protein NIP5-1-like	NM_001287359
CCACAGCCATAGTGAACCAGAA GCCAGTTGACAGAATCACAATCA	Aquaporin NIP6-1	NM_001302923

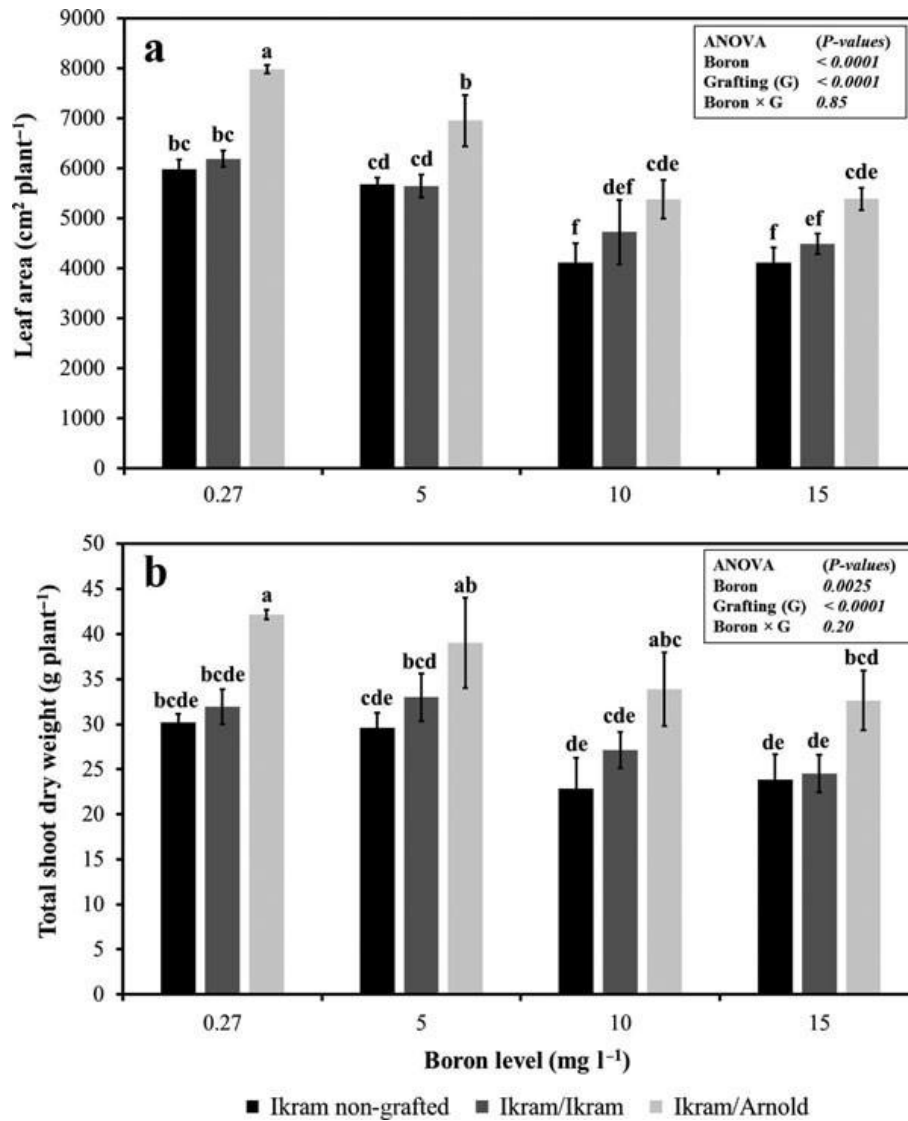


Fig. 1. Effect of boron (B) level on (a) leaf area and (b) total shoot DW of tomato plants (*S. lycopersicum* L.) cv 'Ikram' non-grafted, self-grafted or grafted onto 'Arnold', an interspecific hybrid (*S. lycopersicum* × *S. habrochaites*) rootstock and grown in hydroponic culture under greenhouse conditions for 28 days. Different letters indicate significant differences at $P = 0.05$ according to Duncan's multiple range test.

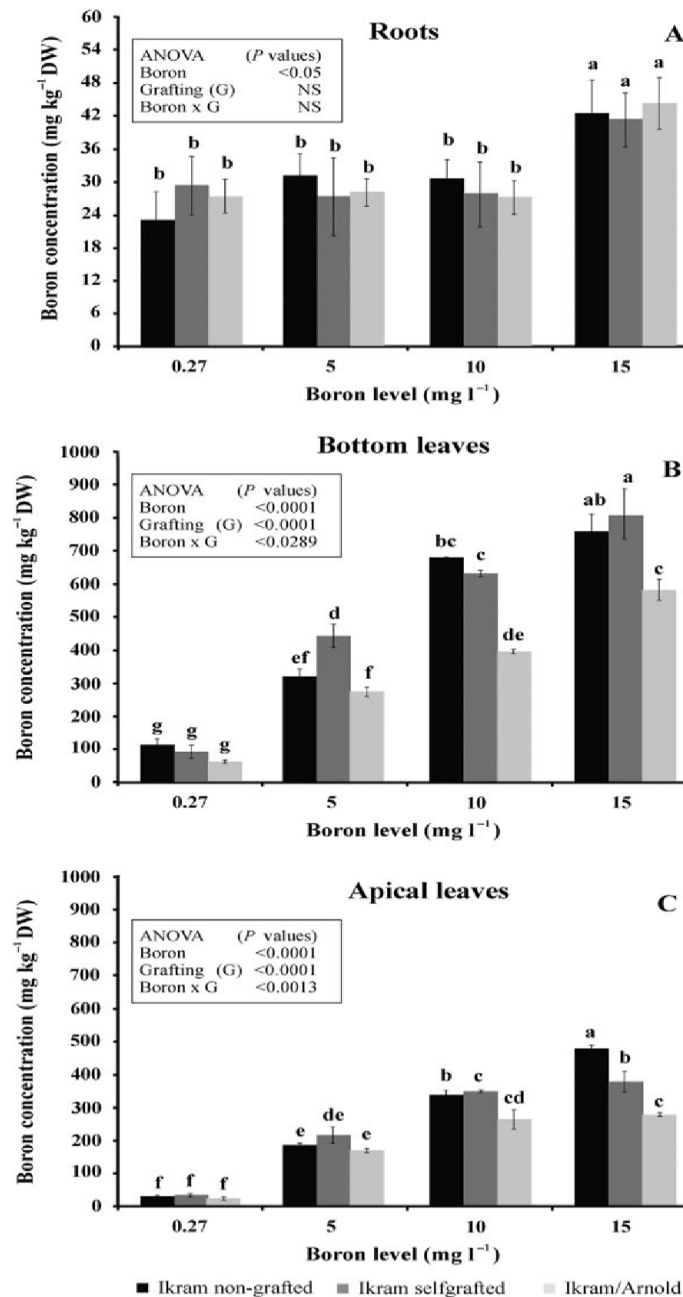


Fig. 2. Effect of boron level on B accumulation in roots (A), bottom (older) (B) and apical (younger) (C) leaves of tomato plants (*S. lycopersicum* L.) cv 'Ikram' non-grafted, self-grafted or grafted onto 'Arnold' an interspecific hybrid (*S. lycopersicum* x *S. habrochaites*) rootstock and grown in hydroponic culture under greenhouse conditions for 28 days. Different letters indicate significant differences at $P = 0.05$ according to Duncan's multiple range test.

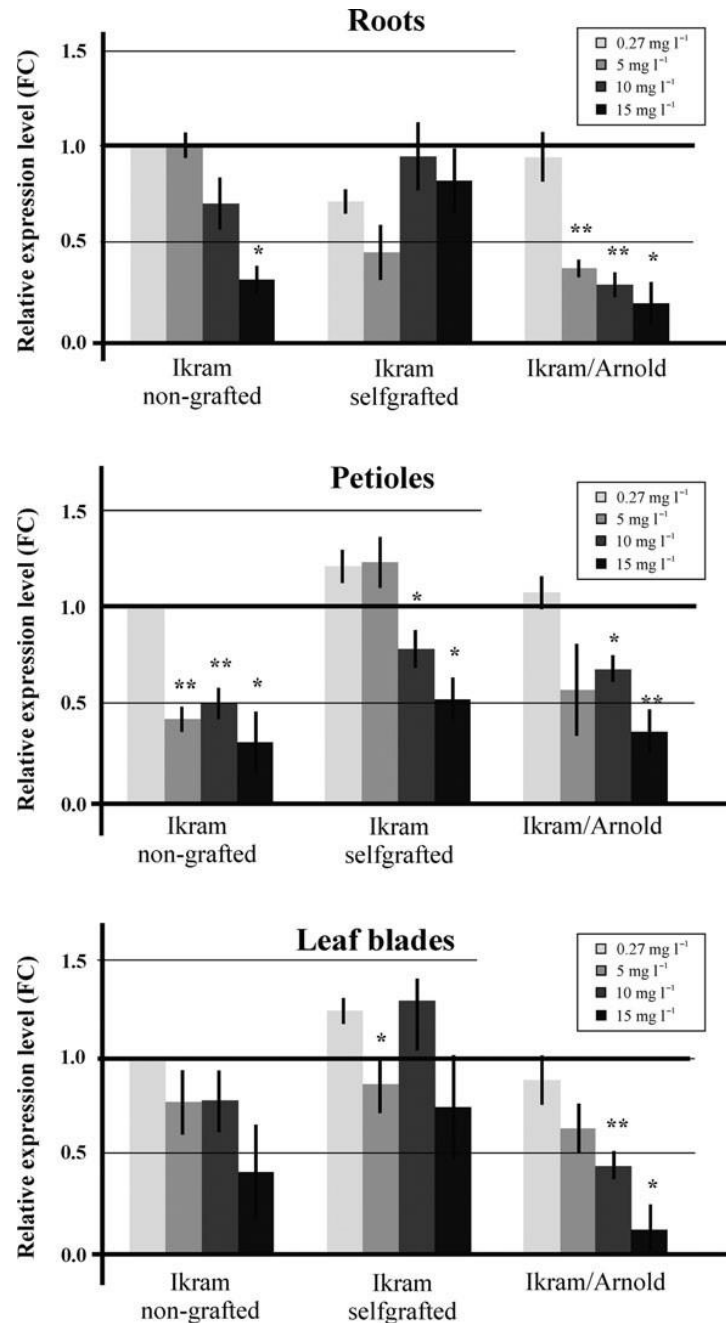


Fig. 4. Effect of boron (B) level on the relative expression of *SINIP5* in different portions of tomato plant tissue (*S. lycopersicum* L.) cv 'Ikram' non-grafted, self-grafted, or grafted onto 'Arnold' an interspecific hybrid (*S. lycopersicum* x *S. habrochaites*) rootstock. The bar charts show the expression differences between control (Ikram non-grafted with 0.27 mg L⁻¹ B) and relative treated samples. Data on gene expression are presented as fold change with respect to the controls for each group of plants. Error bars represent SE of mean of three replicates. Significance calculated using parametric paired t-test: *P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

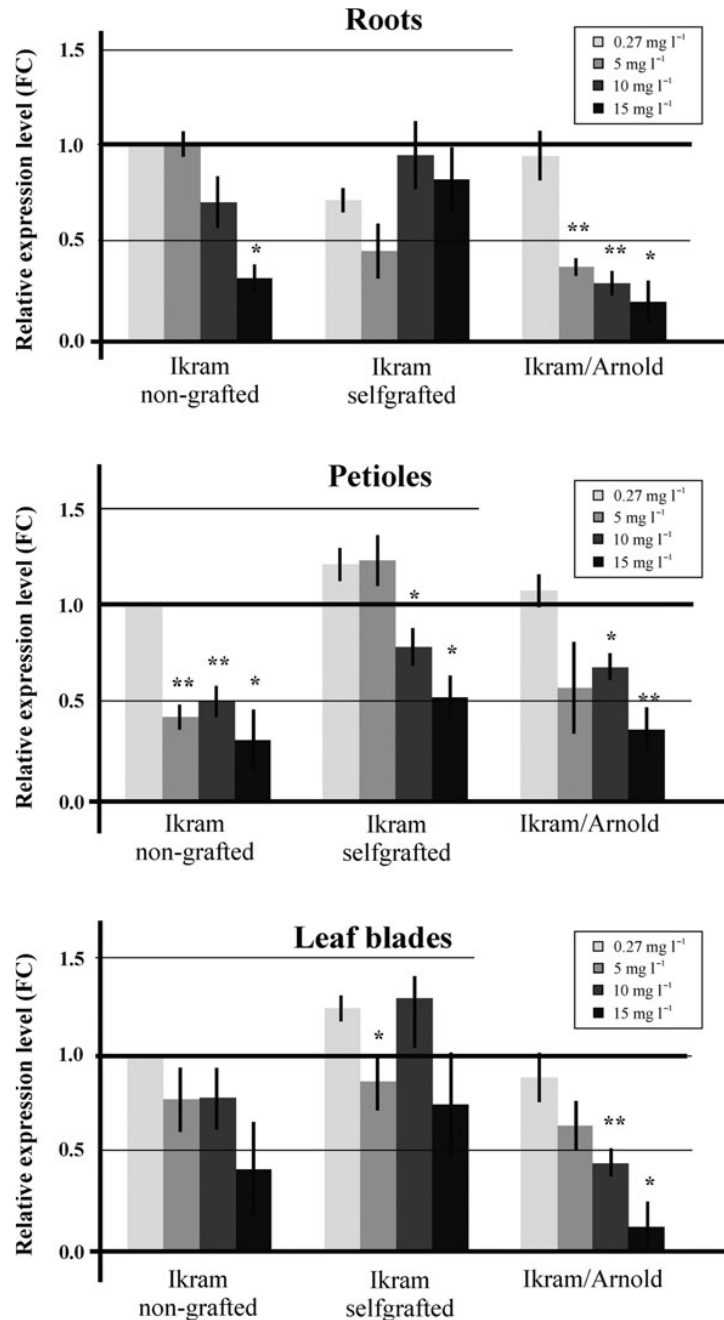


Fig. 5. Effect of boron (B) level on the relative expression of *SIBORI* in different portions of tomato plant tissue (*S. lycopersicum* L.) cv 'Ikram' non-grafted, self-grafted or grafted onto 'Arnold' an interspecific hybrid (*S. lycopersicum* x *S. habrochaites*) rootstock. The bar charts show expression differences between the control (Ikram non-grafted with 0.27 mg L⁻¹ B) and relative treated samples. Data on gene expression are presented as fold change with respect to the controls for each group of plants. Error bars represent SE of mean of three replicates