1 **Can the transcriptional regulation of *NHX1*, *SOS1* and *HKT1* genes handle the**
2 **response of two pomegranate cultivars to moderate salt stress?**

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13 *Running title*: Salt-tolerance of two pomegranate cultivars

**ABSTRACT:** Molecular mechanisms underlying plant functioning under salt conditions have not been completely elucidated, especially in a recalcitrant and less studied fruit trees such as pomegranate (*Punica granatum* L.). Here, we identified and characterized the expression of *NHX1*, *HKT1* and *SOS1* to understand their role in mediating $Na^+$ and $K^+$ transport, translocation and intracellular compartmentation in two pomegranate cultivars (Wonderful and Parfianka) during the first hours of a moderate salt stress (100 mM). In Wonderful, salt treatment significantly increased the $Na^+$ content only in mature leaves (ML) at 3 h after the beginning of the irrigation (2-fold higher than controls), however a concomitant decrease of $K^+$ content was observed (-33%). A significant decrease of *NHX1* and *SOS1* levels was observed in ML of Wonderful starting from 10 h. Salt irrigation significantly increased expression levels of these genes at all time points in young leaves of Wonderful (YL; with the exception of *NHX1* at 24 h) and led to a 7-fold induction of *HKT1* in roots. In Parfianka, salt treatment did not affect the $Na^+$ content, irrespective of leaf age. A significant increase of $K^+$ content was observed only in ML at 3 h (+46%). However, *NHX1* gene expression was downregulated at the same time in ML of Parfianka, while it was upregulated in YL. An opposite trend was observed in relation to *SOS1* expression. Our finding reinforces the idea that difference between cultivars in ion homeostasis and salt tolerance is associated with transcriptional regulation of *NHX1*, *HKT1* and *SOS1* genes, these being members of three major $Na^+$ transporters gene families.

**Keywords:** Ion compartmentalization, salt tolerance, *Punica granatum*, transporter gene families.

## 1. Introduction

Abiotic stresses such as drought, high temperature and salinity increasingly harm agriculture by negatively affecting the yield of a variety of crops (Onvekachi et al., 2019). Where salinity is concerned, several strands of evidence indicate that high concentrations of salt in the soil alter the capacity of plant roots to take up water (Zhu, 2001; Pennella et al., 2016). In addition, high concentrations of salt within plant tissues can be toxic, causing a wide range of physiological,

39 metabolic and genomic alterations which lead to impairments of plant processes such as
40 photosynthesis, nutrient and reactive oxygen species (ROS) balance (Arif et al., 2020).

41 In most plant species, it is $Na^+$ (rather than $Cl^-$) that causes toxic effects within cells via its
42 accumulation in the cytoplasm (Maathius et al., 2014). Metabolic toxicity of $Na^+$ is a result of its
43 ability to compete with $K^+$ for binding sites essential for cellular function. In particular, high levels
44 of $Na^+$ in the cytosol (or high $Na^+/K^+$ ratio) can inhibit $K^+$ uptake and/or disrupt various enzymatic
45 processes that require $K^+$ for functioning (e.g., $K^+$ dependent pyruvate kinase; Maathius, 2009). In
46 addition, cellular toxicity of $Na^+$ causes osmotic imbalance (i.e., osmotically driven removal of water
47 from cells) at the root-soil interface but also in other tissues (Flowers and Yeo, 1986). To
48 mitigate/detoxify these effects, plants can (1) sequester $Na^+$ in vacuoles within each plant cell and/or
49 (2) efflux $Na^+$ from cells. In the first case, $Na^+$ enters leaf cells and is then pumped into the vacuole
50 before concentrations increase over a tolerance threshold in the cytoplasm. This process is primarily
51 catalysed by a vacuolar $Na^+/H^+$ antiporter belonging to NHX gene family (*AtNHX1-4*), which utilizes
52 the $H^+$ gradient as a driving force for vacuolar $Na^+$ sequestration (Tester and Davenport, 2003;
53 Barragán et al., 2012; Yarra, 2019). The intercellular compartmentation not only protects the
54 cytoplasm, it also allows the plant to lower its cellular water potential and as such prevent water loss.
55 The increased osmolarity in the vacuole is paralleled in the cytoplasm by the synthesis and
56 accumulation of compatible solutes which not only maintain water relations but also act as ROS
57 scavenger (Maathuis et al., 2014). In the second case, plants can export $Na^+$ back to the growth
58 medium or to apoplastic spaces to avoid its cytosolic accumulation. This mechanism is at least
59 partially mediated by the "Salt Overly-Sensitive-1" (SOS1) protein (Hamam et al., 2016). This
60 putative $Na^+/H^+$ antiporter in the plasma membrane is considered essential for controlling long-
61 distance $Na^+$ movement in plants (Shi et al., 2002), being capable of xylem loading and/or regulating
62 cytosolic $Na^+$ extrusion from plant cells (Maathuis et al., 2014).

63 In addition to features of every cell within the plant that promote cellular survival and thus
64 contribute to the tolerance of the whole plant to salinity, plants can also have a wide range of other
65 mechanisms that involve particular activities of specific cell types. Other processes that contribute to
66 salt tolerance are the reabsorption of $Na^+$ from the xylem (Apse and Blumwald, 2007; Maathuis et
67 al., 2014) and its recirculation in the phloem (Berthomieu et al., 2003) to prevent excessive
68 accumulation of $Na^+$ in the aboveground tissues. This process is mediated by members of the HKT
69 (high-affinity $K^+$ transporter) gene family.

70 Pomegranate (*Punica granatum* L.) from the family Punicaceae, is a deciduous shrub widely
71 cultivated in Iran, Central Asia and Mediterranean area. Pomegranate has gained widespread
72 popularity as a functional food and nutraceutical source. The health effects of the whole fruit, as well
73 as its juices and extracts, have been studied since then in relation to a variety of chronic diseases, not
74 to mention therapeutic health benefits in aging due to its antioxidant properties (Johanningsmeier and
75 Harris, 2011). Currently, pomegranate is considered as a promising crop for its good adaptation to a
76 wide range of environmental conditions: it grows well in semi-arid regions by tolerating several
77 abiotic stresses, such as drought, elevated temperature and salinity (Teixeira da Silva et al., 2013;
78 Calzone et al., 2019). However, tolerance is highly cultivar-dependent and related to macronutrient
79 uptake, $Na^+$ and $Cl^-$ distribution, and osmolyte production (Karimi and Hasanpour, 2014). Indeed, in
80 a previous paper focused on the physiological and biochemical responses of the widely grown
81 cultivars Wonderful and Parfianka to salinity, we reported a differential salt tolerance, this being
82 moderate for Wonderful and elevated for Parfianka (Calzone et al., 2020). However, very few studies
83 have investigated the molecular mechanisms underlying pomegranate salt tolerance. Here, we wanted
84 to address the following questions: (i) What kind of $Na^+$ translocation and organ
85 compartmentalization occurs in these cultivars during the early phase of salt stress? and (ii) How does
86 the transcriptional regulation of *NHX1*, *HKT1* and *SOS1* contribute to the inter-cultivar variation in
87 salt tolerance? We postulated that difference in ion homeostasis and salt tolerance between cultivars
88 could be associated with transcriptional regulation of these genes.

89 **2. Materials and methods**

90 2.1. Plant material and experimental design

91 Two-year-old pomegranate plants of the commercial cultivars Wonderful and Parfianka were
92 purchased from a local nursery, transplanted into 5-L plastic pots filled with sandy soil and kept well-
93 watered under field conditions at the San Piero a Grado field station of the University of Pisa
94 (Tuscany, Italy, 43°40’48’’ N, 10°20’46’’ E, 2 m a.s.l.) until the beginning of the experiment. In the
95 greenhouse (April 2019), 48 plants were selected for height and weight uniformity, and subjected to
96 two irrigation regimes: 200 ml of 0 mM or 100 mM NaCl solutions (prepared using deionized water;
97 EC: 0.5 and 8.36 mS $cm^{-1}$, respectively), provided once to each pot.

98 At the beginning of the experiment, mature (ML) and young (YL) leaves were identified
99 according to Calzone et al. (2020). Plants were then harvested at 0.5, 3, 10 and 24 h after the irrigation,
100 according to Swindell (2006). At each sampling time, three plants per cultivar per salt treatment were
101 randomly selected, and their roots, ML and YL were separately flash-frozen and ground in liquid
102 nitrogen and stored at -80 °C until molecular analysis. Unlike the leaves, only fine roots (diameter ≤
103 3 mm) were analyzed at 0.5 h after the beginning of the irrigation, as the initial perceivers of osmotic
104 stress, stimulating the underground and aboveground plant defense (Gruber et al., 2009; Yang et al.,
105 2020).

106 2.2. Sodium and potassium determination

107 Sodium and $K^+$ contents were determined in both leaves (ML and YL) and roots. According to Cataldi
108 et al. (2003), these cations were extracted by suspending about 13 mg of oven-dried samples in 4 ml
109 of HPLC-grade water. Mixtures were shaken for 15 min and centrifuged at 1000 *g* for 10 min. Each
110 supernatant was collected and filtered through 0.2 µm Minisart® SRT 15 aseptic filters. Sodium and
111 $K^+$ contents were determined by a Dionex™ Aquion™ Ion Chromatography System (Dionex CDRS
112 600 4 mm suppressor, 4×50 mm Dionex IonPac™ CG12A pre-column, 4×250 mm Dionex IonPac™

113 CS12A column; Thermo Fisher Scientific, Waltham, MA, USA) using 20 mM methanesulfonic acid
114 as eluent and a flow rate of 1.0 ml $min^{-1}$.

115 2.3. RNA extraction and cDNA synthesis

116 Total RNA was extracted from nitrogen-frozen leaves using the RNeasy® Plant Mini Kit (Qiagen,
117 Hilden, Germany) according to the manufacturer's protocol. To avoid genomic DNA contamination,
118 DNAse treatment was performed with the Amplification Grade DNase I Kit (Sigma-Aldrich, Saint
119 Louis, MO, USA) according to manufacturer's instructions. The RNA quality was assessed by
120 separation on a 1% agarose run in 1× Tris acetate-EDTA (TAE) buffer at 40 V for 5 min and 100 V
121 for 20 min. The final concentration of the isolated RNA was quantified at 260 nm using a
122 NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).
123 Complementary DNA (cDNA) was synthesized from 0.5 µg of purified total RNA with the iScript™
124 cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The
125 reaction mix was sequentially incubated at 25 °C for 5 min for primer annealing and at 46 °C for 20
126 min for reverse transcription. The reaction was then stopped by heating at 95 °C for 1 min and then
127 chilled on ice. Leaf cDNA was stored at -80 °C until needed.

128 The mRNA was directly extracted from nitrogen-frozen roots using the Oligotex Direct
129 mRNA Mini Kit (Qiagen) according to the manufacturer's protocol. The final concentration of
130 mRNA was quantified spectrophotometrically as reported above for total RNA extracted from leaves.
131 The mRNA was retrotranscribed into cDNA using the SuperScript IV VILO Mastermix (Thermo
132 Fisher Scientific) following the manufacturer's instructions. The reaction mix was sequentially
133 incubated at 25 °C for 10 min for primer annealing and at 50 °C for 10 min for reverse transcription.
134 The reaction was stopped by heating at 85 °C for 5 min and then chilled on ice. Root cDNA was
135 stored at -80 °C until needed.

136 2.4. qPCR primers design

137 For the initial identification of *NHX1*, *SOS1*, *HKT1* and *ACT7* (the selected endogenous reference

138 gene) in *P. granatum*, representative protein sequences of *Arabidopsis thaliana* were obtained from
139 the Aramemnon protein membrane database (http://aramemnon.uni-koeln.de/; Schwacke et al. 2003),
140 and were used as query sequences (see Table 1 for the accession number) in tBLASTn for translated
141 nucleotide databases, searching the *P. granatum* genomic database (taxid: 22663; Ford et al., 2012).
142 Hits with the lower E-values were selected and examined further. For each gene, the corresponding
143 mRNA sequence was obtained from the coding sequence and used for the quantitative PCR (qPCR)
144 primers design. For PCR amplicons 70-150 bp long, qPCR primers were designed on these sequences
145 using the Primer3Plus online software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and the
146 following criteria: 18-23 bp primers size, primers melting temperature (Tm) 58-62 °C with 1 °C as
147 max Tm difference, primers GC content 30-60%, 3 max Poly-X and 1 GC clamp (Quellhorst and
148 Rulli, 2008; Thornton and Basu, 2011). The specificity of the resulting primer pair sequences was
149 checked against the green plants database (taxid: 33090) using BLAST analysis. Quality and
150 efficiency of primers were analysed using the Beacon Designer
151 (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1), Eurofins Oligo-Analysis
152 (https://www.eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/) and Thermo Fisher Multiple
153 Primer Analyzer softwares (https://www.thermofisher.com/us/en/home/brands/thermo-
154 scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-
155 library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Secondary structures of
156 amplicons were checked using the DNA folding form of the MFold Web Server
157 (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form). Amplicons with secondary structures
158 with a Tm lower than the qPCR annealing temperature were discarded (Thornton and Basu, 2011).

159 2.5. Primer efficiency test and qPCR assay

160 Equal amounts of cDNA were used for the qPCR to analyse transcript levels, and actin gene (*ACT7*,
161 AT5G09810.1, with an amplicon length of 72 bp; Table 1) was selected as the housekeeping gene to
162 normalise the data (forward 5′- GGGAATGAGCGTCGAGAATTG -3′ and reverse 5′-

163 TTCACGGACTCAAACAAGCC -3′ primers, Table 1). Before its use as reference gene, it was
164 verified that the level remained unchanged throughout the time in controls and salt-irrigated plants.
165 Specific primers of *NHX1*, *SOS1* and *HKT1* (with an amplicon length of 85, 150 and 79 bp,
166 respectively; Table 1) were synthesized by the Integrated DNA Technologies, Inc. (Coralville, IA,
167 USA), and their sequences are reported in Table 1. For each cDNA sample, three technical replicates
168 were analysed for each biological sample (i.e. three biological samples per cultivar per treatment per
169 time of analysis) using the Fast SYBR™ green Master Mix in a QuantStudio 3 system (Thermo Fisher
170 Scientific). Each 20 µl of qPCR assay dispensed in 0.1 ml cells of 96-well plates contained 2 µl
171 (corresponding to 500 ng) of the initial RNA used for cDNA synthesis, 1 µl of each primer (350 nM),
172 6 µl of nuclease-free water and 10 µl of Fast SYBR™ green Master Mix following the manufacturer's
173 instruction (Hartley et al., 2020). The two-step thermal profile comprised: (1) 95 °C/20 s to activate
174 the AmpliTaq® Fast DNA Polymerase, (2) 40 cycles of 95° C/1 s, 60 °C/20 s. Gene-specific primers
175 were tested to determine the optimal number of PCR cycles required to be within the linear range of
176 amplification, using dilutions of cDNAs. Five-point standard curves of different cDNA
177 concentrations (1:5, 1:10, 1:100, 1:1000, 1:10000 of initial cDNA) were used to verify the efficiency
178 of each primer (Ahmad et al., 2016), calculated with a linear regression analysis. The cycle threshold,
179 the efficiency and the coefficient determination were determined automatically by the QuantStudio
180 Software (Thermo Fisher Scientific). Melting curve analysis and agarose gel electrophoresis (1%
181 w/v) of the products were used to confirm the specificity of the qPCR and the absence of primer
182 dimers or non-specific amplification products (Döring et al., 2014). Using actin gene as the internal
183 reference gene, relative gene expression values were calculated as $2^{-\Delta\Delta Ct}$ by following the calculation
184 described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied
185 Biosystems, Thermo Fisher; Baccelli et al., 2015). Expression data were presented as $\log_2$ fold change
186 of selected genes of interest (*NHX1*, *SOS1* and *HKT1*) in gene expression normalized to *ACT7* and
187 relative to the control (represented by the baseline of the figure) (Livak and Schamittgen 2001;
188 Vangelisti et al, 2019).

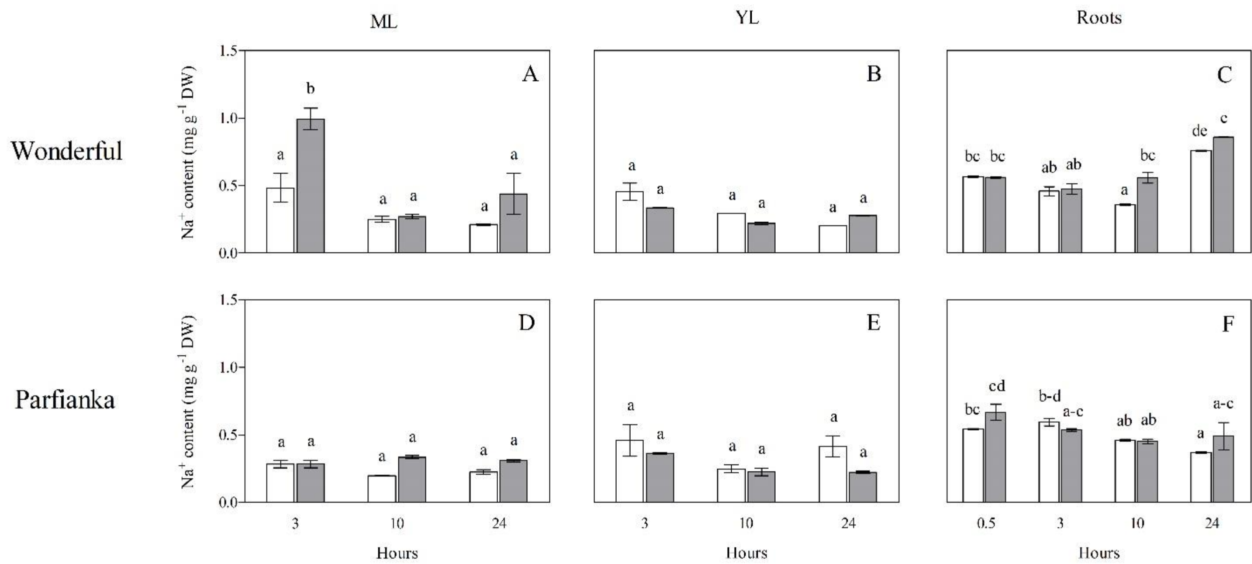
189 2.6. Statistical analysis

190 The Shapiro-Wilk test was firstly used to assess the normal distribution of data. The effects of
191 cultivars, salt treatment, time, leaf age and their interactions on leaf parameters were tested using a
192 four-way analysis of variance (ANOVA). The effects of cultivars, salt treatment, time and their
193 interactions on root parameters were tested using a three-way ANOVA. Significant differences
194 among means were assessed by the Tukey's *post-hoc* test ($P \leq 0.05$). All analyses were performed
195 using JMP 13.2.0 software (SAS Institute Inc., Cary, NC, USA).

196 **3. Results**

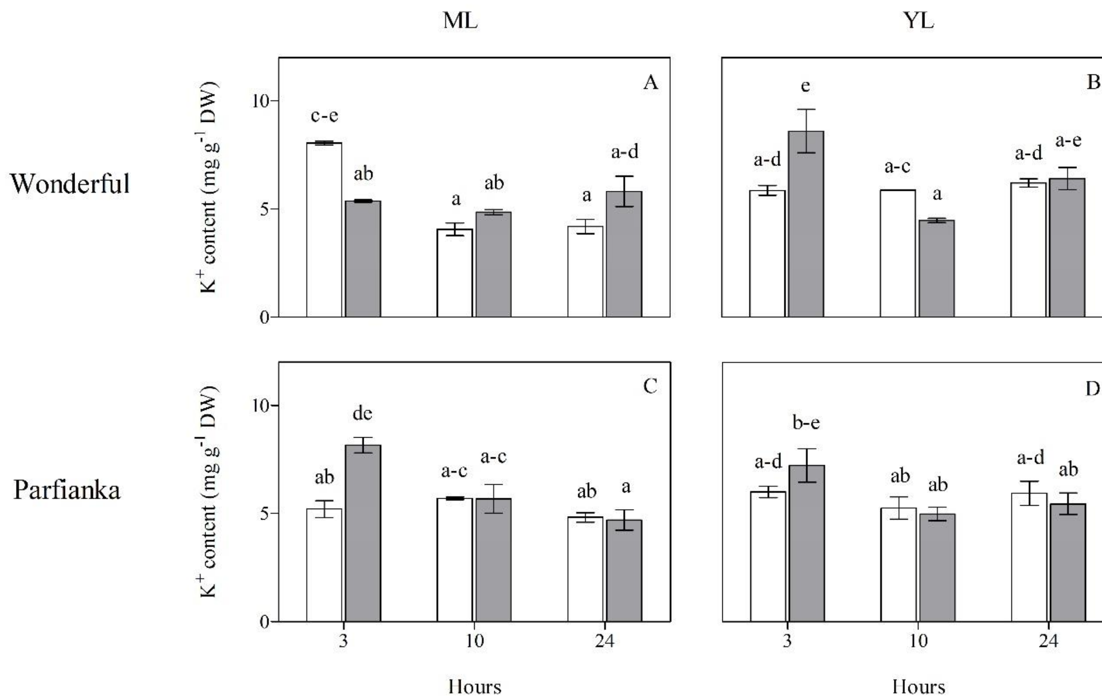
197 3.1. Sodium and $K^+$ contents

198 The four-way ANOVA revealed significant interactions among cultivars × leaf age × time × salt
199 treatment for $Na^+$ and $K^+$ contents in leaves (and most of the tested effects were significant as well,
200 Table S1). In Wonderful plants, salt treatment significantly increased the $Na^+$ content only in ML at
201 3 h (2-fold higher than controls; Fig. 1A), while an opposite trend was observed for ML in terms of
202 $K^+$ content (-33%; Fig. 2A). No other significant differences were observed in Wonderful YL (Fig.
203 1B), except for $K^+$ at 3 h (+46% compared with controls; Fig. 2B). In Parfianka plants, salt treatment
204 did not affect the leaf $Na^+$ content, irrespective to leaf age (Fig. 1 D-E), while a significant increase
205 of $K^+$ content was observed in ML at 3 h (+57% compared with controls; Fig. 2C). No other
206 significant differences were observed for Parfianka YL in terms of $K^+$ content (Fig. 2D).




207

208 **Fig. 1 Sodium distribution in leaves and roots.** Sodium ($Na^+$) content in mature (ML, A-D) and
209 young (YL, B-E) leaves and roots (C-F) of two pomegranate cultivars (Wonderful, A-B-C, and
210 Parfianka, D-E-F) subjected to two different irrigations: 0 mM NaCl (controls, white bars) and 100
211 mM NaCl (grey bars). The analyses were carried out at 0.5 (only for roots), 3, 10 and 24 h after the
212 beginning of the irrigation. Data are shown as mean ± standard error (n = 3). The four-way (Table
213 S1) and three-way (Table S2) ANOVA were performed for leaves (A-B, D-E) and roots (C-F),
214 respectively. Different letters indicate significant differences among means ($P \leq 0.05$), according to
215 Tukey's *post-hoc* test (if means are represented by more than three letters, only starting and ending
216 letters are reported, separated by "-").



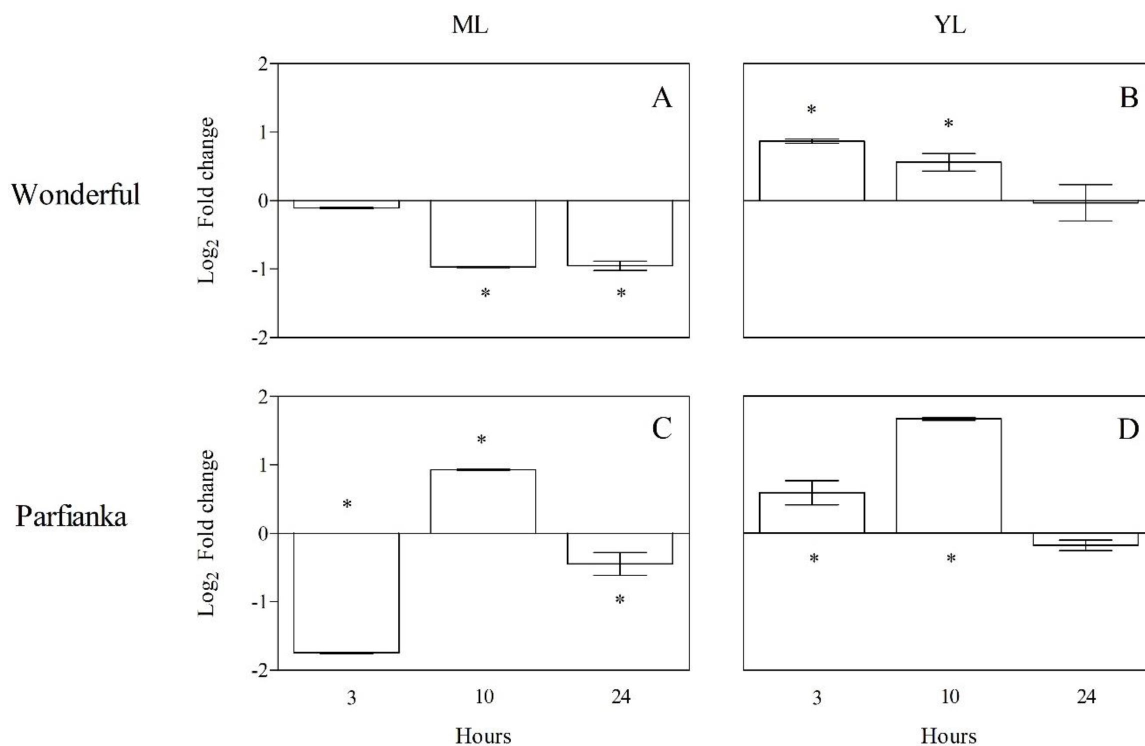

217

218 **Fig. 2 Potassium distribution in leaves and roots.** Potassium ($K^+$) content in mature (ML) and
219 young (YL) leaves of two pomegranate cultivars (Wonderful, A-B, and Parfianka, C-D) subjected to
220 two different irrigations: 0 mM NaCl (controls, white bars) and 100 mM NaCl (grey bars). The
221 analyses were carried out at 3, 10 and 24 h after the beginning of the irrigation. Data are shown as
222 mean ± standard error (n = 3). The four way ANOVA were performed (Table S1). Different letters
223 indicate significant differences among means ($P \leq 0.05$), according to Tukey's *post-hoc* test (if means
224 are represented by more than three letters, only starting and ending letters are reported, separated by
225 "-").

226 The three-way ANOVA revealed significant interactions among cultivars × time × salt treatment for
227 $Na^+$ content in roots (all other tested effects were significantly different as well, except for time × salt
228 treatment on $Na^+$ and cultivars × salt treatment on $Na^+$). Conversely, only significant time and
229 cultivars × salt treatment effects were found on $K^+$ root content (Table S2). In Wonderful roots, salt
230 treatment significantly increased $Na^+$ content only at 10 h after the beginning of the irrigation (Fig.
231 1C). No other significant differences were observed for root $K^+$ content (*data not shown*). In Parfianka
232 plants, no significant differences were observed in terms of root $Na^+$ (Fig. 1F). The interaction

233 cultivars × salt treatment on $K^+$ content in Parfianka root revealed that salt treatment induced an
234 increase of this cation at all the times investigated (+19% compared with controls).
235
236 3.2. Expression patterns of genes

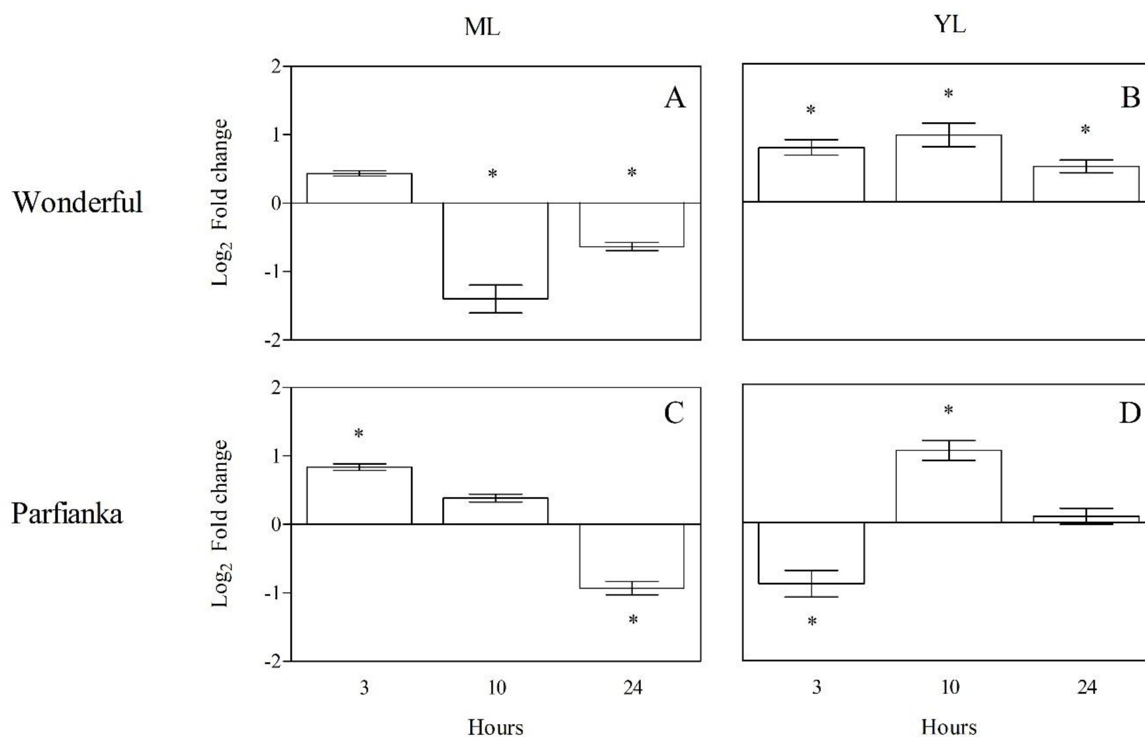
237 Based on a general analysis of the qPCR data, Figures 3-4 report the relative expression levels of the
238 investigated genes in salt-irrigated plants compared to controls (baseline) in ML, YL and roots at 3,
239 10 and 24 h after the beginning of the irrigation. A significant four-way interaction, cultivar × leaf ×
240 time × salt, was found with the expression levels of *NHX1* and *SOS1* in leaves, while most other
241 tested effects were also significant (Table S3). Interestingly, while in Wonderful ML a significant
242 decrease of *NHX1* levels was observed at 10 and 24 h after the beginning of the irrigation (Fig. 3A),
243 salt irrigation significantly increased expression levels in Wonderful YL (3 and 10 h; Fig. 3B). In
244 Parfianka ML, *NHX1* gene expression was downregulated at 3 h after the beginning of the irrigation
245 and upregulated at 10 h (Fig. 3C) while it was upregulated at both time points in YL (Fig. 3D).




246 **Fig. 3 Transcript levels of *NHX1* in leaves.** Logarithm (Log2) of fold change expression of *NHX1*
247 gene in mature (ML, A-C) and young (YL, B-D) leaves of two cultivars (Wonderful, A-B, and
248 Parfianka, C-D) of *Punica granatum* plants subjected to salt treatment (100 mM NaCl). The analyses

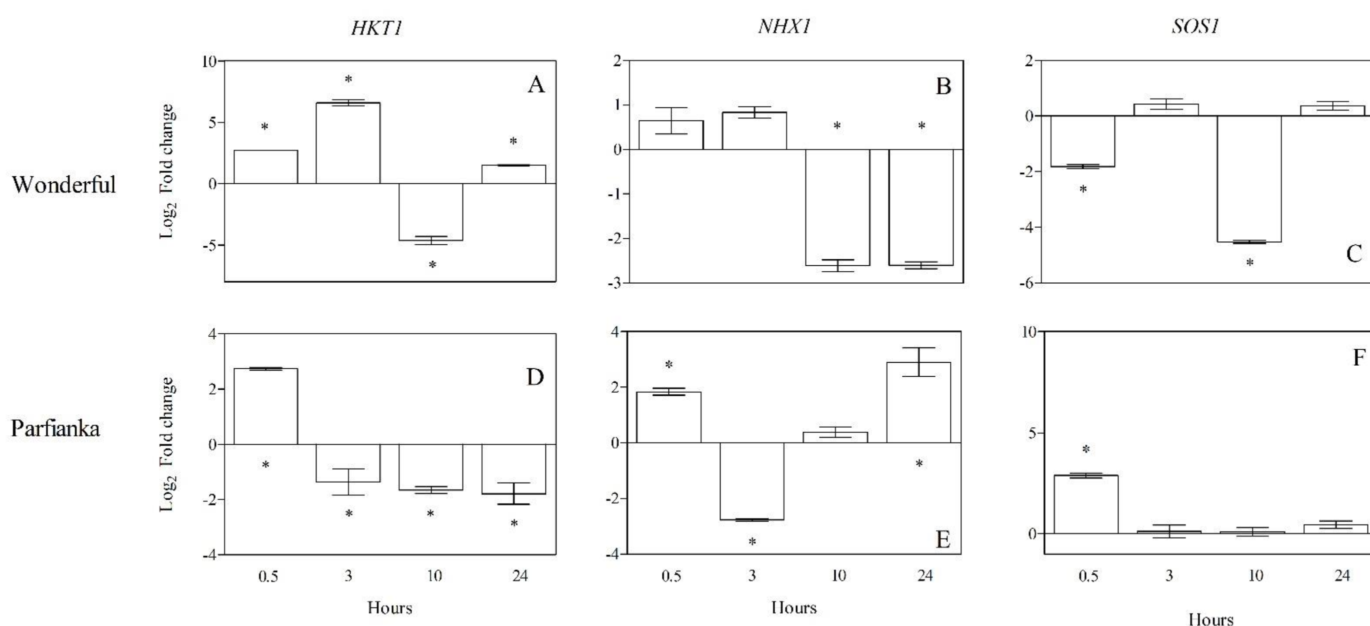
249 were carried out at 3, 10 and 24 h after the beginning of the irrigation. Bars (n = 9) show means ± standard error. Baseline represents controls plants (0 mM NaCl). Asterisks indicate significant differences ($P \leq 0.05$), according to Tukey's *post-hoc* test.

252 In Wonderful ML, a significant decrease of *SOS1* levels was observed but only at 10 and 24 h (Fig. 4A). By contrast, salt irrigation significantly increased the relative expression levels of *SOS1* at all time points in YL of Wonderful (Fig. 4B). In Parfianka ML, *SOS1* gene expression was upregulated at 3 h, downregulated at 24 h (Fig. 4C) and not significantly altered at 10 h after the beginning of the irrigation. In Parfianka YL too, a highly variable patter was observed with *SOS1* downregulation at 3 h (Fig. 4D), upregulation at 10 h (2-fold higher than controls), and no differences at 24 h.




259 **Fig. 4 Transcript levels of *SOS1* in leaves.** Logarithm (Log2) of fold change expression of *SOS1* gene in mature (ML, A-C) and young (YL, B-D) leaves of two cultivars (Wonderful, A-B, and Parfianka, C-D) of *Punica granatum* plants subjected to salt treatment (100 mM NaCl). The analyses were carried out at 3, 10 and 24 h after the beginning of the irrigation. Bars (n = 9) show means ± standard error. Baseline represents controls plants (0 mM NaCl). Asterisks indicate significant differences ($P \leq 0.05$), according to Tukey's *post-hoc* test.

265 A significant three-way interaction, cultivar × salt × time, was found for the expression levels
266 of *HKT1*, *NHX1* and *SOS1* in roots (Table S4). In Wonderful roots, *HKT1* gene expression was
267 upregulated at 0.5 and 3 h, downregulated at 10 h and again upregulated at 24 h after the beginning
268 of the irrigation (Fig. 5A). In Parfianka too, a highly variable pattern was observed with *HKT1*
269 upregulation at 3 h and downregulation starting from 3 h onwards (Fig. 5D). In Wonderful roots, a
270 significant decrease of *NHX1* levels was observed only at 10 and 24 h (Fig. 5B). In Parfianka, a highly
271 variable pattern was observed with *NHX1* upregulation at 0.5 h, downregulation at 3 h, not
272 significantly altered at 10 h after the beginning of the irrigation and again upregulation at 24 h (Fig.
273 5E). In Wonderful roots, a significant decrease of *SOS1* levels was observed only at 0.5 and 10 h (Fig.
274 5C), whereas only a brief upregulation was found in Parfianka roots at 0.5 h (Fig. 5F).




275

276 **Fig. 5 Transcript levels of *HKT1*, *NHX1* and *SOS1* in roots.** Logarithm (Log2) of fold change
277 expression of *HKT1* (A and D), *NHX1* (B and E) and *SOS1* (C and F) genes in roots of two cultivars
278 (Wonderful, A-C, and Parfianka, D-F) of *Punica granatum* plants subjected to salt treatment (100
279 mM NaCl). The analyses were carried out at 0.5, 3, 10 and 24 h after the beginning of the irrigation.
280 Bars (n = 9) show means ± standard error. Baseline represents controls plants (0 mM NaCl). Asterisks
281 indicate significant differences ($P \leq 0.05$).

282 **4. Discussion**

283 In a previous report (Calzone et al., 2020), we characterized the tolerance of the Wonderful and
284 Parfianka pomegranate cultivars to a range of salt treatments (100-200 mM NaCl), concluding that
285 they can be considered tolerant to moderate levels of salt (i.e. 100 mM NaCl). Molecular mechanisms
286 and genes underlying pomegranate functioning under salt conditions have not been completely
287 elucidated so far. In the present study, we identified and characterized the expression of *NHX1*, *HKT1*
288 and *SOS1* genes, members of three major $Na^+$ transporter gene families that play pivotal roles in
289 salinity tolerance. This is a first step towards understanding the role of these genes in mediating $Na^+$
290 and $K^+$ transport, translocation and intracellular compartmentation in pomegranate plants during the
291 first hours (i.e., osmotic phase; Munns and Tester, 2008) of moderate salt stress.

292 A major mechanism contributing to salt tolerance involves the control of $Na^+$ root uptake and
293 its distribution within the plant in order to avoid toxic $Na^+$ accumulation in shoots (Tester and
294 Davenport, 2003; Munns, 2005). Therefore, the first question we wanted to address in the present
295 study was: "What kind of $Na^+$ translocation and organ compartmentalization occurs in Wonderful
296 and Parfianka pomegranate cultivars during the early phase of salt stress"? Constitutive $Na^+$ levels in
297 roots were comparable between cultivars. After irrigation with salt only a very modest increase in
298 $Na^+$ was observed in Wonderful roots whereas no changes in $Na^+$ was reported in Parfianka. In shoots
299 too, overall levels of tissue $Na^+$ were very similar between cultivars although a transient spike in ML
300 of Wonderful was registered in the early (3 h) time point. This transient increase of $Na^+$ levels did not
301 inhibit the uptake of $K^+$ (with the exception of ML at 3 h) supporting the assumption that Wonderful
302 plants quickly allocated the required amount of $Na^+$ to the shoots to achieve full osmotic adjustment
303 and maintain their growth rate (Shabala, 2013). In both cultivars, shoot $Na^+$ levels were about half
304 the values reported in roots (0.28±0.02 *vs.* 0.61±0.04 in Wonderful, 0.27±0.02 *vs.* 0.54±0.04 mg $g^{-1}$
305 in Parfianka; shoots *vs.* roots). Overall, these data suggest that both cultivars were able to adopt robust
306 mechanisms to keep low shoot $Na^+$, so preventing ion toxicity occurring during the initial phases of

307 osmotic stress due to moderate levels of salinity. Conversely, $Na^+$ concentrations in both leaves and
308 roots of Parfianka plants were never affected by salt treatment, suggesting that they benefited from
309 avoiding $Na^+$ accumulation (Katschning et al., 2015). A stable internal shoot/root $Na^+$ concentration
310 is a characteristic that has been associated to salt tolerant species that are often capable of minimizing
311 $Na^+$ accumulation and/or maximizing $K^+$ accumulation, as confirmed by the significant and constant
312 rise of $K^+$ concentrations observed in roots Tester and Davenport, 2003). Our results clearly showed
313 that the two cultivars differed substantially in their response to salt irrigation in terms of $Na^+$ and $K^+$
314 accumulation, and this could be one of the reasons for their differential salt tolerance (Calzone et al.,
315 2020). The different performance in $Na^+$ and $K^+$ homeostasis between cultivars could be associated
316 to contrasting expression levels of $Na^+$ transporters (Munns, 2005; Maathuis, 2006).

317 In the light of the above, the second question was: "How does the transcriptional regulation
318 of *NHX1*, *HKT1* and *SOS1* contribute to the inter-cultivar variation in salt tolerance"? In Wonderful
319 plants, salt irrigation never up-regulated *SOS1* expression in roots. It is well known that the $Na^+/H^+$
320 antiporter SOS1 mediates $Na^+$ efflux and controls long-distance roots-to-shoots $Na^+$ delivery (Shin et
321 al, 2002). Conversely, a significant increase in *HKT1* expression was observed throughout the whole
322 period of the experiment (with the exception at 10 h) in roots of Wonderful. This result indicates
323 higher $Na^+$ unloading from the xylem stream (and its preferential accumulation in roots; Garcia-
324 Abellan et al., 2014). Remarkably, *HKT1* was not identified in pomegranate leaves, suggesting that
325 it is not involved in $Na^+$ recirculation in this species, but it could be responsible for the rise in $Na^+$
326 content observed in ML and roots at 3 and 10 h, respectively, in Wonderful plants. A different
327 expression pattern of *SOS1* and *NHX1* was observed in relation to leaf age: in ML of Wonderful, low
328 transcript levels of these genes were observed starting from 10 h after the treatment; in YL of
329 Wonderful, increased *SOS1* and *NHX1* expression was observed throughout the whole experiment
330 (with the exception of *NHX1* at 24 h). This result indicates that these genes can coordinate the
331 (re)direction of $Na^+$ to specific tissues that need $Na^+$ for osmotic adjustment playing an important role
332 in salt tolerance (Katschnig et al., 2015). Thus, $Na^+$ was preferentially removed from organs that the

333 plant needed to protect (i.e., YL) and redirected towards ‘sacrificial’ parts, such as ML of Wonderful
334 (Tester and Davenport, 2003; Calzone et al., 2020). In fact, our results indicate an increased capacity
335 of Wonderful plants in extruding $Na^+$ from the cytosol toward leaf apoplast, thus maintaining low
336 concentrations of $Na^+$ (and improving $K^+$ content) in YL cells during the first hour of salt irrigation
337 (Olìas et al., 2009; Gao et al., 2016).

338 In Parfianka roots, salt irrigation induced a significant increase in the expression of the
339 examined genes as early as at 0.5 h, probably indicating that *SOS1*, *HKT1* and *NHX1* synergistically
340 regulate $Na^+$ and $K^+$ homeostasis by controlling $Na^+$ transport systems at root level, as observed in
341 *Puccinellia tenuiflora* plants (Zhang et al., 2017). At foliar level, a different expression pattern of
342 *SOS1* and *NHX1* was observed in relation to leaf age and timing in Parfianka plants. In ML, *SOS1*
343 levels did not exhibit a clear trend showing a significant increase only 3 h after the beginning of the
344 irrigation. The transient increased expression of *NHX1* (observed only at 10 h) indicates a greater
345 capacity of ML to enhance activity of intracellular $Na^+$ compartmentation (without any change in
346 cellular $Na^+$ concentrations; Tester and Davenport, 2003). In Parfianka plants under optimal growth
347 conditions, YL showed much higher expression levels of *NHX1* and *SOS1* than ML, suggesting that
348 the differential expression of these genes may underpin the avoiding $Na^+$ accumulation (and $K^+$ loss)
349 during the first hours of salt treatment. Under salt conditions, high *SOS1* and *NHX1* expression was
350 observed in YL of Parfianka only at 10 h, indicating that these genes can coordinate the (re)direction
351 of $Na^+$ to specific tissues and the $Na^+$ vacuolar compartmentation in order to avoid cytoplasmatic $Na^+$
352 toxic accumulation (Galvez et al., 2012). It is worth to note that salt irrigation induced an increase of
353 *NHX1* transcript levels also at 3 h indicating its involvement in $Na^+$ compartmentation into vacuoles
354 of YL cells. This might be a consequence of the “accumulation” strategy for salt tolerance in
355 Parfianka plants, which implicates the use of $Na^+$ as osmolyte to lower water potential during the first
356 hours of salt irrigation (Maathius, 2014).

357 **5. Conclusions**

358 In the present study, the transcriptional regulation of *NHX1*, *HKT1* and *SOS1* in two commercial
359 cultivars subjected to a moderate salt concentration have been analysed. The expression induction of
360 these genes in leaves and roots underlined their potential role in mediating $Na^+$ and $K^+$ transport,
361 translocation and intracellular compartmentation during the first hours of salt treatment. Our data
362 confirmed that Wonderful plants preferred to redirect $Na^+$ to roots and ML, so maintaining low $Na^+$
363 concentration in YL (in order to prevent $Na^+$ accumulation and/or $K^+$ loss). On the other hand,
364 Parfianka plants seemed to be able to avoid $Na^+$ accumulation, especially improving high $K^+$
365 concentration in ML and maintaining unchanged $Na^+$ concentration in YL, although there were no
366 substantial differences in ML and root $Na^+$ distribution. Our findings reinforce the idea that difference
367 in ion homeostasis and salt tolerance between cultivars (or species) is associated with the expression
368 regulation of *NHX*, *HKT* and *SOS* family genes. However, it is worth to note that the levels of these
369 genes did not exhibit a clear trend straightforwardly explaining the dynamics of $Na^+$ and $K^+$ content
370 in different organs. This was likely due to the moderate severity and the short duration of the salt
371 treatment. Further investigations are required to better understand the role of $Na^+$ ($K^+$)/$H^+$ transporters
372 in mediating $Na^+$ and $K^+$ translocation and intracellular compartmentation in pomegranate cultivars
373 under harsher salt stress conditions (salt concentrations and time of treatment).
374

375 **CrediT authorship contribution statement**

376 **Antonella Calzone:** Conceptualization, Formal analysis, Investigation, Data Curation, Writing -
377 Original Draft; **Lorenzo Cotrozzi:** Formal analysis, Writing - Review & Editing; **Elisa Pellegrini:**
378 Conceptualization, Writing - Original Draft, Supervision; **Giacomo Lorenzini:** Writing – Review &
379 Editing, Supervision; **Cristina Nali:** Conceptualization, Writing - Review & Editing, Funding
380 acquisition; **Frans Maathius:** Conceptualization, Writing - Review & Editing Supervision

381 **Declaration of Competing Interest**

382 The authors declare no competing financial interest.

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476

477 **Figure Legends**

478 Fig. 1 Sodium distribution in leaves and roots. Sodium ($Na^+$) content in mature (ML, A-D) and young
479 (YL, B-E) leaves and roots (C-F) of two pomegranate cultivars (Wonderful, A-B-C, and Parfianka,
480 D-E-F) subjected to two different irrigations: 0 mM NaCl (controls, white bars) and 100 mM NaCl
481 (grey bars). The analyses were carried out at 0.5 (only for roots), 3, 10 and 24 h after the beginning
482 of the irrigation. Data are shown as mean ± standard error (n = 3). The four-way (Table S1) and three-
483 way (Table S2) ANOVA were performed for leaves (A-B, D-E) and roots (C-F), respectively.
484 Different letters indicate significant differences among means ($P \leq 0.05$), according to Tukey's *post-*
485 *hoc* test.

486 Fig. 2 Potassium distribution in leaves and roots. Potassium ($K^+$) content in mature (ML) and young
487 (YL) leaves of two pomegranate cultivars (Wonderful, A-B, and Parfianka, C-D) subjected to two
488 different irrigations: 0 mM NaCl (controls, white bars) and 100 mM NaCl (grey bars). The analyses
489 were carried out at 3, 10 and 24 h after the beginning of the irrigation. Data are shown as mean ±
490 standard error (n = 3). The four way ANOVA were performed (Table S1). Different letters indicate
491 significant differences among means ($P \leq 0.05$), according to Tukey's *post-hoc* test.

492 Fig. 3 Transcript levels of *NHX1* in leaves. Logarithm (Log2) of fold change expression of *NHX1*
493 gene in mature (ML, A-C) and young (YL, B-D) leaves of two salt-treated (100 mM NaCl) cultivars
494 (Wonderful, A-B, and Parfianka, C-D) of *Punica granatum* plants. The analyses were carried out at
495 3, 10 and 24 h after the beginning of the irrigation. Bars (n = 9) show means ± standard error. Baseline
496 represents controls plants (0 mM NaCl). Asterisks indicate significant differences ($P \leq 0.05$),
497 according to Tukey's *post-hoc* test.

498 Fig. 4 Transcript levels of *SOS1* in leaves. Logarithm (Log2) of fold change expression of *SOS1* gene
499 in mature (ML, A-C) and young (YL, B-D) leaves of two salt-treated (100 mM NaCl) cultivars
500 (Wonderful, A-B, and Parfianka, C-D) of *Punica granatum* plants. The analyses were carried out at
501 3, 10 and 24 h after the beginning of the irrigation. Bars (n = 9) show means ± standard error. Baseline

502 represents controls plants (0 mM NaCl). Asterisks indicate significant differences ($P \leq 0.05$),
503 according to Tukey's *post-hoc* test.

504 Fig. 5 Transcript levels of *HKT1*, *NHX1* and *SOS1* in roots. Logarithm (Log2) of fold change
505 expression of *HKT1* (A and D), *NHX1* (B and E) and *SOS1* (C and F) genes in roots of two salt-
506 treated (100 mM NaCl) cultivars (Wonderful, A-C, and Parfianka, D-F) of *Punica granatum* plants.
507 The analyses were carried out at 0.5, 3, 10 and 24 h after the beginning of the irrigation. Bars (n = 9)
508 show means ± standard error. Baseline represents controls plants (0 mM NaCl). Asterisks indicate
509 significant differences ($P \leq 0.05$).

510

511 Table 1. *ACT7*, *NHX1*, *SOS1* and *HKT1* genes (*Arabidopsis thaliana* and *Punica granatum* locus tag) and primers pairs (written in 5’-3’) for the assay

512 used in this study.

| Gene | *A. thaliana* | *P. granatum* | Primer Forward | Primer Reverse | Amplicon length |
|---|---|---|---|---|---|
| *ACT7* | AT5G09810.1 | MTKT01003207.1 | GGGAATGAGCGTCGAGAATTG | TTCACGGACTCAAACAAGCC | 72 bp |
| *NHX1* | AT5G27150.1 | MTKT01006319.1 | TCAGCATCATCAGTGCAACC | TTGACGGGATTGCTTAGTGC | 85 bp |
| *SOS1* | AT2G01980.1 | MTKT01006319.1 | GCTTCAAATGCTGCAACGTC | AGCAGCTTTGTTGCCTTCTC | 150 bp |
| *HKT1* | AT4G10310.1 | MTKT01001080.1 | ACAATCGTGTCCACCTTTGC | TGCCCGAGTTGTTCTTGAAC | 79 bp |

513

514 Table S1. *P* values of four-way analysis of variance (ANOVA) for the effects of cultivars, salt
515 treatment, time, leaf age and their interactions on leaf $Na^+$ and $K^+$ content of *Punica granatum*
516 cultivars (Wonderful and Parfianka). d.f., represents the degrees of freedom.

| **Effects** | **d.f.** | **$Na^+$** | **$K^+$** |
|---|---|---|---|
| Cultivars | 1 | 0.002 | 0.864 |
| Salt treatment | 1 | 0.048 | 0.032 |
| Time | 2 | <0.001 | <0.001 |
| Leaf age | 1 | 0.037 | 0.016 |
| Time × Salt treatment | 2 | 0.559 | 0.027 |
| Cultivars × Salt treatment | 1 | 0.009 | 0.416 |
| Time × Cultivars × Salt treatment | 2 | 0.008 | 0.002 |
| Time × Cultivars | 2 | 0.001 | 0.036 |
| Time × Leaf age | 2 | 0.149 | 0.038 |
| Cultivars × Leaf age | 1 | <0.001 | 0.058 |
| Time × Cultivars × Leaf age | 2 | 0.001 | 0.646 |
| Salt treatment × Leaf age | 1 | <0.001 | 0.886 |
| Time × Salt treatment × Leaf age | 2 | 0.101 | 0.002 |
| Cultivars × Salt treatment × Leaf age | 1 | 0.209 | 0.048 |
| Time × Cultivars × Salt treatments × Leaf age | 2 | 0.007 | <0.001 |

517

518 Table S2. *P* values of three-way analysis of variance (ANOVA) for the effects of cultivars, salt treatment and time on roots $Na^+$ and $K^+$ content of

519 *Punica granatum* cultivars (Wonderful and Parfianka). d.f. represents the degrees of freedom.

| **Effects** | **d.f.** | **$Na^+$** | **$K^+$** |
|---|---|---|---|
| Cultivars | 1 | 0.001 | 0.072 |
| Salt treatment | 1 | 0.001 | 0.030 |
| Time | 3 | <0.001 | <0.001 |
| Time × Cultivars | 3 | <0.001 | 0.589 |
| Time × Salt treatment | 3 | 0.054 | 0.526 |
| Cultivars × Salt treatment | 1 | 0.363 | 0.001 |
| Time × Cultivars × Salt treatment | 3 | 0.012 | 0.857 |

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521

522 Table S3. *P* values of four-way analysis of variance (ANOVA) for the effects of cultivars, salt
523 treatment, time, leaf age, and their interactions on expression levels of genes *NHX1* and *SOS1* at leaf
524 level of *Punica granatum* cultivars (Wonderful and Parfianka). d.f. represents the degrees of freedom.

| **Effects** | **d.f.** | ***NHX1*** | ***SOS1*** |
|---|---|---|---|
| Cultivars | 1 | <0.001 | 0.575 |
| Salt treatment | 1 | <0.001 | <0.001 |
| Time | 2 | <0.001 | <0.001 |
| Leaf age | 1 | <0.001 | <0.001 |
| Time × Salt treatment | 2 | <0.001 | <0.001 |
| Cultivars × Salt treatment | 1 | <0.001 | 0.575 |
| Time × Cultivars × Salt treatment | 2 | <0.001 | <0.001 |
| Time × Cultivars | 2 | <0.001 | <0.001 |
| Time × Leaf age | 2 | <0.001 | <0.001 |
| Cultivars × Leaf age | 1 | 0.358 | <0.001 |
| Time × Cultivars × Leaf age | 2 | 0.014 | 0.001 |
| Salt treatment × Leaf age | 1 | <0.001 | <0.001 |
| Time × Salt treatment × Leaf age | 2 | <0.001 | <0.001 |
| Cultivars × Salt treatment × Leaf age | 1 | 0.358 | <0.001 |
| Time × Cultivars × Salt treatment × Leaf age | 2 | 0.014 | 0.001 |

525

526 Table S4. *P* values of four-way analysis of variance (ANOVA) for the effects of cultivars, salt
527 treatment, time, and their interactions on expression levels of the genes *HKT1*, *NHX1* and *SOS1*
528 and at root level of *Punica granatum* cultivars (Wonderful and Parfianka). d.f. represents the
529 degrees of freedom.

| **Effects** | **d.f.** | ***HKT1*** | ***NHX1*** | ***SOS1*** |
|---|---|---|---|---|
| Cultivars | 1 | <0.001 | 0.014 | 0.001 |
| Salt treatment | 1 | <0.001 | 0.006 | 0.001 |
| Time | 3 | <0.001 | 0.014 | 0.001 |
| Time × Cultivars | 3 | <0.001 | 0.002 | <0.001 |
| Time × Salt treatment | 3 | <0.001 | 0.019 | 0.001 |
| Cultivars × Salt treatment | 1 | <0.001 | 0.019 | 0.001 |
| Time × Cultivars × Salt treatment | 3 | <0.001 | 0.002 | <0.001 |

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