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# Comparative expression of candidate genes involved in sodium transport and compartmentation in citrus

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ABSTRACT

Plants possess a number of mechanisms to cope with sodium ($Na^+$) under salt stress conditions that include minimizing $Na^+$ influx, maximizing efflux back to the growth medium or to apoplastic spaces via $Na^+/H^+$ antiporters in the plasma membrane, intracellular compartmentation of $Na^+$ into the vacuole, as well as recirculation of $Na^+$ out of the shoot via the phloem. $Na^+$ transport in plants constitutes a complex system, in which different $Na^+$ transporters are closely related and their functions are matched tightly. The fact that in citrus under salt stress does chloride appear to be the more toxic ion has led to little attention being paid to $Na^+$ uptake and transport mechanisms in citrus. The aim of this study was to provide insight into the links between the expression levels of candidate $Na^+$ transporter genes (*SOS1*, *NHX1*, *HKT1*), as well as tonoplast proton pumps (*V-ATPase, V-PPiase*), and $Na^+$ tolerance in two citrus rootstocks, Cleopatra mandarin and trifoliate orange, differing in their $Na^+$ exclusion capacity under salt stress. According to the results of this preliminary study, we hypothesize that higher root $Na^+$ concentration in trifoliate orange genotype, and thus lower allocation of this ion in the shoots, is the result of an enhanced retrieval of $Na^+$ from xylem stream, and an impaired translocation to the shoot tissues, probably as a consequence of the overexpression of putative *SOS1* (in roots) and *HKT1* (both in roots and shoots). Moreover, the higher transcriptional levels of putative *NHX1* found in roots and shoots of trifoliate orange plantlets compared to those of Cleopatra mandarin, together with the enhanced activity of the tonoplast proton pumps in the former, might reveal the preferential sequestration into vacuole of retrieved $Na^+$ from xylem mainly in roots.



## 1. Introduction

When grown in saline environment all plants accumulate sodium ions ($Na^+$) to some extent, due to the strong driving force for its entry (Rodríguez-Rosales et al., 2009). $Na^+$ influx into root cells occurs via plasma-membrane non-selective cation channels or $Na^+$ may also passively enter via anatomical 'leaks' in the root endodermis (Hasegawa et al., 2000; Munns and Tester, 2008), and it is transported to the shoot via the transpiration stream (Amtmann et al., 1997; Roberts and Tester, 1997). This transport triggers ion toxicity as a result of the elevation of cytoplasmic sodium concentration (Kingsbury and Epstein, 1986).

Plants possess a number of mechanisms to prevent accumulation of $Na^+$ in the cytoplasm that include minimizing $Na^+$ influx, maximizing sodium efflux back to the growth medium or to apoplastic spaces via $Na^+/H^+$ antiporters in the plasma membrane, intracellular compartmentation of $Na^+$ into the vacuole, as well as recirculation of $Na^+$ out of the shoot via the phloem (Apse et al., 1999). Except for some halophytic species that are able to effectively maintain very low $Na^+$ net influx (Munns and Tester, 2008), one of the key responses to salt stress is to maintain cellular ion homeostasis by restricting the accumulation of $Na^+$ (Tester and Davenport, 2003). The salt overly sensitive (SOS) signaling pathway (SOS3, SOS2, and SOS1) has been proposed to mediate cellular signaling under salt stress, maintaining ion homeostasis and conferring this way salt tolerance. In *Arabidopsis thaliana* the SOS pathway is comprised of a $Ca^{2+}$ sensor, SOS3, that transduces a $Ca^{2+}$ signal to recruit and activate an effector kinase, SOS2, which in turn phosphorylates and stimulates the $Na^+/H^+$ exchange activity of the ion transporter SOS1 at the plasma membrane to re-establish ion homeostasis (Blumwald et al., 2000; Quintero et al., 2002; Shi et al., 2002). Moreover, SOS1 is involved in controlling long-distance $Na^+$ transport in plants from root to shoot. The expression of SOS1 in cells surrounding the stele indicates a role of

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this transporter in controlling the $Na^+$ load of the vascular system in *A. thaliana* (Shi et al., 2002). In xylem parenchyma cells, reversibility in the $Na^+$ transport direction of SOS1 is hypothesized (Shi et al., 2002). SOS1 would retrieve $Na^+$ from the xylem stream under severe salt stress, whereas under mild salt stress it may function in loading $Na^+$ into the xylem.

The sequestration of excessive $Na^+$ inside root or stem cell vacuoles is a strategy used by many plants to survive salt stress (Blumwald et al., 2000). Plant NHX genes encode $Na^+/H^+$ antiporters, localized in the vacuolar membrane, that are involved in the compartmentation of sodium ions into the vacuole (Blumwald and Poole, 1985; Barkla et al., 1995; Apse and Blumwald, 2007), thus protecting essential enzymatic reactions in the cytoplasm from excess $Na^+$ levels while contributing to salt tolerance and ion homeostasis within the cells (Hasegawa et al., 2000; Serrano and Rodríguez-Navarro, 2001). Anyhow, the operation of NHX exchangers has to be energized by the vacuolar proton gradient established by vacuolar $H^+$ pumps. In this sense, both tonoplast $H^+$-adenosine triphosphatase (V-ATPase) that acidifies the vacuolar lumen (Ayala et al., 1996; Wang et al., 2001 among others) and $H^+$-inorganic pyrophosphatase (V-PPiase; Parks et al., 2002; Vera-Estrella et al., 2005; Krebs et al., 2010) are involved in $Na^+$ compartmentation.

Moreover, some members of the high-affinity potassium transporters protein family (HKT) are proposed to play a major role in $Na^+$ uptake and recirculation within salt-stressed plants (Apse and Blumwald, 2007), thus being critical for salinity tolerance (Berthomieu et al., 2003). HKTs are segregated into two subgroups based on their transport selectivity. Group 1 are described as $Na^+$ uniporters, while group 2 are thought to allow $Na^+$ and $K^+$ transport as well as $Na^+$ uniport at high $Na^+$ concentrations (see review by Waters et al., 2013). HKT genes from dicot species fall within the first major subfamily (HKT1), while graminaceous species often present HKT genes in both (HKT1, HKT2) families (Platten et al., 2006). The best characterized member of group 1 HKTs is *AtHKT1*; 1 from *A. thaliana* (Uozumi et al., 2000; Mäser et al., 2002), which is thought to be present around xylem and phloem tissues in the root and shoot/leaves, and it also increases $Na^+$ retention in the root directly (Mäser et al., 2002; Sunarpi et al., 2005).

*Citrus* is considered to be a salt sensitive crop (Maas, 1990); however, the ability of citrus trees to tolerate salinity varies among different species and depends on the rootstock (Maas, 1993). Moreover, citrus rootstocks widely differ in their ability to restrict uptake and/or transport of $Cl^-$ and $Na^+$ (Grieve and Walker, 1983; Walker, 1986; Bañuls and Primo-Millo, 1995). In this sense, there are indeed rootstocks exhibiting high absorption of $Na^+$ and $Cl^-$ exclusion, such is the case of Cleopatra mandarin, while others behave the other way around, i.e., *Poncirus trifoliata* and its hybrids, which accumulate $Cl^-$ but are efficient $Na^+$ excluders at low salinities (Elgazzar et al., 1965; Walker, 1986; Zekri and Parsons, 1992; Levy and Syvertsen, 2004).

There is abundant literature dealing with the effect of salinity on citrus growth and yield (Maas, 1993; Syvertsen et al., 1993 among many others), on the uptake and allocation of both $Cl^-$ and $Na^+$ at the whole plant level in different rootstocks with varying salt tolerances (García-Sánchez and Syvertsen, 2006; Zekri and Parsons, 1992) and in scion–rootstock combinations (Behboudian et al., 1986; Bañuls et al., 1990); also on the physiological effects of salinity (Syvertsen et al., 1988; Bañuls and Primo-Millo, 1992; Iglesias et al., 2008; López-Climent et al., 2008; García-Sánchez and Syvertsen, 2009). In addition, salt tolerance has been somehow explained in terms of plant growth (rootstock vigor, shoot/root ratio), water use and transpiration (Castle and Krezdorn, 1975; Syvertsen et al., 1989, 2010; Moya et al., 1999, 2003). Nevertheless, it remains unclear the way citrus cope with salinity at cell level. In this sense, little is known concerning gene responses to chloride excess. Brumós et al. (2009) observed a differential regulation in a number of uncharacterized membrane transporter genes, like *NRT1-2*, in the tolerant and the sensitive genotypes, suggesting its potential implication in $Cl^-$ homeostasis. However, the mechanisms involved in primary acquisition, subcellular distribution, and long distance transport of $Cl^-$ is still poorly documented and remains unclear. Moreover, the fact that in citrus (such in *vitis*) does chloride appear to be the more toxic ion (reviewed in White and Broadley, 2001) has led to even less attention being paid to $Na^+$ uptake and transport mechanisms in citrus. In a first approach to explain the mechanisms of $Na^+$ exclusion in citrus, Walker (1986) suggested the ability of xylem parenchyma cells to extract $Na^+$ from the xylem stream and sequester it in the woody root and stem tissues. González et al. (2012) stated the role of vacuolar sequestration of $Na^+$ ions in the roots as a mechanism to maintain lower $Na^+$ levels in the leaves. These mechanisms probably rely on membrane bound carriers; nevertheless, knowledge dealing with $Na^+$ membrane transporters in citrus is negligible. Divergence among rootstocks in relative accumulation of $Na^+$ will thus become clearer once the processes involving uptake and transport of this ion are better understood.

The aim of this study was to provide insight into the links between the expression levels of candidate $Na^+$ transporter genes (*SOS1*, *NHX1*, *HKT1*), as well as tonoplast proton pumps (*V-ATPase*, *V-PPiase*), and $Na^+$ tolerance in two citrus rootstocks, Cleopatra mandarin and trifoliate orange, differing in their $Na^+$ exclusion capacity under salinity stress. It is hypothesized that pathways of $Na^+$ influx and efflux, such as $Na^+/H^+$ antiports, may vary in activity depending on the rootstock.

## 2. Materials and methods

### 2.1. Plant material

The genotypes that were studied in this work were Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) a poor $Na^+$-excluder, and trifoliate orange (*P. trifoliata* (L.) Raf. cv. Rubidoux) an efficient $Na^+$-excluder (Walker, 1986; Levy and Syvertsen, 2004).

Seeds of Cleopatra mandarin and trifoliate orange were surface sterilized by immersion in 30% commercial bleach for 15 min and then rinsed four times in sterile distilled water. Seeds were sown into test tubes (one seed per tube) containing agar media ($4\,g\,L^{-1}$). Because of the difference in growth rate of both genotypes (trifoliate orange grew slightly slower than Cleopatra mandarin did), root length was chosen as a criteria for transplanting, in order to ensure that salinity was applied at a similar physiological stage. After emergence, when roots were approximately 2 cm length (approximately two weeks old), uniform plantlets were individually transplanted to test tubes containing half-strength Hoagland's agar ($2.5\,g\,L^{-1}$) medium (pH 6) either with (60 mM) or without NaCl (0 mM). The experimental design was a two rootstock (Cleopatra mandarin and trifoliate orange) × two salinity levels (0, 60 mM NaCl), with four replicates per treatment, each replication comprising 20–30 pooled plantlets. Plants were grown in a growth chamber, at 28 °C/23 °C (light/dark), photoperiod of 16/8 h (light/dark), 80% relative humidity, and white fluorescent tubes.

Thirty days after transplanting plantlets were harvested; at this time $Na^+$ had almost reached a steady state level, thus assuming that the mechanisms for $Na^+$ stress tolerance had been triggered, and visible symptoms of salt injury (leaf burn) in the $Cl^-$ sensitive trifoliate orange rootstock were about to appear. In citrus seedlings, $Na^+$ reaches a steady state level in leaves within few weeks (Storey, 1995), whereas in citrus trees, with large buffering capacity, this may take 4–5 months (Lloyd and Howie, 1989). Harvested plantlets were segregated into roots and shoots and