

## High-affinity potassium uptake in seedlings of two citrus rootstocks Carrizo citrange (*Citrus sinensis* [L.] Osb. × *Poncirus trifoliata* [L.] Raf.) and Cleopatra mandarin (*Citrus reshni* Hort. ex Tanaka)

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### Abstract

Plant growth and K<sup>+</sup> influx responses to K<sup>+</sup> supply were studied in two citrus rootstocks (Carrizo citrange, *Citrus sinensis* [L.] Osb. × *Poncirus trifoliata* [L.] Raf.; Cleopatra mandarin, *Citrus reshni* Hort. ex Tanaka) to determine mechanisms of K<sup>+</sup> acquisition. Seedlings of Carrizo citrange (CC) and Cleopatra mandarin (Cleo) were grown in aerated nutrient solutions for 40 days with either no K<sup>+</sup> added (2–5 μM K<sup>+</sup>), or with additional 25, 50 or 100 μM K<sup>+</sup>. Growth parameters, mineral composition and K<sup>+</sup> influx by K<sup>+</sup> depletion from solutions were determined. The rootstock CC showed greater growth than Cleo, but the effect of K<sup>+</sup> treatments was similar in both, reaching a similar growth at 25, 50 and 100 μM K<sup>+</sup>. There was a decrease in total biomass and leaf K<sup>+</sup> concentration (21% and 38%, respectively) in non-K<sup>+</sup> seedlings when compared to rest of the K<sup>+</sup> treatments. Seedlings treated with low K<sup>+</sup> having root K<sup>+</sup> concentrations below 29 mg g<sup>-1</sup> root dry weight induced the high-affinity K<sup>+</sup> uptake that depleted K<sup>+</sup> from a 50-μM K<sup>+</sup> solution within 7 h. In this experiment, genes encoding putative high-affinity K<sup>+</sup> transporters were isolated and characterised. The study reported that these genes have a high homology with the Arabidopsis AtKUP3, which encodes a putative K<sup>+</sup> transporter of the phylogenetic group II of the KT/HAK/KUP family of K<sup>+</sup> transporters. Therefore, the data suggest that citrus seedlings possess efficient high-affinity K<sup>+</sup> uptake systems that supply K<sup>+</sup> for growth even at very low K<sup>+</sup> concentrations and that genes of KT/HAK/KUP family of K<sup>+</sup> transporters are apparently present in these citrus genotypes.

**Keywords:** Carrizo citrange, Cleopatra mandarin, Plant growth, K<sup>+</sup> depletion, K<sup>+</sup> transporter, HAK, Phylogenetic tree.

**Abbreviations:** Dw\_dry weight; Cleo\_Cleopatra mandarin; CC\_Carrizo citrange; HAK\_high affinity K<sup>+</sup> transporter; HAK/KUP/KT\_high-affinity K<sup>+</sup> symporter family; AtKUP1\_Potassium Transporter from Arabidopsis.

### Introduction

One of the most abundant components of plant cells is potassium (K<sup>+</sup>) which composes up to 10% of total plant dry weight (dw) (Leigh and Jones, 1984). K<sup>+</sup> fulfils important functions and when K<sup>+</sup> is deficient, growth is not only retarded but also it is more sensitive to biotic and abiotic stresses increases and the plant life cycle can be altered (Marchner, 1995). Plants grow well over a wide range of K<sup>+</sup> concentrations in the root medium (approx. 10 μM–10 mM), which contrasts with the relatively constant and low K<sup>+</sup> concentration of the cytosol (about 100 mM, Walker et al., 1996; Leigh, 2001). At low external K<sup>+</sup> concentrations, high-affinity K<sup>+</sup> uptake systems enable root cells to accumulate K<sup>+</sup> to support growth in some plant species (Martínez-Cordero et al., 2004, 2005). Originally characterised in barley, this high-affinity K<sup>+</sup> uptake had a K<sub>m</sub> of 18 μM for K<sup>+</sup>, no discrimination between K<sup>+</sup> and Rb<sup>+</sup> and was weakly inhibited by Na<sup>+</sup> (Epstein et al., 1963). Molecular approaches led to the identification of gene families encoding K<sup>+</sup> transporters and channels (Maser et al., 2001). High-affinity K<sup>+</sup> uptake has been postulated to be mediated mainly by HAK1-type transporters (Rodríguez-Navarro and Rubio, 2006) that belong to group I of the KT/HAK/KUP family (Rubio et al., 2000). AKT1-like inward-rectifier K<sup>+</sup> channels may also

contribute to K<sup>+</sup> uptake at low external K<sup>+</sup> concentrations (Hirsch et al., 1998; Spalding et al., 1999; Rubio et al., 2008; Aleman et al., 2011). Genes encoding HAK1-type transporters have been isolated from several crop species (Santa-María et al., 1997; Bañuelos et al., 2002; Martínez-Cordero et al., 2004; Nieves-Cordones et al., 2007) and *Arabidopsis thaliana* (Rubio et al., 2000). The expression of these HAK1-type genes in yeast showed that they encode high-affinity K<sup>+</sup> transporters with kinetic characteristics similar to the high-affinity K<sup>+</sup> uptake present in other plant roots that are induced by K<sup>+</sup> starvation (Santa-María et al., 1997; Bañuelos et al., 2002; Ahn et al., 2004; Armengaud et al., 2004; Martínez-Cordero et al., 2004; Shin and Schachtman, 2004; Gierth et al., 2005; Nieves-Cordones et al., 2007). Other factors may also regulate the expression of high-affinity K<sup>+</sup> uptake in other species. For example, the expression induced by K<sup>+</sup> starvation is reduced in pepper *CaHAK1* by the presence of NH<sub>4</sub><sup>+</sup> (Martínez-Cordero et al., 2005) but is enhanced by NH<sub>4</sub><sup>+</sup> in the tomato *LeHAK5* (Nieves-Cordones et al., 2007, 2008). In addition, the presence of NaCl prevents the induction of *LeHAK5* by K<sup>+</sup> starvation in tomato (Nieves-Cordones et al., 2007, 2008). Although it has been shown that some transporters of group

**Table 1.** Total biomass, shoot to root relationship, leaf and root K<sup>+</sup> concentration and K<sup>+</sup> uptake of the citrus seedling rootstocks CC and Cleo under different supplies of K<sup>+</sup>.

Main effects	Total biomass (g dw)	Shoot:root	Leaf K concentration (mg g <sup>-1</sup> dw)	Root K concentration (mg g <sup>-1</sup> dw)	K uptake (μg g <sup>-1</sup> root dw day <sup>-1</sup> )
Rootstock					
CC	0.61 A	0.36	9.26	26.07	2.29
Cleo	0.46 B	0.34	9.56	20.57	2.63
K treatments (μM)					
0	0.44 b	0.29 b	4.19 b	11.42	0.95
25	0.61 a	0.40 a	11.37 a	29.00	3.15
50	0.56 a	0.37 a	10.85 a	23.66	2.77
100	0.51 ab	0.33 ab	10.73 a	27.73	2.83
K treatment x Rootstock interaction					
0	0.46	0.26	3.72	10.53 c	0.71 d
25	0.72	0.43	11.93	34.59 a	3.17 ab
50	0.61	0.36	9.94	24.27 b	2.26 c
100	0.61	0.36	10.34	31.79 a	2.72 bc
CC					
0	0.42	0.31	4.56	12.13 c	1.15 d
25	0.50	0.37	10.82	23.42 b	3.13 ab
50	0.51	0.37	11.76	23.05 b	3.29 a
100	0.41	0.30	11.11	23.68 b	2.93 ab
Cleo					
0	0.42	0.31	4.56	12.13 c	1.15 d
25	0.50	0.37	10.82	23.42 b	3.13 ab
50	0.51	0.37	11.76	23.05 b	3.29 a
100	0.41	0.30	11.11	23.68 b	2.93 ab
ANOVA					
Rootstock	**	Ns	Ns	***	**
K treatment	*	*	***	***	***
R x K	Ns	Ns	Ns	***	*

ns, \*, \*\* and \*\*\* indicate non-significant or significant differences at  $P < 0.05$ ,  $0.01$  or  $0.001$ , respectively, for the two way interaction rootstock x potassium supplement treatments. Significant differences ( $P < 0.05$ ) between treatments are denoted with different lower case letters. Differences between rootstocks are indicated by different upper case letters.

II may be involved in low-affinity K<sup>+</sup> uptake or in K<sup>+</sup> movements across the tonoplast (Senn et al., 2001; Bañuelos et al., 2002), little information is available about members of other plant groups of the KT/HAK/KUP family. Citrus is an important crop worldwide that is very often cultivated in arid and semiarid regions with low soil K<sup>+</sup> concentrations, so K<sup>+</sup> fertilizer is an important component in citriculture. Citrus trees are grown on rootstocks, which vary in their ability to extract K<sup>+</sup> from fertilized soils (Wutscher, 1989). Mechanisms involved in K<sup>+</sup> acquisition in citrus were however not studied, and little is known about K<sup>+</sup> uptake especially under low supply. We have undertaken the physiological and molecular characterisation of K<sup>+</sup> uptake at low external concentrations [0–100 μM] in two citrus rootstock cultivars, Carrizo and Cleopatra, which are widely used in the Mediterranean area.

## Results and discussion

### Plant growth and K<sup>+</sup> concentration in the plant tissue

At the end of the experiment, CC seedlings had higher total biomass dry weight but similar shoot to root ratio than Cleo. Seedlings that were not treated with K<sup>+</sup> grew an average of 21% less than those treated with K<sup>+</sup> (Table 1). In addition, this parameter was greater when external K<sup>+</sup> concentrations were 25 and 50 μM for Cleo, and 25 μM for CC, relative to the control treatment, although interaction rootstock x potassium treatment was not significant. Because in the 100-μM K<sup>+</sup> treatment there were no significant differences in the total biomass with the rest of treatments (Table 1); it could be suggested that citrus plants possess efficient high-affinity K<sup>+</sup> uptake systems that supply K<sup>+</sup> for growth, even at very low K<sup>+</sup> concentrations. This idea is also supported by the leaf K<sup>+</sup> concentration data, as these values were similar in plants treated with 25, 50 and 100 μM K<sup>+</sup> and they were in an optimum range for citrus (7–11 mg g<sup>-1</sup> dw; Embleton et al.,

1973), whereas only the plants treated with 0-μM K<sup>+</sup> were K<sup>+</sup> deficient. It is well known that plant species possess physiological and molecular mechanisms to cope with low K<sup>+</sup> concentration in the root medium. These mechanisms were described in several horticultural crops, such as rice (Yao et al., 2010), tomato (Nieves-Cordones et al., 2008) and pepper (Rubio et al., 2010; Pacheco-Arjona et al., 2011), however, they have not been described till date in woody plants including citrus trees. The rates of K<sup>+</sup> absorption in the control treatment (0 μM K<sup>+</sup>) were averaged  $< 1 \mu\text{g g}^{-1} \text{ root d}^{-1}$  but K<sup>+</sup> absorption rates did not reach zero (Table 1) due to the traces of K<sup>+</sup> (2–5 μM) supplied by the other salts in the nutrient solution. When external K<sup>+</sup> was increased to 25 μM K<sup>+</sup>, the absorption rates increased. Further increases in external K<sup>+</sup> tended to reduce the K<sup>+</sup> absorption rates in CC and did not produce increases in the absorption rates in Cleo. Root K<sup>+</sup> concentrations in Cleo seedlings were similar for plants treated with 25, 50 and 100 μM K<sup>+</sup> and were significantly higher than those from the control treatment. In CC, however, the highest root K<sup>+</sup> concentration was obtained with 25- and 100-μM K<sup>+</sup>, followed by 50-μM and control treatment. These results in CC are intriguing and need further characterisation especially because CC is considered to be an Na<sup>+</sup> excluder under salinity stress (Syvertsen et al., 2010) and high-affinity K<sup>+</sup> uptake may allow K<sup>+</sup> to play a role in maintaining ionic balance during excess Na<sup>+</sup> stress (García-Sánchez et al., 2000). Maximal rates of K<sup>+</sup> absorption and K<sup>+</sup> concentration were achieved at a K<sup>+</sup> concentration as low as 25 μM K<sup>+</sup> in both genotypes.

### Potassium depletion in the nutrient solution

To evaluate the effect of K<sup>+</sup> starvation on K<sup>+</sup> uptake by citrus rootstock, the seedlings grown in the 0-, 25-, 50- and 100-μM KCl and maintained for a time period of 40 days were rinsed with cold K<sup>+</sup>-free solution, at time zero, and were transferred to containers filled with 180 ml of nutrient solution with 50

$\mu\text{M K}^+$ . The analysis showed that only plants grown with 0- $\mu\text{M K}^+$  depleted external  $\text{K}^+$  from the 50- $\mu\text{M K}^+$  solution, whereas plants grown with 25- $\mu\text{M K}^+$  or more did not deplete external  $\text{K}^+$  (Fig. 1). CC plants grown in 0  $\text{K}^+$  were able to deplete external  $\text{K}^+$  to zero within 7 h, while  $\text{K}^+$  Cleo plants depleted external  $\text{K}^+$  to about 20  $\mu\text{M}$ . This difference between CC and Cleo could be due to the higher root dry weight of CC, rather than due to a different functionality of the high-affinity  $\text{K}^+$  transporters. In fact, when the average  $\text{K}^+$  absorption rates were calculated from these depletion experiments, a value of  $1.48 \mu\text{g K}^+ \text{g}^{-1} \text{root dw min}^{-1}$  was obtained for both rootstocks (data not shown). These data support the hypothesis that citrus rootstocks possess an efficient high-affinity  $\text{K}^+$  uptake system that is induced in  $\text{K}^+$ -deficient plants with a root  $\text{K}^+$  concentration below  $29 \text{ mg g}^{-1} \text{root dw}$  (Table 1). When the root  $\text{K}^+$  concentration was above that value, (for the 25, 50 or 100  $\mu\text{M K}^+$  treatments,  $\text{K}^+$  depletion in the nutrient solution was not observed. Previous studies on other plant species have shown that there is a threshold  $< 30 \text{ mg g}^{-1} \text{root dw}$  in root  $\text{K}^+$  concentration below which high-affinity  $\text{K}^+$  uptake is induced (Martínez-Cordero, 2005).

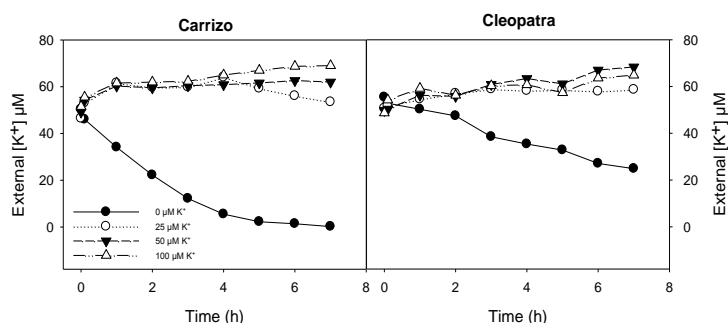
#### Phylogenetic tree of transporters of the family KT/HAK/KUP

HAK transporters of the phylogenetic group I have been suggested as major contributors to high-affinity  $\text{K}^+$  uptake (Rodríguez-Navarro and Rubio, 2006; Aleman et al., 2011). In this experiment, our results suggest that citrus plants have developed efficient high-affinity  $\text{K}^+$  uptake when grown in very low  $\text{K}^+$ . Therefore, we pursued the isolation of cDNAs corresponding to genes encoding HAK transporters from CC roots grown in low  $\text{K}^+$ . Total RNA from roots was purified, and cDNA fragments were isolated using RT-PCR. After sequencing several cDNA fragments, it was observed that all of them corresponded to the same gene. The translated amino acid sequence encoded in this cDNA showed the highest homology with the Arabidopsis AtKUP3 (Fig. 2), a putative  $\text{K}^+$  transporter of group II and it was named CsHAK3. Most high-affinity  $\text{K}^+$  transporters belong to group I of the KT/HAK/KUP family (Rodríguez-Navarro and Rubio, 2006). It is still not elucidated whether or not transporters of group II in citrus mediate high-affinity  $\text{K}^+$  uptake. Further studies with a full-length cDNA of the transporter reported here by its expression in heterologous systems are required to clarify this point. However, it is clear that these citrus seedlings possess efficient high-affinity  $\text{K}^+$  uptake systems that can be induced by low  $\text{K}^+$  supply to support growth.

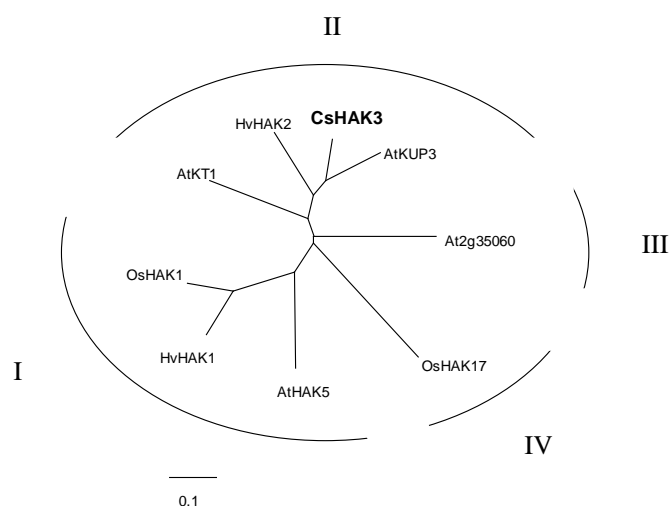
#### Materials and methods

##### Plant growth and $\text{K}^+$ uptake

Seeds of Carrizo citrange (*Citrus sinensis* [L.] Osb.  $\times$  *Poncirus trifoliata* [L.] Raf.; CC) and Cleopatra (*Citrus reshni* Hort. ex Tanaka; Cleo) were surface-sterilised briefly in 70% ethanol, followed by 20% (v/v) commercial bleach for 15 min and washed with sterilised water. Seeds of both the rootstocks were germinated in trays containing sterilised vermiculite wetted with  $0.5 \text{ mmol l}^{-1} \text{CaSO}_4$ , in the dark at  $29^\circ\text{C}$ . When the radicles were 3 to 4 cm long, the seedlings were transferred to 300-ml containers filled with a continuously aerated nutrient solution (1/5 Hoagland) containing the following macronutrients (mM): 1.4  $\text{Ca}(\text{NO}_3)_2$ , 0.35  $\text{MgSO}_4$  and 0.1  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ , and the micronutrients ( $\mu\text{M}$ ): 50  $\text{CaCl}_2$ , 12.5  $\text{H}_3\text{BO}_3$ , 1  $\text{MnSO}_4$ , 1



**Fig 1.** Potassium depletion of the citrus rootstocks CC and Cleo under different supplies of  $\text{K}^+$ . The plants grown in Hoagland solution containing the indicated concentrations of  $\text{K}^+$ . Plants were transferred at time zero to a solution containing 50  $\mu\text{M K}^+$  and at different time points the external  $\text{K}^+$  was determined. A representative experiment is shown.



**Fig 2.** Phylogenetic tree of transporters of the KT/HAK/KUP family. A phylogenetic tree was constructed with representative HAK transporters of the four phylogenetic groups of the family. According to this tree, the putative HAK transporter corresponding to the cDNA fragment isolated from CC roots (CsHAK3) belongs to group II of the family.

$\text{ZnSO}_4$ , 0.5  $\text{CuSO}_4$ , 0.1  $\text{H}_2\text{MoO}_4$ , 0.1  $\text{NiSO}_4$  and 10  $\text{Fe-EDDHA}$ . Four  $\text{K}^+$  treatments were established by adding 0, 25, 50 and 100  $\mu\text{M KCl}$  to the nutrient solution. The presence of traces of  $\text{K}^+$  in the other nutrient salts used to prepare the nutrient solution did that in the 0  $\text{K}^+$  treatment contained 2–5  $\mu\text{M K}^+$  as determined by atomic spectrophotometry. Five replicate plants per  $\text{K}^+$  treatment of each rootstock were grown for 40 days in a controlled environment chamber at a day/night temperature of  $25/20^\circ\text{C}$ , a day/night relative humidity of 65/85%, and a 16-h photoperiod with a photon flux density of  $550 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The solution was renewed every 3 days, and the pH was adjusted daily to 6.0–6.5. In addition, before starting the  $\text{K}^+$  treatments, six seedlings of each rootstock were harvested to establish dry mass and  $\text{K}^+$  status. After 40 days of  $\text{K}^+$  treatments, a short-term  $\text{K}^+$  depletion experiment was carried out. The roots of six plants from each  $\text{K}^+$  treatment were rinsed in a cold  $\text{K}^+$ -free solution and immediately transferred to individual containers of 180 ml of  $\text{K}^+$ -free Hoagland solution supplemented with 50  $\mu\text{M}$

K<sup>+</sup>. K<sup>+</sup> depletion in the nutrient solution was monitored during 7 h by hourly determining the K<sup>+</sup> concentration of solutions. The plants were then separated into root, stem and leaves, and fresh weights were determined. Plant material was dried at 65°C for 4 days and dry weights were determined. Chemical analyses of powdered tissues were carried out after digestion with HNO<sub>3</sub>:HClO<sub>4</sub> (2:1, v:v). The K<sup>+</sup> concentrations were determined using ICP (Iris Intrepid II, Thermo Electron Corporation, Franklin, USA). In addition, K<sup>+</sup> absorption rates by roots were calculated from the K<sup>+</sup> contents in the plants as  $[(\ln(R_{40}) - \ln(R_0)) / (R_{40} - R_0)] \times [(C_{40} - C_0) / (t_{40} - t_0)]$ , where  $R$  is root dry weight and  $C$  is total K<sup>+</sup> content of the whole plant at harvest times 0 ( $t_0$ ) and 40 days ( $t_{40}$ ) after beginning the K<sup>+</sup> treatments.

### High-affinity K<sup>+</sup> genetics

A subset of three plants from the control treatment were separated into roots and leaves and frozen in liquid nitrogen. Total RNA (2 µg) from roots was isolated and reverse-transcribed (RT) using an anchored oligo-dT primer and avian myeloblastosis virus transcriptase (Amersham Pharmacia Biotech, Uppsala), following standard protocols (Sambrook et al., 1989). The reverse transcription products were amplified by polymerase chain reaction (PCR) with the Pfu polymerase (Invitrogen, Carlsbad, CA), using the degenerate sense primer 5'-GAYAA YGGNGANGNG-GNACNTTYGC-3' and the degenerate antisense primer 5'-AANTGNCCNARRTCNGCRAA-3' deduced from the conserved regions DNG[D/E]GGTFA and FADLGHF, respectively, present in HvHAK1 and HvHAK2 (Rubio et al., 2000). PCR products were cloned into the PCR2.1 vector, using the TA cloning kit (Invitrogen, Carlsbad, CA), and sequenced.

### Statistical analysis

Data were subjected to analysis of variance using a two-way ANOVA (SPSS Statistical Package; SPSS Inc., Chicago, IL, USA) with two rootstock genotypes  $\times$  four K<sup>+</sup> treatments and five replicate plants per treatment. Treatment means were separated using Duncan's multiple range tests (Little and Hills, 1987).

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