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ORIGINAL ARTICLE

Genetic analysis of salt tolerance in a progeny derived from the citrus rootstocks Cleopatra mandarin and trifoliate orange

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Abstract A total of 60 traits that could be related to salt tolerance were genetically analyzed using nucellar plants as repetitions of apomictic hybrids in a reference population derived from two common citrus rootstocks, Cleopatra mandarin (salt tolerant) and trifoliate orange (salt sensitive), in two experiments differing in duration (1 versus 3 years) [NaCl] (30 versus 25 mM) and environmental control (greenhouse versus screenhouse). In both experiments, the trifoliate parent always showed less aerial vegetative growth than Cleopatra, and under salinity, the trifoliate parent showed higher Na^+ and Cl^- leaf concentrations than the salt-tolerant parent. Salinity affected the relationships among traits, particularly those involving leaf water potential; leaf concentrations of Cl^-, K^+, B and Fe; and root [Na^+]. Most traits showed heritabilities below 0.6, and their quantitative trait locus (QTL) analyses were carried out using three mapping procedures to obtain complementary genetic information on trait inheritance. A total of 98 QTLs were detected by interval mapping and multiple QTL mapping procedures. Fresh and dried weights of the leaf, studied in both experiments, showed common QTLs, remarking their repeatability. A cluster of QTLs governing plant vigour and leaf boron concentration pointed a genomic region in linkage group 3 as the most relevant one to improve salt tolerance using the Cleopatra parent as donor. Besides, a QTL genotype in linkage group 7, associated with the smallest leaf water potential and defoliation index under salinity, corresponded to the highest leaf [Na^+] and the largest leaf area, suggesting the presence of a putative tissue salt tolerance QTL.

Keywords QTL analysis · Plant vigour · Root · Leaf water potential · B nutrition · Leaf Fe · Candidate genes

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Introduction

Six Mediterranean countries, Spain, Turkey, Egypt, Morocco, Italy and Israel, were among the major producers for mandarins, sweet oranges, grapefruits, lemons and/or limes in the world in 2012 (FAOSTAT 2015). However, this region is affected by water scarcity what forces the use of marginal water for irrigation (Oron et al. 2002) making it more vulnerable to salinization. In the future, this situation is expected to get worse in the Mediterranean region due to the effects of climate change on precipitation, evaporation, runoff and soil moisture storage (Paranychianakis and Chartzoulakis 2005). Citrus is classified among the most salt-sensitive tree crops (Maas 1993). Tree growth and fruit yield of citrus species are impaired at a soil salinity of approximately 2 dS m^{-1} soil saturation, without the concomitant expression of leaf symptoms (Bingham et al. 1974; Cerdá et al. 1990). Therefore, the adoption of both intense management practices and the use of salt-

tolerant genotypes are generally recommended to maintain citrus productivity and to ensure land sustainability.

Salt tolerance in a crop species is conceived as the ability to reduce yield losses when exposed to salinity. Since citrus cultivars are always vegetatively propagated by bud grafting on a seedling rootstock, salt tolerance is usually one of the target traits in rootstock breeding programs taking advantage of the wide genetic diversity and adaptations of *Citrus* spp. and citrus relatives (Herrero et al. 1996). Thus, Cooper et al. (1956) early classified citrus species used as rootstocks in three groups: *Citrus reshni* (Cleopatra mandarin) as good salt tolerant, *Citrus volkameriana* (Volkamer lemon) and *Citrus aurantium* (sour orange) as medium salt tolerant and *Poncirus trifoliata* (trifoliate orange) as poor salt tolerant. However, salt tolerance in citrus can display large variation from one area to another (Maas 1993) probably the cause of direct and indirect interactions between salinity and other physical abiotic stresses like poor soil drainage, drought, irradiance, leaf temperature and atmospheric evaporative demand (Syversten and Levy 2005). Moreover, genetic diversity for salt tolerance among accessions may also exist within each species, as it was recently found in the "salt-sensitive" *P. trifoliata* regarding Cl^- and Na^+ excluding capacities (Sykes 2011). From the agronomic point of view, salt tolerance should be considered in terms of fruit yield; however, this type of evaluation is extremely lengthy and difficult in citrus progenies (Raga et al. 2014, García et al. 2002). Therefore, it is necessary to approach the genetic analysis of salt tolerance in non-grafted progenies derived from salt-tolerant citrus rootstocks where it is reasonable to expect segregation for genes controlling Cl^- and Na^+ homeostasis, vegetative growth, water potential and other traits possibly related to the salt tolerance conferred by the citrus rootstock. Since these traits are quantitative, citrus rootstock breeding could benefit from their quantitative trait locus (QTL) analyses. In fact, QTL analysis of salt tolerance is considered a prerequisite to allow cost-effective applications of genomic-based approaches to breeding programs (Collins et al. 2008) which are particularly long and expensive in the case of citrus rootstocks. The detection of genomic regions containing QTLs controlling salt tolerance would be useful in the search for candidate genes and the implementation of marker-assisted selection schemes in breeding programs of citrus rootstock. An early attempt of QTL analysis of salt tolerance was reported by Tozlu et al. (1999a, 1999b) using a clonally propagated BC1 progeny derived from a citrus rootstock (from *P. trifoliata*) and a citrus variety (from *Citrus grandis*). However, *C. grandis* shows no apomictic reproduction and citrus rootstocks must be able to reproduce asexually through seed by apomixis (adventitious embryony from nucellar cells) to ensure the cheap establishment of well-rooted, uniform orchards. Therefore, a citrus progeny derived from two citrus rootstocks, being at least one of them salt tolerant, would be a more realistic approach. Besides, genotype replication through seed would be possible here by selecting apomictic hybrids within the progeny for the genetic analysis. We have chosen this approach by using a segregating population derived from two citrus rootstocks: *P. trifoliata* and *C. reshni* (the well-known salt-tolerant rootstock Cleopatra mandarin). However, two problems had to be faced in the experimental design: (1) the assessment of nucellar seedlings and (2) the limited number of apomictic hybrids in the progeny. The former can be solved by marker analysis (Ruiz et al. 2000), but the latter, which greatly affects the power of detection of QTLs (Asins 2002), is limited by the size of the original population and the accompanying, maintaining costs. To counteract this limitation, we used replicated progenies which can bring about a major reduction in the number of genotypes that need to be scored, and we carried out two different salt tolerance experiments to test the repeatability of QTLs detected for leaf weight traits.

The objective of this study is the genetic analysis of salt tolerance in terms of vegetative and physiological traits in a progeny derived from two well-known citrus rootstocks, Cleopatra mandarin and trifoliate orange. Both show apomictic reproduction but are genetically distant and differ in many morphological, physiological and agronomic traits, including salt tolerance.

Materials and methods

A reference population that consists of 151 hybrids (RxPr) which had been previously genotyped (Raga et al. 2012) was used for two salt tolerance experiments. This population was obtained at IVIA (Valencia, Spain) by controlled crosses between *Citrus reshni* Hort. ex. Tan. (Cleopatra mandarin) as female (salt tolerant and apomictic) parent and two apomictic and disease-resistant varieties of *P. trifoliata* (L.) Raf. (trifoliate orange): Flying Dragon (83 hybrids) and Rich (68 hybrids) as pollinators.

Plants were cultured into pots (3.5 L), and repetitions (nucellar plants) of each genotype were randomly selected to establish two treatments: control (no NaCl added) and saline irrigation (25 or 30 mM NaCl, depending on the experiment). Zygotic seedlings were identified by analysis of molecular markers (Ruiz et al. 2000) and discarded to keep nucellar seedling only.

The first experiment (Exp. 1) was run under a screenhouse during 3 years (2006–2008). It was started with 1-year-old seedlings from 24 apomictic hybrids in 2006. To increase the number of apomictic genotypes in the progeny that was limited by the juvenility and alternate bearing of the reference population, 1-year-old seedlings from new apomictic RxPr hybrids were added to this experiment in 2007 (18) and in 2008 (28). Thus, there was a total of 533 plants in experiment 1 during 2008 that corresponded to the nucellar progeny from

70 RxPr hybrids and two parents, Cleopatra mandarin and Flying dragon, as controls. Plants were grown with a sterilized substrate mix (50 % peat, 30 % coconut fibre, 15 % sand and 5 % perlite) and placed following a completely randomized design with three to five repetitions per hybrid and treatment. Salt treatment (25 mM NaCl) was applied through irrigation between June and September. Plants were irrigated with 250 ml each of just tap water (0.1 dS/m; pH 6.0, as control) or salted water (salt treatment) three times a week during the salinity treatment. NaCl concentration was increased gradually from 10 to 25 mM (3.2 dS/m; pH 7.5) in a week (conductivity meter HANNA HI9033 multi-range instruments). Both control and salinity-treated plants received the same pest, disease and weed control; fertilization; and pruning work. Fertilizer was proportioned automatically by mixing in a 1:100 proportion the stock solution A (200 mM $NH_4H_2PO_4$) and solution B. The stock solution B contained 12 M $Ca(NO_3)_2$, 2.6 M KNO_3, Sequestrene 138Fe [36 g L^{-1}; (Syngenta, Madrid, Spain)] and 5 % of microelement solution. This solution contained 0.3 mM Cu $SO_4{\cdot}5H_2O$, 3.1 mM Zn $SO_4{\cdot}7H_2O$, 109 mM Mn $Cl_2{\cdot}4H_2O$, 92 mM BO_3H_3, 2 mM NH_4MoO_4 and 0.4 mM V_2O_5. Salt-treated plants were grown similarly to control plants from October 2006 to June 2007 and from October 2007 to June 2008. The following vegetative and physiological characters were evaluated (Table 1): dried and fresh leaf weight in grams (DLW and FLW, respectively); accumulated dry matter percentage (%DMA) measured as the difference between dry leaf weight at the beginning and at the end of salt treatment; leaf water content (LWC) evaluated as the difference between leaf fresh and dried weights; leaf area (LA) in square centimetre and the increment in LA during the saline treatment (LAG) in cm^2; and leaf colour parameters LCL*, LCa* and LCb* defined by Hunter (L* a* b*) coordinates arranged in a Cartesian system and Cr* and Hue* defined by a cylindrical coordinate system (L* C* h), where Cr* (Chroma) represents colour intensity (0 to 60) $\{Cr^* = \sqrt{[(a^*)^2 + (b^*)^2]}\}$ and Hue* (Hue angle) represents leaf colour (0° to 360°) [$Hue^* = \tan^{-1}(b^*/a^*)$] (HunterLab 1996); leaf Cl^- concentration (mg/L) was measured as described by Gilliam (1971) using a Sherwood chloride analyzer 926.

The second experiment (Exp. 2) was run under a greenhouse with controlled humidity and temperature during 3.5 months. Plants were distributed in six blocks following a split-plot design (one repetition per block and treatment) using salinity treatment as the main plot. Seventeen-month-old, nucellar seedlings from 41 apomictic RxPr hybrids were grown in substrate until the experiment was started. Then, substrate was changed to sand and the salt treatment (half diluted Hoagland solution plus 25 mM NaCl) started on June 28, 2010, and reached 30 mM NaCl on July 5, 2010. Plants were harvested for evaluation at the end of the experiment (October 14, 2010).

Several vegetative and physiological traits were evaluated in Exp. 2 (Table 1): defoliation index (DI, visually assigned from 0 to 10), dried and fresh leaf weight in grams (DLW and FLW, respectively), plant height (H) in centimetre, stem diameter (SD) in millimetre and leaf water potential (Ψ) in megapascal measured around solar noon using a Scholander pressure chamber (Scholander et al. 1965) and following the precautions described in Turner (1981). Leaf water potential was measured 7 and 80 days after salinity treatment imposition (Ψ7 and Ψ80, respectively). The difference between both values Ψ 7 and Ψ 80 was named d Ψ. Tissue samples of leaf and root were fresh weight determined, oven dried for 48 h at 80 °C, weighted (dry weight) and prepared for mineral analysis by digestion in a $HNO_3/HClO_4$ (2:1, *v*/*v*) solution. Inorganic solutes (Al, B, Ca, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, Ti, V, Zn) were determined in parts per million by inductively coupled plasma (ICP) spectrometry (Ionomic Service; CEBAS–CSIC, Murcia, Spain) in leaf (L) and root (R) tissues. The differences between leaf and root concentrations (L-R) of some elements (Na, K, Ca, P) and the fresh and dried weights of the root system (total fresh root weight (TFRW) and total dried root weight (TDRW)) were also considered; leaf and root water contents were estimated as the difference between fresh and dried weights (LWC and root water content (RWC), respectively).

For comparative purposes, three traits were evaluated in both experiments as salt tolerance indicators: FLW, DLW and LWC. Other traits are specific of experiment 1 or 2 (Table 1).

For the first experiment, fixed effects for genotype (G), treatment (E) and year (Y) and all their possible interactions, i.e. GxE (genotype by treatment), GxY (genotype by year), ExY (treatment per year) and GxExY (genotype by treatment and year), were analyzed by a repeated measures approach using trees (within each genotype and treatment) as random subject factor and first-order autoregressive covariance structure between measurements taken from the same tree over the years. Pearson correlation coefficients and principal component analysis based on the correlation matrix for the adjusted means were used to study the relations between the different traits. The statistical analysis of the second experiment followed its experimental design; i.e. blocks were random, and to study the GxE interaction, the effects of genotype and treatment were classed as fixed. Considering RxPr hybrid genotypes as a random effects factor, broad-sense heritability (H^2) was estimated for all traits (those evaluated in 2008 in experiment 1 and all evaluated in experiment 2) for nucellar seedlings (repetitions) derived from apomictic RxPr hybrids under control (H^2c) or salinity (H^2s) conditions, based on the genotypic (V_G) and environmental (V_E) variance estimators calculated by minimum variance quadratic unbiased estimator (MIVQUE), as previously reported (Villalta et al. 2007).

QTL analyses were carried out using genotypic and map data from Raga et al. (2012) and adjusted means of traits by interval mapping (IM), multiple QTL mapping (MQM) and

Table 1 Correspondence between the full trait names and their abbreviations

Trait full name	Abbreviation	Experiment
Accumulated dry matter percentage	%DMA	1
Leal Cl^- concentration	Cl	1
Chroma (Cr*) index for colour intensity	Cr	1
Defoliation index	DI	2
Dried leaf weight	DLW	1 and 2
Change in leaf water potential	dΨ	2
Fresh leaf weight	FLW	1 and 2
Plant height	H	2
Hue angle (Hue*) for leaf colour	Hue	1
Leaf Al concentration	L_Al	2
Leaf B concentration	L_B	2
Leaf Ca concentration	L_Ca	2
Leaf Cu concentration	L_Cu	2
Leaf Fe concentration	L_Fe	2
Leaf K concentration	L_K	2
Leaf Li concentration	L_Li	2
Leaf Mg concentration	L_Mg	2
Leaf Mn concentration	L_Mn	2
Leal Na concentration	L_Na	2
Leaf P concentration	L_P	2
Leaf S concentration	L_S	2
Leaf Sr concentration	L_Sr	2
Leaf Ti concentration	L_Ti	2
Leaf V concentration	L_V	2
Leaf Zn concentration	L_Zn	2
Leaf area	LA	1
Increment of leaf area	LAG	1
Leaf colour parameter LCa*	LCa	1
Leaf colour parameter LCb*	LCb	1
Leaf colour parameter LCL*	LCL	1
Difference between leaf and root Ca concentrations	L-R_Ca	2
Difference between leaf and root K concentrations	L-R_K	2
Difference between leaf and root Na concentrations	L-R_Na	2
Difference between leaf and root P concentrations	L-R_P	2
Leaf water content	LWC	1 and 2
Root Al concentration	R_Al	2
Root B concentration	R_B	2
Root Ca concentration	R_Ca	2
Root Cr concentration	R_Cr	2
Root Cu concentration	R_Cu	2
Root Fe concentration	R_Fe	2
Root K concentration	R_K	2
Root Mg concentration	R_Mg	2
Root Mn concentration	R_Mn	2
Root Mo concentration	R_Mo	2
Root Na concentration	R_Na	2
Root Ni concentration	R_Ni	2
Root P concentration	R_P	2
Root S concentration	R_S	2

Table 1 (continued)

Trait full name	Abbreviation	Experiment
Root Sr concentration	R_Sr	2
Root Ti concentration	R_Ti	2
Root V concentration	R_V	2
Root Zn concentration	R_Zn	2
Root water content	RWC	2
Stem diameter	SD	2
Total dried root weight	TDRW	2
Total fresh root weight	TFRW	2
Leaf water potential after 7 days of salt treatment	Ψ_7	2
Leaf water potential after 80 days of salt treatment	Ψ_80	2

Kruskal-Wallis (KW) procedures in MapQTL® 6 (Van Ooijen 2009). Following the strategy explained by Asins et al. (2015), two different QTL detection procedures were used. First, we analyzed the data for each parental meiosis separately i.e. a "two-way pseudo-testcross" analysis (Grattapaglia and Sederoff 1994) what provides the computation advantages of the two genotype QTL models, but the disadvantage of losing power because intralocus interaction is ignored (Van Ooijen 2009). And second, to consider intralocus interaction and unlinked markers and to avoid computation difficulties derived from abundance of dominant markers and segregation distortion, the KW QTL mapping procedure was also used providing complementary genetic information on the highly significant genotypic means. For IM and MQM, a 5 % experimentwise significance level was assessed by permutation tests. These logarithm of the odds (LOD) critical values ranged from 1.2 to 2.5 depending on the trait and linkage group. MQM was used whenever IM detected more than a QTL in the linkage group under study. Only QTLs with LOD ≥2.2 are reported here. For the KW procedure, a cross pollination design of marker genotypes was used and the significance level was fixed at the stringent value of $p \leq 0.005$. Both procedures used different map files accordingly to their respective locus files (Raga et al. 2012). Cleopatra maps contained 81 or 80 markers, distributed along 9 or 12 linkage groups, covering 920.390 or 793.892 cM of the genome depending on the pseudo-testcross or cross pollinated genotype coding, respectively. Similarly, *Poncirus* maps contained 72 or 73 markers, distributed along 11 or 12 linkage groups, covering 682.627 or 729.883 cM of the genome.

Results

Tables 2 and 3 summarise the results from the mixed model analysis. As expected, year effects were significant for all traits in Exp. 1 (Table 2). Genotype effects were significant in most traits. The only exceptions were dΨ, L_Al, R_Al, R_Cu and R_Zn. A smaller proportion of traits showed significant treatment (E) and GxE effects in the Exp. 2 than in Exp. 1. In fact, common traits fresh and dried leaf weight (FLW, DLW) and leaf water content (LWC) showed significant E and GXE effects only in the longest experiment (Exp. 1); however, heritability estimates (also in Tables 2 and 3) were similar between experiments, particularly under salinity. The only vegetative traits significantly affected by salinity in Exp. 2 were those involving the root growth (TFRW, TDRW, RWC). Comparing leaf traits in both experiments, trifoliate parent means for FLW, LWC and DLW were always smaller than those of Cleopatra parent under both treatments indicating the existence of clear developmental and vigour differences for the aerial part of the plant between both rootstocks at origin (Fig. 1). The control and salinity distributions of most relevant traits (TFRW, TDRW, RWC, Ψ7, L_Na, R_Na, L_B, R_B, L_Fe, L_K, R_K and Cl) are shown in Fig. 2. Salinity greatly changed the distribution of Cl, L_Na, R_Na, L_B, L_Fe and L_K. Under salinity, the trifoliate parent showed higher means for Ψ7, R_Na and Na^+ and Cl^- leaf concentrations than the salt-tolerant parent (Cleopatra) whereas under control, the lowest Na^+ concentration corresponded to the trifoliate parent and the lowest Cl^- concentration to Cleopatra. A transgressive segregation was clearly observed for root vegetative traits (TFRW, TDRW, RWC) under both conditions.

Correlation between the adjusted means of all traits was investigated within each treatment, and 99 pairs of traits were significantly correlated under both control and salinity conditions (Online Resource 1). Common traits between experiments were significantly correlated under both control and salinity conditions except for LWC that was only significantly correlated under salinity. When Pearson coefficients of these 99 pairs of traits were graphically represented (Fig. 3), only a few pairs involving Cl^-, R_Na and L_K deviated from the diagonal meaning a change of relationship depending on the controls or salinity condition. Five of these trait pairs, involving leaf Cl^- concentration, changed their sign when comparing control and salinity conditions. Principal component analysis of traits in Exp. 2 (greenhouse experiment) allowed the graphical representation of trait relationships (Fig. 4). Comparing both treatments, the positions of Ψ7, Ψ80, L_B,

Table 2 *P* values for the significant effects in the mixed model analysis and heritability estimates (H^2_C and H^2_S, for control and salinity treatments, respectively) of evaluated traits in experiment 1

Trait Exp.1	G	E	G x E	Y	G x Y	E x Y	G x E x Y	H^2_C	H^2_S
%DMA	<0.0001	ns	0.0233	0.0002	ns	ns	ns	0.49	0.75
Cl^-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.35	0.68
Cr*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0023	0.36	0.43
DLW1	<0.0001	0.0051	0.0009	<0.0001	0.0002	0.001	ns	0.32	0.49
FLW1	<0.0001	0.0003	0.0004	<0.0001	<0.0001	ns	0.0143	0.37	0.46
Hue*	<0.0001	<0.0001	0.023	<0.0001	0.0477	<0.0001	0.0356	0.39	0.21
LA	<0.0001	ns	0.0085	<0.0001	0.0006	ns	0.0483	0.36	0.52
LAG	<0.0001	ns	0.0509	<0.0001	0.0222	ns	0.0459	0.39	0.41
LCa*	<0.0001	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	0.0956	0.23	0.35
LCb*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0015	0.37	0.44
LCL*	<0.0001	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	0.0005	0.35	0.42
LWC1	<0.0001	0.0019	0.0009	<0.0001	<0.0001	0.0582	0.0008	0.32	0.37

L_Na and L_Cu remarkably changed. Thus, correlation analysis (Online Resource 2) showed that the change of leaf water potential under salinity (dΨ_S) was mostly related (r = 0.9) to the first evaluation of leaf water potential (Ψ7_S) which appeared highly, negatively associated with the root concentration of B, Mo and Mn (also in Fig. 4). Under control, both measures of leaf water potential were significantly correlated ($r = 0.56$), and again, dΨ_C was closer to Ψ7_C than to Ψ80_C. Differently from salinity, Ψ7_C was related to vigour traits (height and stem diameter), and the closest nutrient was Na^+ (L-R_Na_C, $r = -0.53$, and R_Na_C, $r = 0.51$). Noteworthily, this trait showed a high correlation to TDRW under salinity ($r = 0.75$), i.e. the larger the root, the higher the root [Na^+]. LWC was not directly related to leaf water potential but to its difference between control and salinity, Ψ 80_C–Ψ 80_S ($p = 0.008$, $r = 0.44$ for LWC2_C and $p = 0.029$, $r = 0.36$ for LWC2_S).

A total of 98 QTLs were detected by IM and MQM procedures using the two-way pseudo-testcross codification of genotypes (Table 4) and 135 marker-trait associations by KW procedure using the four-class codification for codominant markers segregating for different alleles at both parents (Online Resource 3). The closest markers to 45 QTLs by IM corresponded to the most significant marker by KW (markers in italics in Table 4). Most of them were located in linkage groups 3 (from Cleopatra) and 7 (from both parents). The position and one LOD QTL interval of the main clusters of QTLs are shown in Fig. 5. To better visualize complementary genetic information from both QTL detection strategies, integrated *C. reshni* and *P. trifoliata* maps of linkage groups 10+5b, 7, 3 and 4c showing the position of QTLs detected by KW are also shown in Fig. 5. The traits studied in both experiments 1 and 2 (FLW, DLW and LWC) showed common QTLs, remarking their repeatability (Table 4, Online Resource 3 and Fig. 5).

The effects of Cleopatra allele substitutions at CMS30,160 and C2iC1i,470 (significant markers within 76.263–89.566 cM of linkage group r3) on significantly associated traits are clear. The same QTL allele at C2iC1i,470 increasing LA under salinity is also increasing FLW, DLW and LWC, in both experiments and under both treatments (Online Resource 3). Therefore, the same QTL allele was found associated with the highest mean for the traits related to plant vigour. In the case of the QTL at CMS30,160, the increasing allele for DLW2 was also associated with a higher defoliation index (DI) and a lower leaf boron concentration. For leaf water potential, only one QTL was detected at CR17,300 in linkage group 7 (Online Resource 3) where the genotype *bd* showing the lowest Ψ 80 under salinity (Fig. 6) also showed the lowest defoliation index (DI_S), the largest leaf area (LA_S, at $p \leq 0.05$) and the highest leaf [Na^+] (L_Na_S) suggesting the presence of a putative Na^+ tissue tolerance QTL. Two QTLs were detected for dΨ in other linkage groups of the *Poncirus* map: in p4b (at Py65C, a *Citrus tristeza* virus resistance marker derived from a resistance analogue gene) and p4c (MIR164, a marker derived from the *miR164* gene).

Discussion

Peculiarities of citrus rootstocks and salt tolerance

In spite of the economic and social importance of salt tolerance for the citriculture, its genetic analysis has been scarcely approached in rootstock segregant populations. These populations commonly derive from intergeneric crosses between genotypes from *Citrus* spp. and *P. trifoliata* (a main source of disease resistances). Such wide crosses are advantageous and usual in rootstock breeding programs of horticultural crops (King et al. 2010). However, contrary to other horticultural crops where parents are extensively homozygous, citrus parents have a certain degree of heterozygosis giving rise to up to four segregating alleles per locus in the first generation what

Table 3 *P* values for the significant effects in the mixed model analysis and heritability estimates (H^2_C and H^2_S, for control and salinity treatments, respectively) of evaluated traits in experiment 2

Trait Exp.2	G	E	GxE	H2_C	H2_S
DI	<0.0001	<0.0001	0.0196	0.1173	0.3861
DLW2	<0.0001	ns	ns	0.6400	0.5000
FLW2	<0.0001	ns	ns	0.4010	0.4475
FPW	<0.0001	<0.0001	ns	0.6477	0.4957
H	<0.0001	ns	ns	0.6262	0.4218
L_Al	ns	ns	ns	0.0000	0.1052
L_B	0.0001	<0.0001	ns	0.1792	0.2575
L_Ca	0.0005	0.0132	ns	0.1522	0.2189
L_Cu	0.0005	ns	ns	0.1739	0.1360
L_Fe	0.0002	0.0226	ns	0.0927	0.3143
L_K	<0.0001	<0.0001	0.0113	0.3533	0.3840
L_Li	<0.0001	0.0201	0.0023	0.3889	0.4153
L_Mg	<0.0001	<0.0001	ns	0.3902	0.5000
L_Mn	<0.0001	<0.0001	ns	0.2927	0.3459
L_Na	<0.0001	<0.0001	<0.0001	0.5000	0.4846
L_P	<0.0001	0.0092	ns	0.1000	0.2000
L_S	<0.0001	0.0419	ns	0.4235	0.5000
L_Sr	0.0001	ns	ns	0.1692	0.2610
L_Ti	<0.0001	<0.0001	ns	0.2690	0.3013
L_V	<0.0001	<0.0001	ns	0.3077	0.4461
L_Zn	<0.0001	0.0325	ns	0.3817	0.1483
L-R_Ca	0.0044	ns	ns	0.1618	0.1397
L-R_K	0.0001	<0.0001	0.0026	0.2719	0.2531
L-R_Na	<0.0001	0.0026	0.0114	0.3428	0.3847
L-R_P	0.0001	ns	ns	0.2647	0.1552
LWC2	<0.0001	ns	ns	0.2462	0.3696
R_Al	ns	ns	ns	0.0316	0.1119
R_B	<0.0001	<0.0001	ns	0.2843	0.2236
R_Ca	0.0292	<0.0001	ns	0.1289	0.0497
R_Cr	0.0232	ns	ns	0.0148	0.0681
R_Cu	ns	<0.0001	ns	0.0877	0.0445
R_Fe	0.0146	ns	ns	0.0732	0.0549
R_K	0.0042	0.0189	ns	0.2539	0.0451
R_Mg	<0.0001	<0.0001	ns	0.3077	0.5000
R_Mn	0.0243	ns	ns	0.1391	0.0487
R_Mo	0.0071	<0.0001	ns	0.2000	0.1233
R_Na	<0.0001	<0.0001	ns	0.2851	0.3077
R_Ni	0.0243	ns	ns	0.0001	0.0738
R_P	0.0039	ns	ns	0.2000	0.0755
R_S	<0.0001	<0.0001	0.0211	0.7353	0.4336
R_Sr	0.0004	<0.0001	ns	0.1934	0.1468
R_Ti	0.0004	0.0207	ns	0.0882	0.1916
R_V	<0.0001	<0.0001	ns	0.4430	0.2612
R_Zn	ns	ns	ns	0.0978	0.0031
RWC	<0.0001	<0.0001	0.0191	0.5346	0.3524
SD	<0.0001	0.0321	ns	0.6416	0.4509
TDRW	<0.0001	<0.0001	0.0036	0.7105	0.6073
TFRW	<0.0001	<0.0001	0.0025	0.6735	0.4926
Ψ_7	0.0041	ns	ns	0.16	0.24
Ψ_80	0.0011	0.0193	ns	0.08	0.03
dΨ	ns	0.0059	ns	0.00	0.28

makes genetic analysis possible but prevents the estimation of additive gene effect. Other difficulties of genetic studies in citrus rootstock populations come from their partial apomicitic reproduction. The yield of nucellar seedlings through apomixis makes uniform citrus orchards possible, easy and cheap. Therefore, hybrids with no apomixis or low yield of nucellar seedlings are generally discarded for further agronomic evaluation, drastically reducing the size of the target population. Given that nucellar reproduction is not at random, but genetically determined (García et al. 1999; Nakano et al. 2008; Raga et al. 2012), some genotypes are observed in a lower frequency than expected causing segregation distortion in some genomic areas. There are other genetic factors responsible for segregation distortion in citrus populations (Bernet et al. 2010; Raga et al. 2012), all of them make QTL analysis less powerful. Therefore, to obtain more robust conclusions, two salt tolerance experiments using replications (nucellar seedlings) from mature, apomictic hybrids of the same population were run and compared here. Salt tolerance was evaluated in terms of vegetative traits, and for comparison purposes, FLW, LWC and DLW were measured in both experiments (Fig. 1) showing that both parents were clearly different, with or without salinity, for the aerial part of the plant; i.e. no salt tolerance behaviour can be deduced for any particular parent. On the contrary, in Exp. 2 where leaf and root weight traits were evaluated under the same environmental conditions, the trifoliate parent showed a slightly sensitive behaviour in comparison to the Cleopatra root weight changes (Fig. 2). In fact, FLW2, LWC2 and DLW2 were not significantly affected by salinity in this experiment while TFRW, TDRW and RWC were indeed (Table 3). Besides, salinity effects on ionomic and physiological traits such as Cl, L_Na, R_Na, L_K, L_B, L_Fe and Ψ 7 may further explain Cleopatra salt tolerance in terms of trait mean changes which were smaller than those for the trifoliate orange (Fig. 2). Therefore, the joint analysis of these traits in the progeny will help us to understand the multiple effects of salinity on the citrus plants beyond the comparison of the parental genotypes.

Salinity effects on trait distributions and relationships

Salinity changed the relationships between (Fig. 3) and among (Fig. 4) traits. Thus, Fig. 3 shows how Cl^- association with leaf colour parameters LCb, LCL and Hue or with LWC and

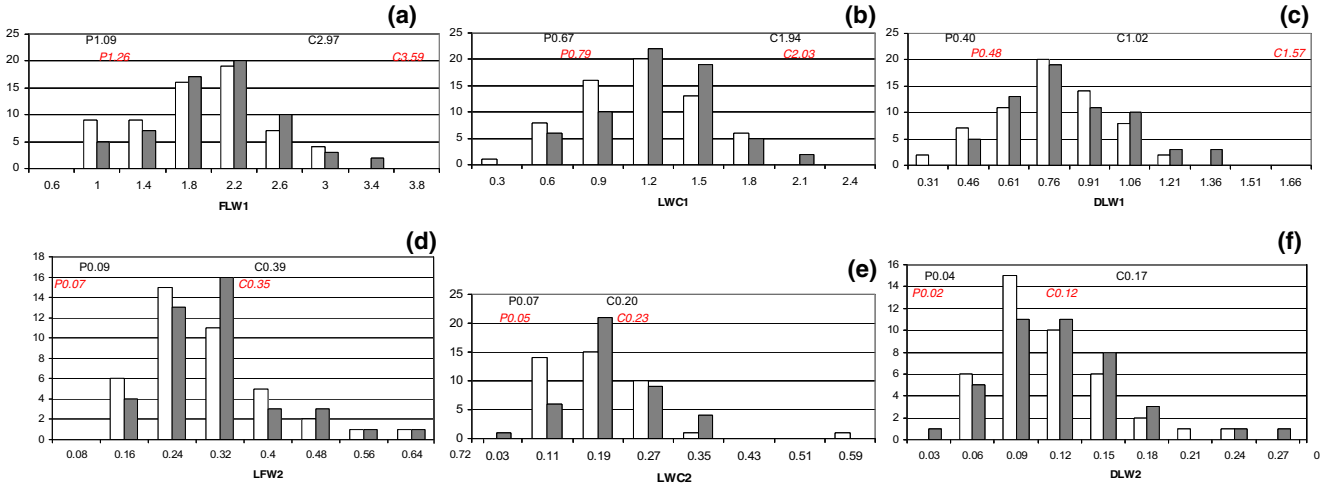


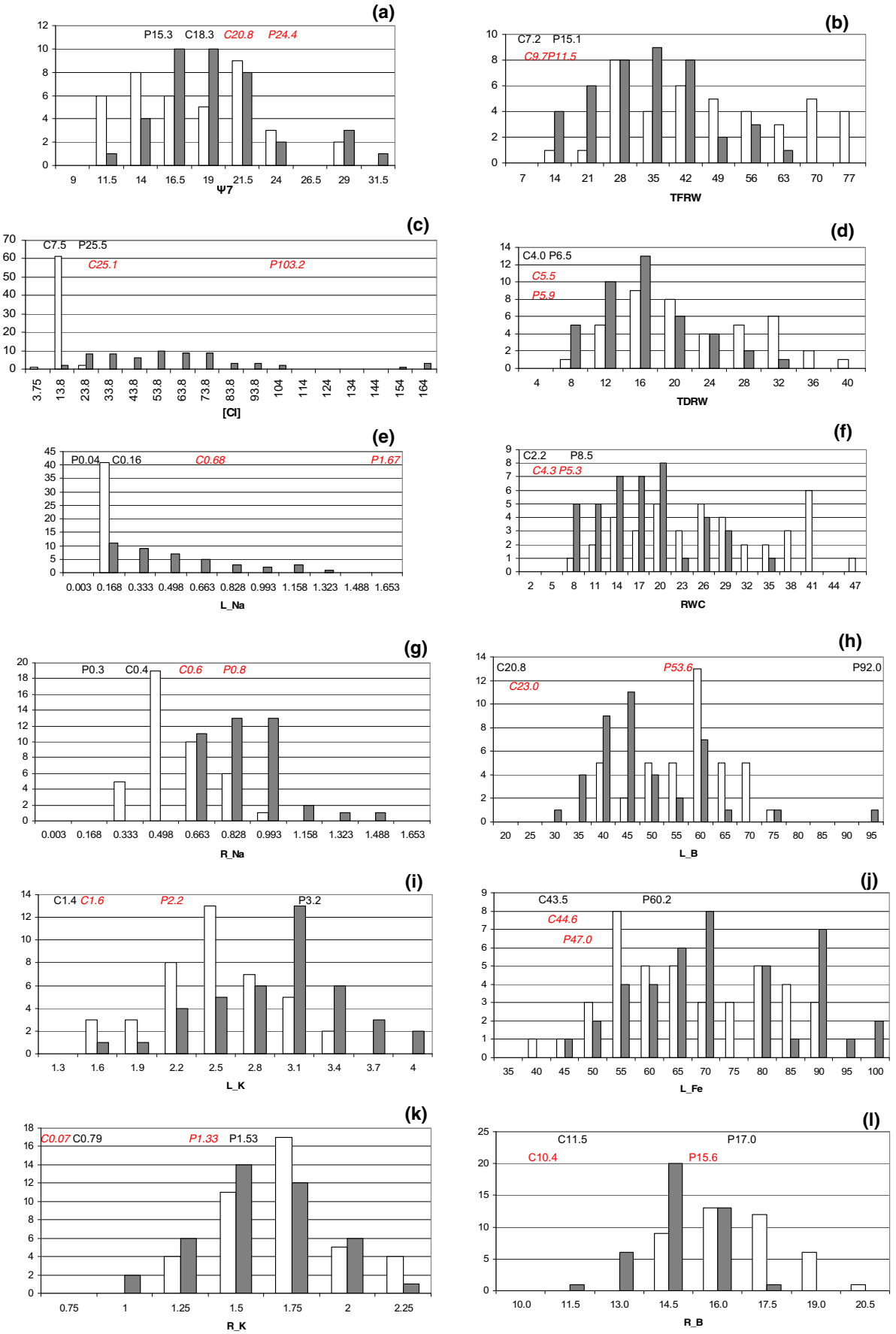
Fig. 1 Distributions of traits studied in both experiments 1 and 2 depending on the treatment (control, or salinity, indicated by *white* or *grey bars*, respectively). Fresh leaf weight, leaf water content and dried leaf weight of experiment 1 correspond to **a**–**c**, respectively, while those for traits in experiment 2 are depicted in **d**–**f**, respectively. The means for the parents: trifoliate orange (P) and Cleopatra mandarin (C) are indicated for control and salinity (in *italics*). Absolute frequencies of each phenotypic class are indicated at the *Y* axis

fresh leaf weight changed of sign when comparing control and salinity, pointing out the importance of Cl^- homeostasis in the plant under both conditions and not only under salinity. Salinity strengthened the relationship between L_K and DLW, while decreasing that between R_Na and L-R_Na (Fig. 3); i.e. the Na^+ gradient between roots and leaves was not so closely related to R_Na under salinity than under control, suggesting that some rootstock genotypes can keep larger [Na^+] differences between them than others (i.e. significant GxE interaction for L-R_Na). In fact, results from experiment 2 (Table 3) showed significant GxE interaction for L-R_Na and other traits (DI, L_K, L_Li, L_Na, L-R_K, R_S, RWC, TDRW and TFRW). TDRW appeared as a main trait related to plant vigour (DLW, H, SD) under both control and salinity (salt tolerance) and the root Na^+ concentration, or the gradient of Na^+ concentration between leaf and root (L-R_Na^+) could play a relevant role in that relationship as suggested by the correlation analysis (Online Resource 2). The ability of the rootstock to retain the toxic ions in roots as a mechanism of salt tolerance had been previously reported in citrus (Kostopoulou et al. 2014; Balal et al. 2012). On the other hand, the distributions of L_K (and L_Fe) were also clearly displaced towards larger values under salinity (Fig. 2). Salinity stress effect on the K^+ concentration of the aerial part of the citrus plant was previously observed by several authors (Morinaga and Sykes 2001; Balal et al. 2012; Khoshbakht et al. 2015). The only leaf nutrient directly related to leaf dried weight under salinity was Fe, and the others (K, P and B) were indirectly related (Online Resource 2), suggesting that Fe leaf nutrition under salinity (highly related to leaf [Ca^{2+}]) could be a limiting factor for salt tolerance in citrus. The relationship of leaf water potential with traits in experiment 2 showed a major change as deduced from the comparison between the control and salinity graphic representations of principal component analysis (Fig. 4) and correlation analysis (Online Resource 2). Thus, while Ψ7 was mainly related to L-R_Na under control (Online Resource 2), the most significant traits associated with Ψ7 under salinity were R_B, R_Mo and R_Mn (Fig. 4 and Online Resource 2). The changes observed in all these physiological traits could be contributing to the salt tolerance of the progeny inherited from Cleopatra mandarin. How much genetic variation of these traits is available for breeding purposes and where is it located in the genome?

Genetic variation and QTL analysis of traits

A profitable amount of genetic variability has been found for several traits in the RxPr population (Fig. 2 and Online Resource 4). According to Tables 2 and 3, most trait heritabilities were below 0.6 although the estimates for some traits related to salt tolerance were ≥0.50 (Cl^-, %DMA, DLW2, TDRW). This is not the case of leaf water potential, a usual indicator of xylem functionality in many drought and salinity tolerance studies (Pedroso et a. 2014; Rodriguez-Gamir et al. 2011; Forner-Giner et al. 2011; García-Sanchez et al. 2009; Ortuño et al. 2004; Morinaga and Sykes 2001; Savé et al. 1995) but whose heritability had not been previously

Fig. 2 Distributions of most relevant traits depending on the treatment (control or salinity, indicated by *white* or *grey bars*, respectively): leaf water potential after 7 days of treatment (Ψ_7, **a**), total fresh root weight (TFRW, **b**), leaf Cl concentration (Cl, **c**), total dried root weight (TDRW, **d**), leaf Na concentration (L_Na, **e**), root water content (RWC, **f**), root Na concentration (R_Na, **g**), leaf B concentration (L_B, **h**), leaf K concentration (L_K, **i**), leaf Fe concentration (L_Fe, **j**), root K concentration (R_K, **k**) and root B concentration (R_B, **l**). The parent means, trifoliate orange (P) and Cleopatra mandarin (C) are indicated for control and salinity (in *italics*). Absolute frequencies of each phenotypic class are indicated at the *Y* axis


(a)
P15.3 C18.3 C20.8 P24.4
Ψ7
(b)
C7.2 P15.1
C9.7P11.5
TFRW
(c)
C7.5 P25.5
C25.1
P103.2
[Cl]
(d)
C4.0 P6.5
C5.5
P5.9
TDRW
(e)
P0.04 C0.16 C0.68 P1.67
L_Na
(f)
C2.2 P8.5
C4.3 P5.3
RWC
(g)
P0.3 C0.4 C0.6 P0.8
R_Na
(h)
C20.8 P53.6 P92.0
C23.0
L_B
(i)
C1.4 C1.6 P2.2 P3.2
L_K
(j)
C43.5 P60.2
C44.6
P47.0
L_Fe
(k)
C0.07 C0.79 P1.33 P1.53
R_K
(l)
C11.5 P17.0
C10.4 P15.6
R_B

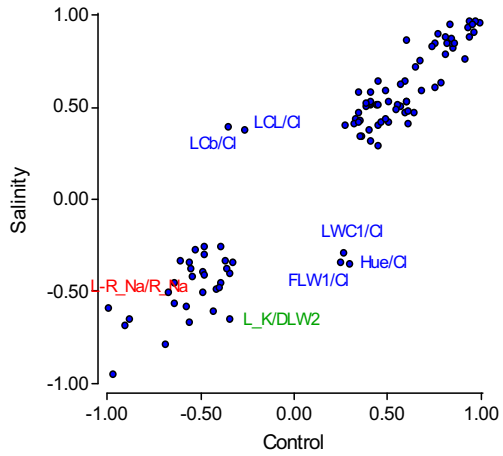


Fig. 3 Dispersion of Pearson's coefficients for significantly ($p < 0.05$) correlated traits under both control and salinity conditions (trait abbreviations in Table 1)

estimated. Present results showed that its utilization for selection may show low predictive value, particularly, its late measure Ψ_{80} (Table 3).

The QTL analysis of the 60 traits evaluated under both environmental conditions, in both experiments, rendered a low number of significant QTLs. In fact, no QTL per trait was the mode class, particularly under control (Online Resource 4). Beyond the significance level fixed for the detection, the reduced number of QTLs detected per trait could be better explained by the reduced number of genotypes rather than by the trait heritabilities. Nevertheless, two facts of the present QTL analysis support a reasonable degree of reliability: (1) some QTLs of traits studied in both experiments showed repeatability, and (2) QTLs governing some traits under both control and salinity mapped together. Thus, from both linkage groups 3 and 7 involved in vegetative growth or plant vigour under control condition, those QTLs in linkage group 3 (Fig. 5) for FLW, DLW, LWC, TDRW and RWC were also detected under salinity and, importantly, in the same genotypic direction than under control, pointing out their relevance for selection in salt tolerance breeding programs of citrus rootstocks. Unfortunately, we cannot relate this region to that where Sahin-Çevik and Moore (2012) located QTLs for trunk diameter, tree high and tree canopy width in linkage group VII because of the lack of common markers.

Forty-five out of 98 QTLs (45.9 %) detected by using the IM procedure were coincident (markers in italics in Table 4) with the most significant markers associated by Kruskal-Wallis (KW, in bold in Online Resource 3). A similar proportion (52.9 %) was reported recently in another citrus progeny (Asins et al. 2015). The complementary nature of QTL information provided by both methods is also displayed in Fig. 5 for the most relevant linkage groups by specifying KW-associated traits in the correspondent integrated *C. reshni-P. trifoliata* linkage groups (7, 10+5b, 3, 4c). This representation is also helpful to connect trait correlations and linkage of QTLs from both parents governing the traits involved. The significant relationships among leaf traits (FLW, LWC, DLW, L_B) and among root traits (RWC, TDRW, R_Na) observed under salinity agreed with genotypic variation at associated markers in linkage group 3 (CL2.26,395, CMS30,160, CR80,260, C2iC11,470 and C8iC1rt,650 in Fig. 5, Table 4 and Online Resource 3). Noteworthily, three QTLs for the duration of the juvenility period (Jp3.2, Jp3.1 and Jp3.3) were previously reported in this same linkage group at

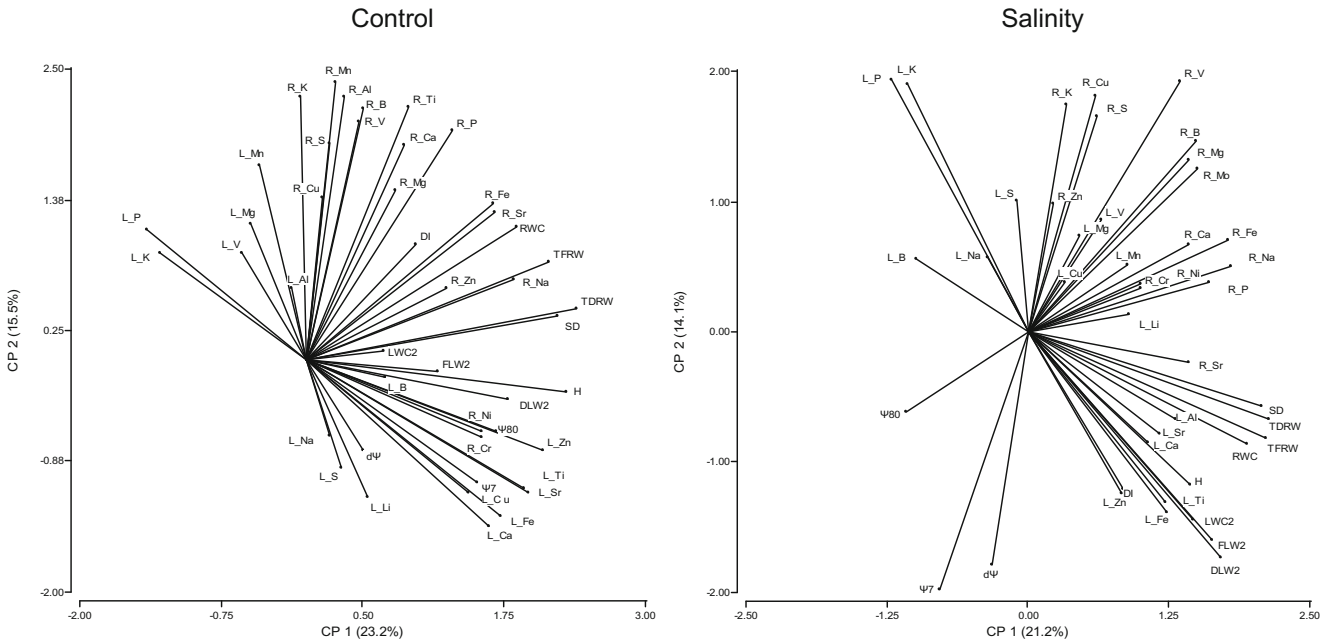


Fig. 4 Graphical representation of principal component (PC) analysis of traits evaluated in experiment 2 under control and salinity (trait abbreviations in Table 1)

Table 4 List of the positions (in cM) and nearest markers (locus) to QTLs detected by IM and MQM in the genetic linkage maps of parents (GM): trifoliate orange (p) or Cleopatra mandarin (r) previously reported by Raga et al. (2012)

Trait	LG	Position	Locus	LOD	μ_1	μ_2	PEV	α	GM	Sig. LOD
Cl_S	7	95.736	C11iC1rt,400	2.31	71.63	41.76	15.3	29.87	p	2.3
Cl_S	4c	20.533	CR23,750	2.31	67.18	40.37	15.3	−26.80	r	1.4
Cr_S	3	5.000	*CR2,190*	2.45	19.21	22.34	16.2	3.13	r	1.8
Dl_C	12	35.232	42C,500	3.96	10.13	7.40	37.30	2.73	p	1.6
Dl_C	12	62.139	CR50,510	2.18	9.37	7.83	16.50	1.53	p	1.6
Dl_C	4b	58.883	C8iC1rt,280	2.20	10.86	7.40	22.8	−3.46	r	1.9
Dl_S	3	88.514	5F4R,600	2.58	8.81	6.40	26.30	2.41	r	1.8
DLW1_C	7	125.539	*C1iC8rt,515*	3.50	0.60	0.81	22.3	0.22	r	2.2
DLW1_S	3	91.566	*C2iC1i,470*	4.45	0.43	1.05	27.4	0.62	r	1.8
DLW1_S	7	100.292	*6F6R,2036*	2.37	0.66	0.84	15.70	−0.18	r	2.2
DLW1_S	7	33.307	CR26,175	2.24	0.63	0.90	14.9	−0.27	p	2.2
DLW2_C	3	89.566	*C2iC1i,470*	3.87	0.12	0.07	36.70	0.05	r	1.9
DLW2_S	3	90.566	*C2iC1i,470*	2.73	0.14	0.06	27.5	0.07	r	1.7
dΨ_S	4b	8.000	Py65C,506	2.58	10.81	1.60	26.2	9.21	p	1.9
dΨ_S	4c	2.000	MIR164	2.29	10.87	5.90	23.7	4.97	p	2.0
FLW1_C	3	83.293	*CR80,260*	2.50	1.46	1.91	16.4	0.45	r	1.7
FLW1_C	7	71.404	CR17,300	2.31	1.90	1.47	15.3	−0.43	r	2.2
FLW1_S	3	90.566	*C2iC1i,470*	4.03	2.70	1.00	23.00	1.70	r	1.8
FLW2_C	3	89.566	*C2iC1i,470*	3.66	0.32	0.18	35.10	0.13	r	1.9
FLW2_S	3	89.514	*C2iC1i,470*	3.86	0.32	0.20	32.1	0.12	r	1.9
H_C	3	52.821	*CMS30,160*	2.71	58.47	89.47	27.4	31.00	r	1.8
H_S	1	16.000	6F6R,850	3.52	46.72	91.78	34.0	−45.06	p	1.4
Hue_C	3	12.000	CR2,190	3.03	118.20	114.38	19.6	−3.82	r	2.0
Hue_C	8+6	87.825	CR14,290	3.73	113.97	117.14	23.5	3.17	r	1.9
Hue_S	3	9.000	CR2,190	2.61	119.83	117.10	17.1	−2.73	r	1.7
L_Al_S	4c	5.000	C2,450	2.28	237.34	123.41	23.6	113.92	p	2.0
L_B_S	3	87.160	5F4R,600	2.51	40.66	54.15	25.60	−13.49	r	2.0
L_Cu_S	9	14.304	COR15,230	2.39	12.98	4.73	24.6	−8.25	r	2.0
L_K_S	4c	11.677	*CR23,750*	3.83	3.04	2.28	36.4	−0.76	r	1.5
L_Na_C	4b	61.460	Acuapor,750	2.26	0.02	0.01	23.4	−0.01	r	1.9
L_Na_C	4c	43.600	C2,500	3.00	0.00	0.02	29.8	−0.02	p	2.0
L_P_C	10+5b	94.935	*SOS1,500*	3.21	0.13	0.16	31.50	−0.02	p	2.3
L_P_C	4c	80.983	CR19,370	2.47	0.15	0.13	25.3	−0.02	r	1.5
L_P_S	4b	110.380	CAT101.140	2.44	0.20	0.11	25.0	−0.09	r	2.0
L_S_C	3	85.160	TAA27,235	2.20	0.24	0.30	22.9	0.06	r	1.8
L_S_S	4d	17.000	Mybg2,210	2.88	0.22	0.30	28.8	−0.09	p	1.4
LA_C	7	43.646	*CL1.35,240*	2.71	7.66	11.20	17.70	−3.53	r	2.2
LA_C	8+6	89.825	CR14,290	2.56	7.41	10.12	16.8	2.70	r	1.9
LA_S	7	101.292	*6F6R,2036*	3.11	8.64	10.59	20.00	−1.95	r	2.3
LA_S	7	31.307	*CR26,175*	2.47	8.90	10.59	16.3	−1.69	p	2.2
LA_S	7	6.000	*CR41,750*	2.85	11.11	8.94	18.5	2.18	p	2.2
LAG_C	7	71.404	CR17,300	2.90	2.49	4.20	19.1	−1.71	r	2.3
LAG_C	4c	19.012	CR23,750	2.87	2.81	4.60	18.9	−1.78	p	1.8
LCb_C	3	13.000	CR2,190	2.29	19.90	25.71	15.2	5.81	r	1.8
LCb_C	8+6	90.825	CR14,290	4.38	26.85	21.37	27.0	−5.48	r	1.9
LCb_S	3	5.000	*CR2,190*	2.90	16.69	19.95	18.8	3.27	r	1.7
LCL_C	4b	18.075	*520AR,350*	2.25	36.87	39.38	14.9	−2.50	p	1.9
LCL_C	8+6	88.825	CR14,290	4.09	41.83	37.07	25.5	−4.76	r	2.0

Table 4 (continued)

Trait	LG	Position	Locus	LOD	μ_1	μ_2	PEV	α	GM	Sig. LOD
LCL_S	3	5.000	*CR2,190*	2.60	34.93	37.29	17.0	2.36	r	1.8
LWC1_C	3	83.293	*CR80,260*	2.50	0.85	1.15	16.5	0.30	r	1.8
LWC1_C	7	80.189	24R,950	2.21	1.13	0.86	14.7	−0.27	r	2.2
LWC1_S	3	89.566	*C2iC1i,470*	3.70	1.47	0.74	21.80	0.72	r	1.7
LWC2_C	3	89.566	*C2iC1i,470*	2.29	1.99	1.19	23.7	0.80	r	1.9
LWC2_S	3	89.566	*C2iC1i,470*	4.22	1.96	1.23	34.1	0.73	r	1.7
LWC2_S	3	94.566	CR71,310	2.15	1.33	1.99	19.5	−0.66	r	1.7
R_Al_S	10+5b	56.907	*CR12,250*	2.70	464.34	669.35	27.3	−205.01	p	2.3
R_B_S	7	79.391	11FrRv,520	2.65	15.75	12.61	26.9	3.14	p	2.3
R_B_S	10+5b	10.000	5R4R,1100	2.70	15.26	13.14	27.3	−2.13	r	2.2
R_Ca_C	8	42.824	*SOS2,800*	3.38	1.20	1.41	32.9	−0.21	p	1.2
R_Ca_C	4d	15.000	Mybg2,210	2.23	1.45	1.22	23.1	0.23	p	1.3
R_Ca_S	4c	12.856	C2,450	2.29	0.72	1.48	23.7	−0.76	p	1.9
R_Cr_S	7	163.250	CR55,520	2.86	4.39	33.04	28.7	−28.66	p	2.4
R_Cr_S	10+5b	160.495	*CMS46,190*	2.79	11.68	28.66	28.0	16.97	r	2.1
R_Cu_S	7	80.189	24R,950	2.43	83.91	115.20	24.9	31.29	r	2.4
R_Fe_C	4c	4.000	C2,450	2.46	382.06	305.12	25.2	76.94	p	1.9
R_Fe_C	4c	19.012	CR23,750	2.74	301.74	370.16	27.6	−68.42	p	1.9
R_Fe_S	10+5b	167.393	CMS46,190	2.47	262.59	341.82	25.3	79.23	r	2.1
R_Fe_S	8+6	19.437	*SOS2,800*	2.69	349.86	264.66	27.2	−85.20	r	2.0
R_K_C	4b	57.883	CR3,320	2.29	1.95	1.27	23.7	−0.68	r	2.1
R_Mg_S	7	199.121	*CAC23,230*	5.01	0.13	0.16	44.7	0.02	r	2.4
R_Mg_S	4b	37.083	*AGG9,125*	2.20	0.15	0.13	22.9	0.02	p	1.8
R_Mn_S	10+5b	38.071	*CCCAluI,650*	5.04	35.76	48.32	44.8	−12.56	p	2.2
R_Mo_S	12	1.000	*C11iC1rt,350*	2.23	2.02	2.56	23.1	−0.54	p	1.6
R_Na_C	12	41.012	6F5R,1200	2.32	0.58	0.42	24.0	−0.16	r	1.7
R_Na_C	4b	30.083	*C8iC2rt,1600*	2.51	0.57	0.39	25.6	0.18	p	1.8
R_Na_C	4d	12.000	Mybg2,210	3.96	0.51	0.38	37.4	0.13	p	1.3
R_Na_S	4b	41.833	*AGG9,125*	2.22	0.90	0.61	23.0	0.29	p	2.0
R_Ni_S	7	163.250	CR55,520	2.72	2.40	14.93	27.5	−12.53	p	2.5
R_Ni_S	10+5b	159.495	*CMS46,190*	2.77	5.47	13.12	27.9	7.64	r	2.1
R_Ni_S	8+6	20.437	*SOS2,800*	3.45	15.79	4.34	33.5	−11.45	r	2.0
R_P_S	10+5b	39.071	*CCCAluI,650*	2.64	0.38	0.48	26.8	−0.11	p	2.2
R_P_S	4c	13.856	CR23,750	3.19	0.27	0.59	31.4	−0.31	p	1.8
R_S_S	10+5b	0.000	*5R4R,1100*	3.43	0.41	0.31	33.3	−0.10	r	2.1
R_Sr_C	4d	16.000	Mybg2,210	5.54	28.71	22.20	48.0	6.52	p	1.3
R_Sr_C	8+6	68.825	TAA52,120	2.23	30.37	23.72	23.2	−6.66	r	2.0
R_Sr_S	7	61.908	CR18,200	2.35	24.33	14.90	24.3	−9.42	r	2.4
R_Sr_S	4c	12.856	C2,450	2.40	10.18	27.30	24.7	−17.12	p	1.9
R_Ti_S	10+5b	40.071	*CCCAluI,650*	3.00	5.26	6.80	24.60	−1.54	p	2.1
R_Ti_S	10+5b	75.852	CR69,230	2.50	5.29	6.76	25.6	1.47	r	2.0
R_V_S	7	199.121	*CAC23,230*	2.50	1.87	2.20	25.5	0.33	r	2.3
R_V_S	10+5b	136.709	*5F6R,260*	2.77	1.72	2.29	27.9	−0.57	p	2.3
R_Zn_C	12	22.000	*6F5R,1200*	2.68	19.94	13.92	27.1	−6.01	r	1.6
R_Zn_S	12	55.787	CMS20,170	2.39	80.64	−26.81	24.6	−107.46	r	1.4
RWC_C	3	47.821	*CL2.26,395*	3.00	16.93	32.25	29.9	15.32	r	1.8
RWC_C	4d	21.143	Mybg2,210	2.32	31.96	19.95	24.0	12.01	p	1.5
RWC_S	3	55.821	*CMS30,160*	2.81	10.14	20.34	28.3	10.20	r	1.8
TDRW_C	3	50.821	CL2.26,395	3.66	12.34	25.95	35.1	13.61	r	1.8

Table 4 (continued)

Trait	LG	Position	Locus	LOD	μ_1	μ_2	PEV	α	GM	Sig. LOD
TDRW_S	3	43.821	CL2.26,395	2.46	9.84	17.68	25.2	7.84	r	1.8

The 5 % (Sig. LOD) significance LOD scores for each trait-linkage group combination estimated from 1000 permutation tests each are also included. The estimated difference between parent alleles is α and the percentage of explained variance (PEV). Genotypic means (μ) are included. Most significant markers by KW ($p < 0.005$) are indicated in italics. Trait abbreviations in Table 1. QTLs for the difference of element concentrations between root and leaf were not included

C8intC1rt650, C2iC1i,470/TAA27 and CL2-26, respectively, in this segregant population (Raga et al. 2012) connecting genetically this trait to plant vigour, a well-known trait association in fruit trees (Visser 1970).

Salt tolerance and candidate genes

As far as salt tolerance is concerned, most QTLs for vegetative growth were detected in linkage group 3 (FLW, LWC, DLW, RWC, TDRW) where a L_B QTL also mapped. Given that they mapped together, is boron nutrition playing an active role in salt tolerance (citrus vigour under salinity)? We suspect it is not; i.e. it is a consequence of the gene substitution effects at these plant vigour QTLs and the salinity limiting effect on B nutrition. Thus, both L_B and R_B distributions moved to the left under salinity (Fig. 2h and l), but B is needed for root and stem growth (R_B_S and SD_S were correlated, Online Resource 2). In fact, the predominant function of B is in the formation of primary cell walls (O'Neill et al. 2004; Miwa and Fujiwara 2010; Wang et al. 2015). Therefore, less B would reach the leaves in large-rooted vigorous genotypes (genotypes with higher RWC_S), and this lower L_B_S would also explain a higher DI_S. These relationships agreed with the Cleopatra-allele substitution effects at CMS30,160 in linkage group 3 where QTLs for L_B, FLW2, DLW2, RWC and DI (Online Resource 3, Fig. 5) were detected under salinity. These results on the salt tolerance of rootstocks derived from Cleopatra mandarin might be useful in order to increase

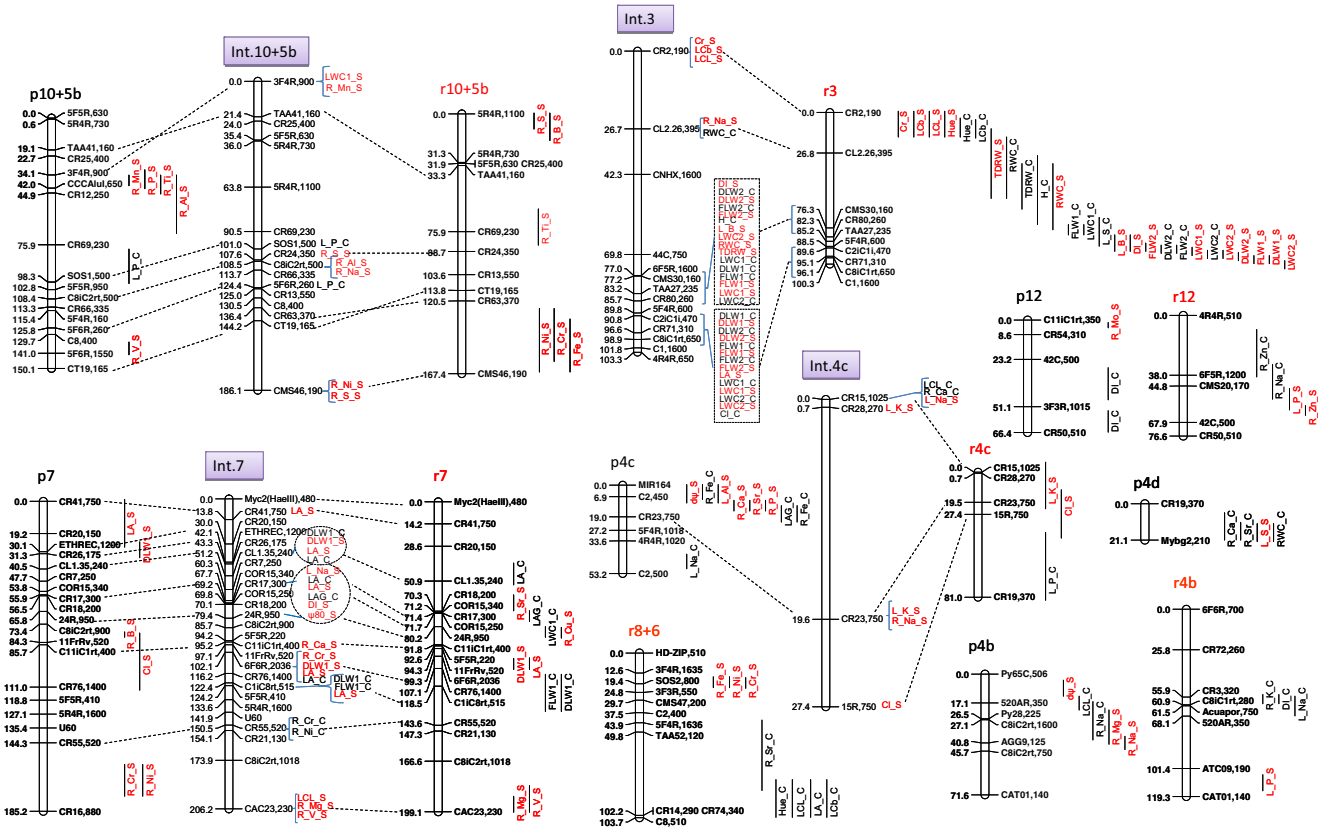


Fig. 5 Distribution of significant QTLs (IM and MQM) from Table 3 along *Poncirus* (p) and Cleopatra mandarin (r) linkage groups. Each QTL is coded as the trait (trait abbreviations in Table 1), and one LOD interval is depicted below. The corresponding Kruskal-Wallis trait-maker associations (Online Resource 3) are displayed in the integrated p+r (Int.) linkage groups 10+5b, 7, 3 and 4c, between the maps of both parents

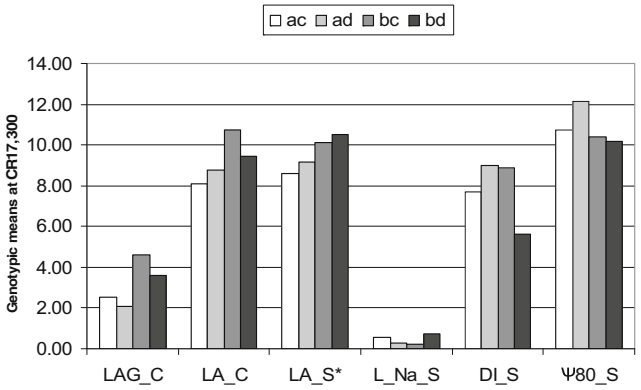


Fig. 6 Comparison of genotypic means at CR17,300 in linkage group 7 for the following traits LAG_C (leaf area growth under control, from Exp.1) and LA_C (leaf area under control), LA_S (leaf area under salinity), L_Na_S (Leaf [Na+] under salinity), DI_S (defoliation index under salinity) and Ψ_{80}_S (leaf water potential after 80 days of salinity treatment) from Exp. 2. *LA_S QTL at marker CR17,300 was at $p \leq 0.05$

citriculture sustainability in the Mediterranean area because they provide a new perspective to the suitability of saline wastewaters (frequently containing elevated B concentration) for irrigation of citrus orchards (Grattan et al. 2015).

Two QTLs for leaf area growth (LAG) and leaf water potential-related traits were detected in linkage groups 7 and 4c (Fig. 5). These LAG QTLs corresponded to control, but none was detected under salinity. However, in the case of linkage group 7, two QTLs for LA under both control and salinity were also detected by KW in the same genomic region (Online Resource 3). Although the most significant marker is not the same for both LA_C and LA_S, if we compare genotypic means for Ψ80_S, DI_S, L_Na_S, LA_S (significant here only at $p \leq 0.05$), LA_C and LAG_C at the CR17,300 marker in linkage group 7 (Fig. 6), the genotype *bd* showed both the largest L_Na_S and LA_S means. Besides, the inverse relationship observed between L_Na_S and DI_S (Online Resource 2) was supported by the genotypic means at this marker. These results suggest the presence of a putative leaf-Na^+ tolerance QTL here. Regarding the cluster of QTLs for dΨ_S, L_K_S, R_Na_S and Cl_S in linkage group 4c (Fig. 5, Table 4 and Online Resource 3), the same marker locus CR23,750 was linked to a QTL for LAG_C (in the *Poncirus* genome) and another for Cl_S (in the Cleopatra genome), suggesting that the genotypic difference in Cl^- accumulation might silence any difference between *Poncirus* alelles at the LAG QTL, what could be reasonable to interpret as a leaf-Cl^- sensitivity QTL. Tozlu et al. (1999b) found a genomic region (NaCl1c) involved in Na^+ and Cl^- accumulation under salinity where growth and dried mass-related QTLs mapped (Tozlu et al. 1999a), but they did not associate it with tissue salt sensitivity. In fact, their linkage group I where they placed NaCl1c corresponded to our linkage group 8+6 (see location of common marker Got1 in Cai et al. 1994 and Ruiz and Asins 2003). Differences could be explained by the salt-tolerant donor used in each case.

Seven markers related to salt tolerance candidate genes (42C, COR15, Acuapor, SOS1, SOS2, CCC and Ethrec), one marker for the microARN *miR164* and two markers for transcription factors (Mybg2 and EREBP1) were found linked to QTLs detected by IM and/or KW for traits evaluated under both control and salinity (Table 4 and Online Resource 3). Regarding candidate genes associated with salt tolerance rootstock effects in the *C. volkameriana* × *P. trifoliata* grafted population reported by Raga et al. (2014), linked markers to *CCC* and *CNHX1* were associated to salt-tolerance-related traits in two cases: 3F-4R,900 (linked to CCCAlu,650 in p10+5b) associated with LWC1_S (and R_Mn_S) and CMS30,160 (linked to CNHX, 1600 in linkage group 3) associated with several LWC traits. No Na^+ QTL was found associated with salt tolerance candidates SOS1, SOS2 or NHX1 but a L_Na_C QTL was detected at Acuapor,750, a marker derived from an aquaporin gene, in r4b. Leaf Cl^- accumulation was not found associated with marker CCCAlu1, 650 (corresponding to a citrus-chloride cotransporter reported by Brumos et al. 2009) in p10+5b; however, the maximum LOD score of QTLs for the root concentrations of Mn (trait significantly related to Ψ7_S), P and Ti under salinity located at this maker by IM (Table 4, Fig. 5). Regarding leaf water potential, two QTLs were detected for leaf water potential change under salinity (dΨ_S in Table 4) that were somehow related to *C. tristeza* virus (CTV) infection. One of those dΨ_S QTLs was detected at miR164 marker (in p4c) which corresponds to a microRNA gene from a CTV-challenged phloem cDNA library (Song et al. 2009). Jones-Rhoades et al. (2006) reported that the *miR164* expression was high in leaf, shoot and root of *Poncirus* and its target genes were NAC transcription factors whose overexpression induces, among other effects, reduced lateral rooting. In the case of salinity, Barciszewska-Pacak et al. (2015) have shown recently that it increased *Arabidopsis* leaf miR164c-3p expression more than four times. The other QTL detected for dΨ _S corresponded to a candidate gene for CTV resistance (resistance analogue *Py65* reported by Yang et al. 2001). Of course, plants of experiment 2 were obtained from seeds and kept under a greenhouse, so they were virus free. This possible connection between both biotic and abiotic stresses and the vascular system deserves a future genomic insight taking advantage of the genome sequences already available for *Citrus* at the web portals http://phytozome.jgi.doe.gov/and https://www.citrusgenomedb.org/.

In conclusion, salt tolerance in terms of vegetative growth, leaf water potential and nutrient distribution has been genetically studied using nucellar seedlings of a population derived from two common but far related citrus rootstocks, trifoliate orange (*P. trifoliata*) and Cleopatra mandarin (*C. reshni*), a very well-known salt tolerance rootstock whose inheritance

had never been studied before. Correlation and QTL analyses of 60 traits in two experiments under both control and salinity conditions have shown that salt tolerance was mostly related to the whole plant vigour and its ionomic profile. The position of QTLs reported for plant vigour traits in linkage groups 3 and 7 might be useful to obtain selection tools to improve rootstocks for salt tolerance and as genomic reference points where to continue searching for candidate genes.

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Data archiving statement The SSR primer sequences are available upon request from http://www.ivia.es/deps/biot/labgen/request.html. The genetic linkage maps have been submitted to the Citrus Genome Database (https://www.citrusgenomedb.org/). Concerning markers derived from salt tolerance candidate genes, COR15 and Aquapor correspond to contig6158 (ATTATCAATT AATTTATAAA AGAAAA TTAG TTTCTTTTTT TTTTTT) and contig2599 (TGGGGAAAAC TGCCTTGAAA GGAACCCCTT TTAATTCTT), respectively, from the KCl-salt1 library at Valencian Implemented Citrus EST and NucleoTide (VICENT sequences database). Mygbg2 and EREBP1 correspond to transcription factors from the NCBI database, accessions EF071983 and FJ544914, respectively. Marker 42C corresponds to a lectin gene obtained by PCR select and overexpressed in Cleopatra roots under salinity (forward primer: AGATCAAGCAGCAGATCC; reverse primer: AGCAAGCTCTTACTGTGACC). The parents of the progeny are kept at the Citrus Germplasm Bank, and the accession references are as follows: IVIA-385 (Cleopatra mandarin), IVIA-537 (Flying Dragon trifoliate orange) and IVIA-236 (Rich trifoliate orange).

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