Inheritance of Rootstock Effects and Their Association with Salt Tolerance Candidate Genes in a Progeny Derived from 'Volkamer' Lemon

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ABSTRACT. A seedling population from hybrids between 'Volkamer' lemon (*Citrus volkameriana*) and 'Rubidoux' trifoliate orange (*Poncirus trifoliata*) was grafted with 'Hashimoto' Satsuma mandarin (*Citrus unshiu*) to study the inheritance of rootstock effects on salt tolerance in terms of fruit yield. Trees were maintained in a screenhouse, and a salt treatment (25 mM NaCl) was applied to 32 genotypes from June to September every year for 5 years. Rootstocks were genotyped for five salt tolerance candidate genes. Significant effects of rootstock genotype (G) and treatment (E) were found for most traits. Salinity decreased yield and juice volume but improved soluble solids concentration (TSS) and rind thickness. Year effects were highly significant in most cases. G × E interactions were found for fruit weight, total fruit weight, juice volume (JV), leaf water content (LWC), and leaf [Na$^+$]. Therefore, rootstocks that induce early fruit maturation under salinity (by increasing TSS and maintaining JV) can be selected to expand the harvesting calendar of mandarin cultivars. Salt tolerance candidate genes SOS1 and NHX1 were associated with fruit yield traits under normal conditions (1.4 dS·m^{-1}), and SOS1 and CCC were associated with LWC under salinity conditions (4 dS·m^{-1}). Only 5% progeny induced higher accumulated yield than 'Volkamer' lemon under salinity. Given the low heritability of rootstock effects on fruit yield under salinity conditions (0.18 at most), marker-assisted selection might be useful.

More than 800 million hectares of land throughout the world are affected by salinity (Food and Agriculture Organization of the United Nations, 2008), which can decrease yield and lead to increased poverty and reliance on imports (Witcombe et al., 2008). Citrus species are among the most salt-sensitive of horticultural crops, although differences in tolerance exist among species in these genera (Maas, 1993). Tree growth and fruit yield of citrus species are impaired at a soil salinity of ≈2 dS·m^{-1} soil saturation without the concomitant expression of leaf symptoms (Bingham et al., 1974; Cerdá et al., 1990). Several lines of evidence indicate that citrus species are predominantly chloride-sensitive (Bañuls et al., 1997; Cooper, 1961; Ruiz et al., 1999; Walker et al., 1982). Cooper et al. (1956) classified citrus rootstocks into three groups: 1) good salt tolerance ('Cleopatra' mandarin *Citrus reshni*); 2) medium salt tolerance ['Volkamer' lemon and sour orange (*Citrus aurantium*)]; and 3) poor salt tolerance [trifoliate orange and 'Troyer' or 'Carrizo' citrange (*Citrus sinensis* × *Poncirus trifoliata*)]. This classification

agrees with the order of these genotypes according to their ability to restrict Cl$^-$ transport to the scion on grafted trees under field conditions (irrigation with saline) and in an experiment involving the culture of Satsuma mandarin (*Citrus unshiu*) rootstock combinations, which evaluated salt tolerance according to fruit and leaf characteristics (García et al., 2002). Chloride concentrations in the leaves of seedlings under salinity conditions have been used by most rootstock breeders to select salt-tolerant genotypes (Sykes, 1985). However, there are many examples of contradictory results related to the ability to exclude Cl$^-$ (Grieve and Walker, 1983); in most cases, it is not possible to determine whether experimental or genetic differences are responsible for these results. Thus, Sykes (2011) showed the presence of within-species diversity for chloride and sodium excluding capacities recommending progeny testing to investigate their inheritance for future rootstock breeding. Quantitative trait loci (QTL) analysis of these traits has been carried out in citrus seedlings (Tozlu et al., 1999).

Salt tolerance is a quantitative trait in plants. The identification of QTL that control salinity tolerance is of great importance for breeding salt-tolerant crops (Cuartero et al., 2006; Flowers, 2004; Koyama et al., 2001). However, QTL analysis of rootstock effects takes a long time as a result of the long juvenility period of citrus species and is very expensive because of the required time and huge cultivation costs for maintaining large segregating progenies. Besides, rootstock

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may affect numerous citrus attributes (Castle, 2010) making interpretation of data analysis very complicated. An alternative strategy is the analysis of candidate genes functionally involved in the salt tolerance response. These genes could serve as useful markers for early selection of rootstocks that confer salt tolerance to the grafted citrus cultivar. The regulation of ion homeostasis is one of the main strategies used by glycophytes for salinity–stress adaptation (Munns and Tester, 2008). It involves a network of transport processes that regulate the uptake, extrusion through the plasma membrane, compartmentalization of salts into cell vacuoles, and recirculation of ions through plant organs. This facilitates plant osmotic adjustment and maintenance of high K^+/Na^+ ratios in the cytosol (Apse and Blumwald, 2007; Pardo and Rubio, 2011). Genes coding for cation transporters such as HKT (high-affinity K^+ transporter), SOS1 (salt overly sensitive), and NHX family (Na^+/H^+ exchangers) are considered candidate genes for salt tolerance because they regulate the internal concentrations of Na^+ in various tissues and indirectly regulate K^+ homeostasis (Munns and Tester, 2008; Yamaguchi et al., 2013). Porat et al. (2002) isolated NHX1 from citrus and found that it was markedly induced by salt stress in a salt-tolerant cell line form 'Shamouti' sweet orange (*C. sinensis*). Brumós et al. (2009) found that a cation-chloride cotransporter (AtCCC ortholog) was induced in 'Carrizo' citrange but not in 'Cleopatra' mandarin seedlings under salt treatment.

Following Shannon and Grieve (1999), salt tolerance values should take into consideration the portion of the plant to be marketed. Salinity effect on fruit yield has been reported almost exclusively by studies that used a single-scion/single-rootstock combination (reviewed by Grieve et al., 2007; Prior et al., 2007; Storey and Walker, 1999) or one cultivar grafted onto two genetically unrelated rootstocks (García-Sánchez et al., 2003, 2006; Navarro et al., 2010; Syvertsen et al., 2010). These studies used 'Cleopatra' mandarin and 'Carrizo' or 'Troyer' citrange. Therefore, the salinity response has been extensively studied in these rootstocks; however, the inheritance of the rootstock-mediated salt tolerance has been hardly approached in citrus. We report an inheritance study of salt tolerance in terms of mandarin fruit yield conferred by a segregating population of rootstocks derived from a medium salt-tolerant *C. volkameriana* and a salt-sensitive *P. trifoliata* cultivar. As possible tools for selection in future rootstock breeding programs, trait correlations and the association of candidate genes (primarily those involved in Na^+ and Cl^- homeostasis) with traits involved in salt tolerance were also investigated.

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. A seedling population was developed by open pollination from an intergenic hybrid between 'Volkamer' lemon [V (female parent)] and 'Rubidoux' trifoliate orange [P (male parent)] obtained by J.B. Forner [Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain]. Twenty-five random seedlings derived from each individual V × P fruit-yielding tree were genotyped for five isozymatic loci (García et al., 1999) to infer their genetic origin (apomictic or sexual; sexual by cross-pollination or self-pollination). Seedlings derived from cross-pollination were discarded. The seedling population, and 10 seedlings each from two control genotypes ('Volkamer' lemon and 'Flying Dragon' trifoliate orange), were grafted with the 'Hashimoto' Satsuma mandarin in 1996.

Eighteen genotypes (13 derived from apomictic V × P hybrids) were grown as control treatment (no NaCl added), and 32 genotypes (20 derived from apomictic V × P hybrids) were grown under salinity conditions. Thirteen genotypes derived from V × P apomictic hybrids (i.e., those yielding seedlings that are genetically identical to the mother hybrid) were present in both treatments. The number of repetitions (nucellar seedlings) from apomictic hybrids varied between two and 25. Eight-year-old trees (163 total) were grown individually in 17-L pots with a sterilized substrate mix (50% peat, 30% coconut fiber, 15% sand, and 5% perlite) and placed in a screenhouse at IVIA according to a completely randomized design. Salt treatments were applied for 5 years (2004–08) between June and September with three irrigations per week of 500 mL per plant. The NaCl concentration in the irrigant was gradually increased during 2 weeks to 25 mM [4 dS·m^{-1}; pH 7.85; conductivity meter (HI9033 multirange; Hanna Instruments, Smithfield, RI)]. Plants in control conditions received the same irrigation regime using tap water (1.4 dS·m^{-1}, pH 7.43; 106 mg·L^{-1} Cl$^-$, 158 mg·L^{-1} SO$_4^{2-}$, 250 mg·L^{-1} CO$_3$H$^-$, 115 mg·L^{-1} NO$_3^-$, 138 mg·L^{-1} Ca^{2+}, 43 mg·L^{-1} Mg^{2+}, 62 mg·L^{-1} Na$^+$, 2 mg·L^{-1} K$^+$). 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$_4$H$_2$PO$_4$) and solution B. The stock solution B contained 12 M Ca(NO$_3$)$_2$, 2.6 M KNO$_3$, 36 g·L^{-1} iron chelate (Sequestrene 138 Fe; Syngenta, Madrid, Spain), and 5% microelement solution. This solution contained 0.3 mM copper SO$_4$·5 H$_2$O, 3.1 mM zinc SO$_4$·7 H$_2$O, 109 mM manganese Cl$_2$·4 H$_2$O, 92 mM BO$_3$H$_3$, 2 mM NH$_4$MoO$_4$, and 0.4 mM V$_2$O$_5$. A record of weather data variables including temperature and relative humidity measured under the net was obtained from the Moncada meteorological station located at IVIA (Supplemental Fig. 1).

TRAIT EVALUATION. Four yield-related traits and 25 vegetative and physiological traits were evaluated for each plant. Eighteen of these traits were measured during the 5 experimental years under control and salinity conditions. The following fruit-yield traits were evaluated annually between October and November ['Hashimoto' Satsuma mandarin collection period (Soler, 1999)]: fruit number per plant (FN); mean fruit weight per plant (FW), measured as the average weight (grams) of 10 randomly sampled fruit; and total yield per plant (TFW), measured as the weight (kilograms) of all fruit produced per plant. The total accumulated production per plant (AcTFW), measured as the total fruit weight (kilograms) produced during the 5 experimental years (2004–08), was also measured.

The 10 randomly sampled fruit per tree also were evaluated every year for the following internal and external fruit-quality traits: fruit diameter [FD (millimeters)] was measured in the transversal section; rind thickness [RT (millimeters)], including flavedo and albedo, was measured in the transversal section; juice volume (milliliters) was quantified as the volume of juice without pulp; acidity (pH) was measured with a digital pH meter (PH25; Crinson, Barcelona, Spain); and total soluble solids (percent) was measured with a digital refractometer (Pallete PR-101; Atago, Tokyo, Japan).

Three fully developed leaves per plant were sampled from vegetative spring shoots to measure the following leaf characteristics after each yearly saline treatment period (June to

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September): final leaf fresh weight [LFW*f* (grams)]; final leaf dry weight [LDW*f* (grams)] measured in samples dried at 100 °C for 3 d; accumulated dry matter percentage (%DMA) calculated as the difference between leaf dry weight at the beginning and at the end of salt treatment [%DMA = (LDW$_f$ − LDW$_i$) × (100/LDW$_i$)]; final leaf water content [LWC*f* (grams)] calculated as the difference between LFW$_f$ and LDW$_f$; leaf area [LA (square centimeter)] measured with a leaf area quantifier (LI-3100C area meter; LI-COR, Lincoln, NE); leaf color parameters LCL*, LCa*, and LCb* defined by Hunter coordinates (L*, a*, b*) arranged in a Cartesian system, where a* (*x*-axis) varies in a green/red scale (–60 to +60), b* (*y*-axis) varies in a blue/yellow scale (–60 to +60), and L* represents brightness in a black/white range (0 to 100) (CR-200 colorimeter; Konica Minolta, Basildon, U.K.); and leaf color parameters Cr* and Hue* defined by the cylindrical coordinate system (L*, C*, h), where Cr* (chroma) represents color intensity (0 to 60) {Cr* = $\sqrt{[(a*)^2 + (b*)^2]}$} and Hue* (hue angle) represents leaf color (0° to 360°) [hue* = tan^{-1} (b*/a*)] (HunterLab, 1996). The trait increment (for LFW, LDW, and LWC) at the end of the treatment (dTrait) also was calculated each year.

The foliar concentration (milligrams per liter) of Cl$^-$ was evaluated (Model 926 chloride analyzer; Sherwood Scientific, Cambridge, U.K.) at the end of each saline treatment period (June to September) using a random sample of three leaves per plant from the vegetative spring shoots (Gilliam, 1971). The foliar concentrations (percentage) of Na$^+$, Ca^{2+}, Mg^{2+}, K$^+$, and phosphorus were evaluated by inductively coupled plasma analysis at G.E. Cota.2 (Valencia, Spain) only for 2005.

At the end of the experiment in 2008, all plants (12-year-old trees) were pulled up, cleaned, and dissected to evaluate the following plant growth traits: trunk diameter [TrunkD (millimeters)] measured 2 cm above the graft line (using a digital caliper); total plant weight (kilograms); foliage weight (kilograms) measured above the graft line; rootstock weight (kilograms) the weight of the roots and trunk below the graft line; and the root weight [RootW (kilograms)]. Four leaf traits were measured only at the end of the experiment: leaflet fresh weight [LFWhj (grams)]; rachis fresh weight [LFWrq (grams)]; leaflet dry weight [LDWhj (grams)]; and rachis dry weight [LDWrq (grams)].

Rootstock genotyping. Genomic DNA was extracted from the bark tissue below the graft union in each plant according to the protocol of Ruiz et al. (2000) with the following minor modifications. One piece of bark (1 × 0.5 cm) was homogenized in liquid N$_2$, and an additional ethanol wash was performed before resuspending the DNA pellet. Polymerase chain reaction (PCR) conditions were specific for each marker, and the resulting product was analyzed by electrophoresis in sequencing-type 10% polyacrylamide gels and revealed by silver staining as described in Ruiz et al. (2000). A set of five primer pairs was used to detect sequence characterized amplified regions targeting the following salinity tolerance candidate genes, all involved in ion Na$^+$ or Cl$^-$ homeostasis: SOS1 and SOS2 [salt overly sensitive primers were derived from those used in Villalta et al. (2008)]; NHX1 [Na$^+$/H$^+$ exchanger primers designed from citrus cDNA sequence AY028416 at the National Center for Biotechnology Information (Porat et al., 2002)]; CCC [cation chloride cotransporter primers designed from the citrus chloride cotransporter DNA sequence kindly provided by J.M. Colmenero-Flores (Colmenero-Flores et al., 2007)]; and Ethrec (ethylene receptor), whose primers were obtained from the

cDNA sequence AF092088 (*C. sinensis* putative ethylene receptor mRNA). In the case of CCC, four segregating alleles were revealed after *Alu*I digestion of PCR products, obtaining a cleavage amplified polymorphism marker. These salt tolerance candidate genes were located at the following linkage groups: 10 + 5b (SOS1 and NHX1), 8 + 6 (SOS2), 7 (CCC), and 3 (Ethrec), as reported by Bernet et al. (2010) and Raga et al. (2012). The identity of the polymorphic amplification products was previously checked by sequence analysis. A citrus primer list is available for scientific purposes only at IVIA Genetic Laboratory (Asins et al., 2009).

Statistical and genetic analyses. The fixed effects for rootstock G, E, year (Y), and all their possible interactions [i.e., genotype per treatment (G × E), genotype per year (G × Y), treatment per year (E × Y), and genotype per treatment per year (G × E × Y)] were analyzed by a repeated measures approach using trees (within each genotype and treatment) as a random subject factor and first-order autoregressive covariance structure between measurements taken from the same tree over the years. Pearson's correlation analyses between fruit yield parameters and all evaluated traits were studied under control and salinity conditions using the 13 apomictic hybrids included in both treatments in all years (InfoStat, 2004). Considering rootstocks as a random effects factor, broad-sense heritability (H^2) was estimated for all traits evaluated in 2008 only for nucellar rootstocks (repetitions) derived from apomictic V × P hybrids under control (H^{2}c) or salinity (H^{2}s) conditions based on the genotypic (V$_G$) and environmental (V$_E$) variance estimators calculated by minimum variant quadratic unbiased estimator, as previously reported (Villalta et al., 2007).

Associations between parental alleles at candidate genes and means per treatment for traits were studied using the Kruskal-Wallis procedure in MapQTL 6 (van Ooijen, 2009). All V × P plants were considered taking into account their genotypes and nucellar or self-pollination origins. The significance level for candidate genes was fixed at $P \leq 0.05$.

Results

A significant effect ($P \leq 0.05$) of the rootstock genotype and treatment was found for most fruit yield and quality traits (Table 1). Salinity decreased yield and juice volume, but improved two quality traits by ≈6% (TSS and RT). Year effects were highly significant in most cases.

Salinity greatly reduced tree growth and accumulated fruit yield. Leaf concentrations of Cl$^-$ and Na$^+$ increased strongly (215% and 149%, respectively). No significant rootstock effect on leaf Cl$^-$ concentration was detected in contrast to that for leaf Na$^+$ concentration. A significant G × E interaction was found for this trait, suggesting that the rootstock effect depends on the presence of salinity (i.e., differences among rootstocks for Na$^+$ translocation to the leaf were not predicted from control conditions). Few traits showed a significant G × E interaction (differential rootstock behavior) that did not depend on the year (no significant G × E × Y); these included FW, TFW, and LWC. There were two additional traits for which a G × E interaction was significant (AcTFW and leaf [Na$^+$]), but these were evaluated in only 1 year. Estimates for trait heritability ranged from low to very low (Table 1). In general, plants under saline treatment had smaller estimates than those under control conditions. Exceptions to this included pH, LDW, LDWrq, LFWrq, and TrunkD.

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Table 1. Probability values of rootstock effects from the three-way repeated analyses using nucellar rootstocks from 13 apomictic hybrids between 'Volkamer' lemon and 'Rubidoux' trifoliate orange.[z]

Trait[y]	G[x]	E	C→S	G×E	Y[w]	G×Y	E×Y	G×E×Y	H²c	H²s
FN	<0.0001	—	—	—	<0.0001	—	—	—	0.29	0.18
FW	0.0016	0.0001	−6	0.0018	<0.0001	—	—	—	0.21	0.10
TFW	<0.0001	<0.0001	−14	0.0251	<0.0001	0.0113	0.0291	—	0.40	0.15
AcTFW	0.0156	<0.0001	−30	0.0079	.	.	.	.	0.33	0.01
TSS	<0.0001	0.0041	6	—	<0.0001	—	—	—	0.18	0.10
JV	0.0302	0.0002	−3	0.0309	<0.0001	0.0002	—	0.0187	0.09	0.06
RT	0.0012	0.0003	−6	—	<0.0001	—	—	—	0.08	—
FD	0.0154	0.0001	−3	—	<0.0001	—	—	—	0.14	—
pH	0.0003	—	—	—	<0.0001	<0.0001	—	—	—	0.21
LDWf	—	—	—	—	<0.0001	—	—	—	—	0.18
LDWhj	—	—	—	—	.	.	.	.	—	—
LDWrq	—	—	—	—	.	.	.	.	—	0.10
% DMA	—	0.0264	−44	—	.	.	.	.	0.05	0.12
LFWf	—	—	—	—	<0.0001	—	<0.0001	—	—	—
LFWhj	—	—	—	—	.	.	.	.	—	0.04
LFWrq	—	—	—	—	.	.	.	.	—	0.14
LWCf	—	—	—	0.0401	<0.0001	—	<0.0001	—	0.03	0.08
LA	—	0.0351	7	—	<0.0001	—	<0.0001	—	0.02	—
LCa*	0.0315	—	—	—	<0.0001	—	0.0010	—	0.13	0.08
LCb*	—	—	—	—	<0.0001	0.0089	0.0006	—	0.19	—
LCL*	0.0145	—	—	—	<0.0001	—	0.0025	—	0.19	—
Cr*	0.0270	—	—	—	<0.0001	0.0052	0.0014	—	0.26	0.01
Hue*	—	0.0102	−1	—	<0.0001	—	0.0055	—	—	—
PLTW	<0.0001	0.0001	−27	—	.	.	.	.	0.38	0.17
FoW	<0.0001	<0.0002	−28	—	.	.	.	.	0.45	0.17
RTSW	0.0003	0.0023	−25	—	.	.	.	.	0.26	0.17
RootW	0.0002	0.0023	−27	—	.	.	.	.	0.30	0.19
TrunkD	—	—	—	—	.	.	.	.	0.08	0.38
Cl	—	<0.0001	215	—	<0.0001	0.0449	<0.0001	0.0223	0.30	0.10
Na	0.0005	<0.0001	149	0.0059	.	.	.	.	0.39	0.22
Ca	0.0025	0.0061	−1	—	.	.	.	.	0.30	0.06
Mg	<0.0001	<0.0001	−11	—	.	.	.	.	0.38	0.28
K	0.0414	0.0021	32	—	.	.	.	.	0.18	0.01
P	0.0091	0.0056	13	—	.	.	.	.	0.05	0.04

[z]'Hashimoto' Satsuma mandarin was the scion in all cases. G, E, and Y correspond to genotypic, salinity treatment (25 mM NaCl), and year effects, respectively. Genotype per treatment (G×E), genotype per year (G×Y), treatment per year (E×Y), and genotype per treatment per year (G×E×Y) are the interactions. The positive or negative increment of trait mean when the treatment was significant is indicated as a percentage in the transition from a control to the saline condition (C→S). H²c and H²s are sensu lato heritability estimates for control and salinity conditions, respectively.

[y]FN = fruit number; FW = mean fruit weight; TFW = total yield; AcTFW = total accumulated fruit yield; TSS = soluble solids concentration; JV = juice volume; RT = rind thickness; FD = fruit diameter; pH = fruit juice acidity; LDWf = final leaf dry weight; LDWhj = leaflet dry weight; LDWrq = rachis dry weight; % DMA = accumulated dry matter percentage; LFWf = final leaf fresh weight; LFWhj = leaflet fresh weight; LFWrq = rachis fresh weight; LWCf = final leaf water content; LA = leaf area; LCa*, LCb*, and LCL* = leaf color parameter (defined by Hunter a*, b* and L*, respectively); Cr* = leaf color function $\sqrt{(a^*)^2 + (b^*)^2}$; Hue* = leaf index $\tan^{-1}(b^*/a^*)$; PLTW = total plant weight; FoW = foliage weight; RTSW = rootstock weight; RootW = root weight; TrunkD = trunk diameter; Cl, Na, Ca, Mg, K, and P = foliar concentrations of Cl^-, Na^+, Ca^{2+}, Mg^{2+}, K^+, and phosphorus, respectively.

[x]Dashes indicate data not significant.

[w]Full stops indicate that the trait was evaluated only 1 year.

Considering accumulative fruit yield under salinity as the most relevant criteria for selection of rootstocks conferring salt tolerance, only 5% of V × P hybrids conferred higher yield than the 'Volkamer' lemon parent when used as rootstock (Fig. 1B) Noteworthy, differences between control genotypes were larger under control than under salinity condition (Figs. 1A and 1B). If only the last 3 years of evaluation are considered (2006–08), and $P > 0.02$ is chosen as significant, then TFW is strongly correlated with the FN component (Table 2), which is quite consistently related to TSS, particularly under salinity. The FW component is related to FD, JV, and RT, and indirectly to TSS and FN, under both control and salinity conditions. Correlation coefficients for traits evaluated under control conditions were usually higher than the same traits under salinity conditions. A significant correlation between both conditions with respect to fruit traits was found only for AcTFW (0.79) and FN (0.73) in
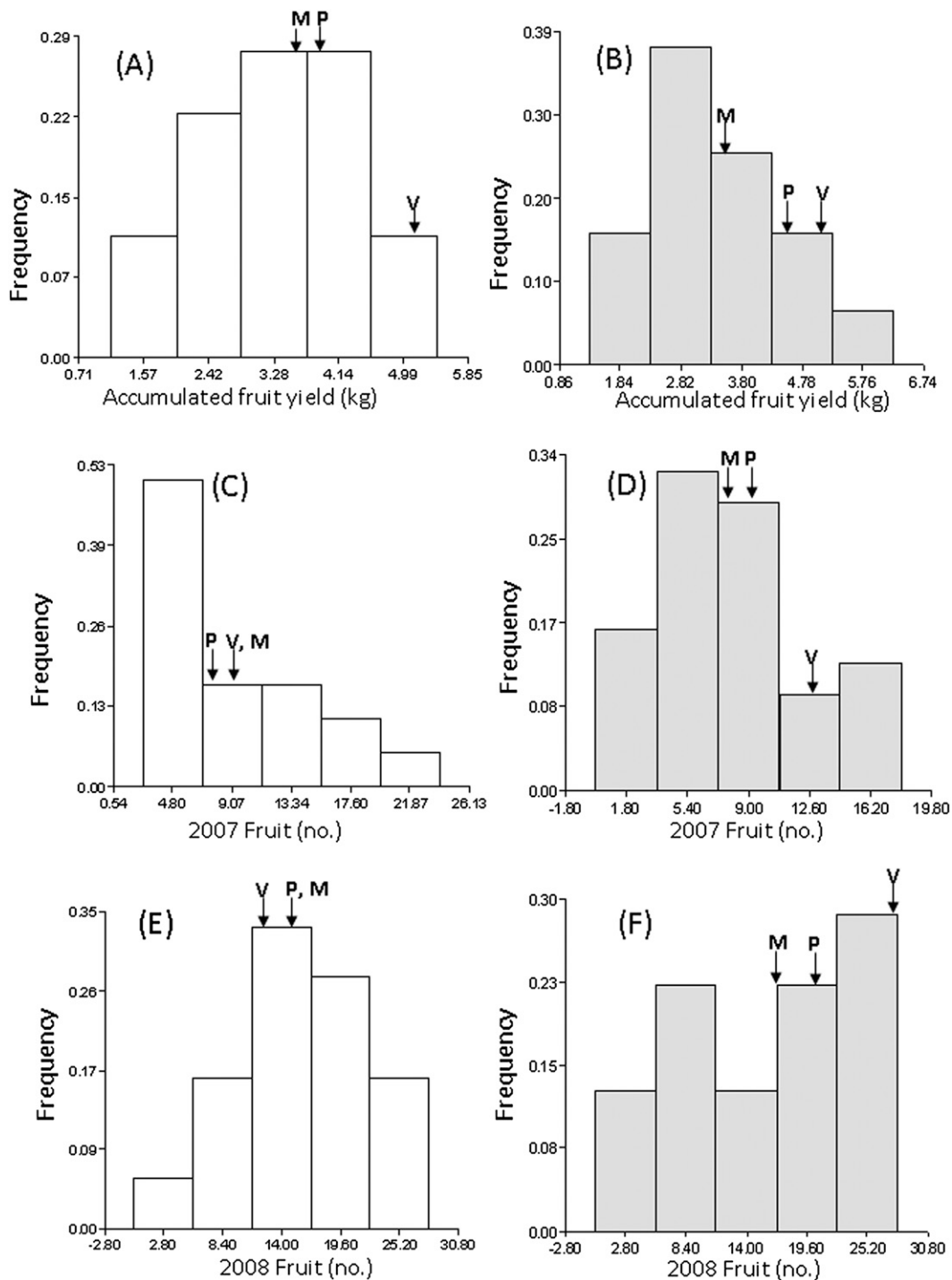
Fig. 1. Frequency distributions of (**A–B**) total accumulated fruit yield (AcTFW) and (**C–F**) fruit number (FN) of 'Hashimoto' Satsuma mandarin grafted on nucellar seedlings from hybrids between 'Volkamer' lemon and 'Rubidoux' trifoliate orange for the two last yielding years under control (**A, C**, and **E**) and salinity (**B, D**, and **F**) conditions. The phenotypic means of the population (M) and the control genotypes ['Volkamer' lemon (V) and 'Flying Dragon' trifoliate orange (P)] are indicated.

2007, despite the FN distribution differences between both treatments (Figs. 1C and 1D).

Fruit yield traits did not show a consistent, significant correlation with any non-fruit (vegetative) trait. Leaf [Cl⁻] appeared related to FN and indirectly to FW under control and salinity conditions in 2007 and 2006, respectively, whereas leaf [Na⁺] was only related to TFW and FN in 2006. The leaf color

hue* index also appeared related to fruit yield traits under control (2006 and 2007) and salinity (2007) conditions.

The poor consistency of correlations through all experimental years indicated the importance of the year effect. Thus, the distribution of FN (under both the control and salinity conditions) changed depending on the year, as shown in Figure 1C–E for the last 2 consecutive years (2007 and 2008).
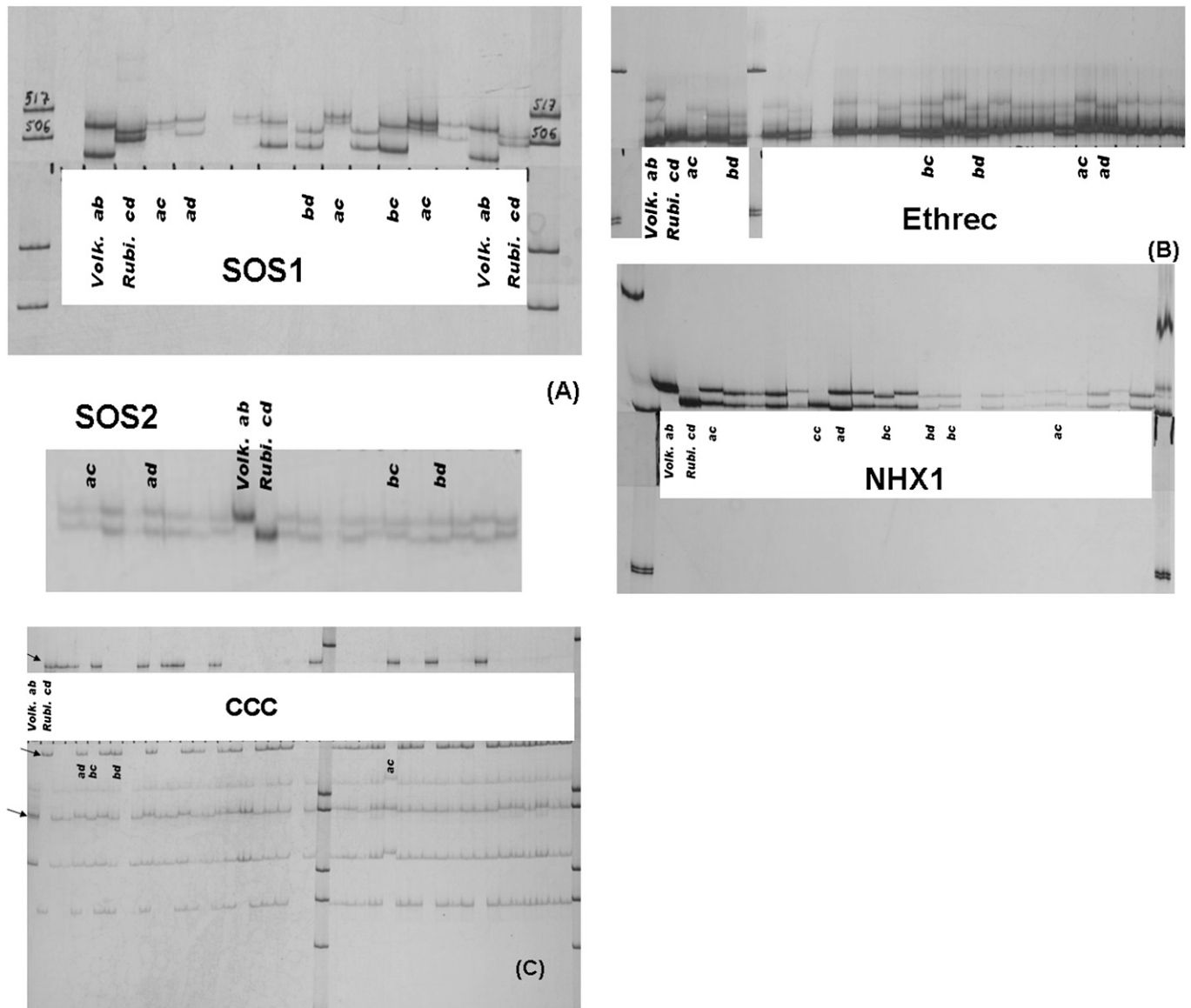
Fig. 2. Silver-stained gel electrophoresis of markers developed for (**A**) salt overly sensitive genes SOS1 and SOS2; (**B**) Ethrec (ethylene receptor) and the Na^+/H^+ exchanger gene NHX1; and (**C**) CCC (cation chloride cotransporter) of V × P hybrids between between 'Volkamer' lemon (V) and 'Rubidoux' trifoliate orange (P). Four possible genotypes of the V × P progeny are indicated as *ab*, *ac*, *bc*, and *bd*, where *a* and *b* correspond to the alleles of 'Volkamer' lemon (Volk.) and *c* and *d* correspond to those of 'Rubidoux' trifoliate orange (Rubi.).

Four genotypes, named *ac*, *ad*, *bc*, and *bd*, were observed for the hybrids derived from the cross between 'Volkamer' lemon (*ab*) and 'Rubidoux' trifoliate orange (*cd*) at each of the five candidate gene loci (Fig. 2). Similar to the results from correlation analyses, candidate genes were associated with some traits only in certain years, except for TFW, which was consistently associated with SOS1 (Fig. 2A) under control conditions (1.4 dS·m^{-1} tap water) for the 3 last experimental years (Table 3). NHX1 (Fig. 2B) was associated with fruit yield traits (FN and FW) only under control conditions. The same allele at NHX1 that correlated with higher FW7 (fruit weight in year 2007) and FW8 also correlated with higher LWC7. The same allele at SOS1 that correlated with higher FN6 and FN7 correlated with higher TFW in 2006, 2007, and 2008. Leaf [Cl$^-$] was found associated with CCC (Fig. 2C) and Ethrec (Fig. 2B), but only under control conditions. For Ethrec, the same allele

that correlated with a higher increment for leaf water content in control (dLWC8_C) was associated with a high leaf [Cl$^-$]. CCC was associated with LWC (but not with leaf [Cl$^-$]) under salinity conditions. Under salinity conditions, variations for LWC were associated with 'Volkamer' lemon alleles (at CCC and SOS1), whereas 'Rubidoux' trifoliate orange alleles in the rootstock at SOS2 were associated with significant variations in leaf [Na$^+$] in Satsuma mandarin.

Discussion

Many non-genetic factors can affect plant responses to salinity such as the composition and concentration of salts (Lauchli and Grattan, 2007), drought, irradiance, leaf temperature, atmospheric evaporative demand, soil type, and agronomic practices (Adnan, 2004; Syvertsen and Levy, 2005). We

Table 2. Pearson correlation coefficients and probability values of significant ($P < 0.02$) correlations between fruit yield traits (last 3 years) and all evaluated traits (Corr. trait) using nucellar rootstocks from 13 apomictic hybrids between 'Volkamer' lemon and 'Rubidoux' trifoliate orange under control and salinity (25 mM NaCl) conditions.[z]

Control				Salinity			
Yield trait[y]	Corr. trait[x]	Pearson	P value	Yield trait[y]	Corr. trait[x]	Pearson	P value
TFW8	TSS8	0.57	0.0171	TFW8	pH8	−0.56	0.0014
TFW8	FN8	0.95	<0.0001	TFW8	FN8	0.93	<0.0001
TFW7	FN7	0.95	<0.0001	TFW7	Hue*7	−0.47	0.0070
TFW6	LCb*6	0.78	0.0001	TFW7	FN7	0.92	<0.0001
TFW6	Cr*6	0.80	0.0001	TFW6	TSS6	0.61	0.0005
TFW6	pH6	−0.76	0.0004	TFW6	RT6	−0.60	0.0006
TFW6	LCL*6	0.73	0.0006	TFW6	FW6	−0.60	0.0006
TFW6	TSS6	0.71	0.0013	TFW6	FD6	−0.54	0.0026
TFW6	Hue*6	−0.57	0.0128	TFW6	Na	−0.69	<0.0001
TFW6	FN6	0.95	<0.0001	TFW6	FN6	0.87	<0.0001
FW8	JV8	0.69	0.0024	FW8	RT8	0.55	0.0018
FW8	dLDWhj	−0.64	0.0058	FW8	TSS8	−0.54	0.0022
FW8	dLDW8	−0.63	0.0066	FW8	pH8	0.48	0.0074
FW8	RT8	0.57	0.0161	FW8	K	0.46	0.0127
FW8	FD8	0.96	<0.0001	FW8	FN8	−0.44	0.0148
FW7	JV7	0.75	0.0005	FW8	JV8	0.73	<0.0001
FW7	FD7	0.75	0.0005	FW8	FD8	0.93	<0.0001
FW7	Cl7	−0.65	0.0034	FW7	JV7	0.49	0.0068
FW7	LA7	0.57	0.0134	FW7	RT7	0.48	0.0079
FW7	RT7	0.58	0.0155	FW7	FN7	−0.48	0.0081
FW6	RT6	0.83	0.0001	FW7	FD7	0.81	<0.0001
FW6	pH6	0.70	0.0024	FW6	Cl6	−0.57	0.0011
FW6	TSS6	−0.68	0.0037	FW6	TSS6	−0.70	<0.0001
FW6	FN6	−0.65	0.0062	FW6	RT6	0.87	<0.0001
FW6	FD7	0.88	<0.0001	FW6	JV6	0.81	<0.0001
FN8	TSS8	0.72	0.0012	FW6	FN6	−0.80	<0.0001
FN7	Cl7	0.69	0.0016	FW6	FD6	0.94	<0.0001
FN7	Hue*7	−0.55	0.0186	FN8	pH8	−0.65	0.0001
FN7	FD7	−0.51	0.0380	FN8	TSS8	0.55	0.0018
FN6	TSS6	0.80	0.0001	FN7	Hue*7	−0.49	0.0049
FN6	LCL*6	0.78	0.0001	FN7	TSS7	0.47	0.0110
FN6	LCb*6	0.78	0.0001	FN7	LCL*7	0.44	0.0142
FN6	Cr*6	0.80	0.0001	FN6	Na	−0.64	0.0002
FN6	FD6	−0.56	0.0185	FN6	JV6	−0.64	0.0002
FN6	pH6	−0.90	<0.0001	FN6	Cl6	0.50	0.0043
				FN6	TSS6	0.78	<0.0001
				FN6	RT6	−0.74	<0.0001
				FN6	FD6	−0.78	<0.0001

[z]'Hashimoto' Satsuma mandarin was the scion in all cases. The number after the trait name indicates the year of harvesting (e.g., six for 2006).
[y]Significant yield traits: FN = fruit number; FW = mean fruit weight; TFW = total yield.
[x]Correlated traits: FN = fruit number; FW = mean fruit weight; TSS = soluble solids concentration; JV = juice volume; RT = rind thickness; FD = fruit diameter; pH = fruit juice acidity; dLDW = increment for leaf dry weight at the end of the treatment; dLDWhj = increment for leaflet dry weight at the end of the treatment; LA = leaf area; LCb* and LCL* = leaf color parameters defined by Hunter b* and L*; Cr* = leaf color function $\sqrt{(a^*)^2 + (b^*)^2}$; Hue* = leaf index $\tan^{-1} (b^*/a^*)$; Cl, Na, and K = foliar concentrations of Cl$^-$, Na$^+$, and K$^+$, respectively.

experimentally tried to minimize non-genetic effects and interactions by growing plants in large pots under a screenhouse and controlling salinity with irrigation. We approached the citriculture practice by evaluating the rootstocks in terms of the fruit yield of the scion during several years. For this purpose, a population of genetically related rootstocks was grafted with the same citrus cultivar. This experimental design allowed us to genetically analyze the rootstock effects on the grafted cultivar. The approach of Tozlu et al. (1999) used a non-grafted segregating progeny derived from *Citrus grandis* (a sexual species not used as rootstock) and *P. trifoliata*. Our

approach intended a more realistic evaluation of the salt tolerance conferred by the rootstock. Nevertheless, our findings, using a semihydroponic system, might differ from those that would have been obtained under real field conditions.

Highly significant year effects were obtained for most traits (Table 1). Differences among years for relative humidity and temperature existed (Supplementary Fig. S1), which might explain the year effects, at least in part. Mandarins are perennial crops with alternate-bearing, and so year effects also can be attributed to this phenomenon and interfere with the salt

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Table 3. List of significant trait candidate gene associations and corresponding genotypic means using seedlings from 13 or 32 hybrids between 'Volkamer' lemon and 'Rubidoux' trifoliate orange as rootstocks under control and salinity (25 mM NaCl), respectively.[z]

Trait[y]	Candidate gene[x]	K*	Mean a-/c-	Mean b-/d-
Cl8_C	CCC_V	4.667 *	11.678	15.471
LWC8_S	CCC_R	4.539 *	1.687	1.438
AcTFW8_C	NHX1_R	4.000 *	4.073	3.107
Na_C	NHX1_R	4.554 *	0.068	0.110
FW8_C	NHX1_V	4.303 *	63.691	78.322
FW7_C	NHX1_V	8.163 ***	106.181	119.938
LWC7_C	NHX1_V	4.930 *	1.304	1.590
dLWC8_C	Ethrec_R	3.927 *	0.514	0.418
Cl8_C	Ethrec_R	4.200 *	15.761	11.972
RootW8_C	SOS1_R	4.022 *	0.585	0.314
RTSW8_C	SOS1_R	4.011 *	0.657	0.379
PLTW8_C	SOS1_R	4.930 *	0.425	0.311
FoW8_C	SOS1_R	4.917 *	1.082	0.689
TFW8_C	SOS1_R	4.303 *	1.362	0.930
TFW7_C	SOS1_R	5.239 *	1.362	0.683
FN7_C	SOS1_R	4.592 *	12.187	6.270
TFW6_C	SOS1_R	6.302 *	1.200	0.797
FN6_C	SOS1_R	6.267 *	15.708	9.336
LWC8_S	SOS1_V	4.539 *	1.438	1.687
Na_S	SOS2_R	5.272 *	0.299	0.394

[z]'Hashimoto' Satsuma mandarin was the scion in all cases. The letters R and V after the marker locus refer to 'Rubidoux' trifoliate orange (c and d) or 'Volkamer' lemon (a and b) alleles, respectively (see Fig. 2). Means of hybrids designated a- and b- correspond to those genotypes receiving allele a (ac and ad) or b (bc and bd), respectively, from 'Volkamer' lemon. Similarly, genotypic means c- and d- correspond to hybrids receiving allele c (ac and bc) or d (ad and bd) from 'Rubidoux' trifoliate orange, respectively. K is the Kruskal-Wallis statistic provided by MapQTL 6 software (van Ooijen, 2009). The number after the trait name indicates the year of harvesting (e.g., six for 2006). Control (1.4 dS·m⁻¹) or salinity (4 dS·m⁻¹) conditions are indicated with the suffix _C or _S after the trait code. *Significant at 5%; ***significant at 0.5%.

[y]Salt tolerance-related traits: FN = fruit number; FW = mean fruit weight; TFW = total yield; AcTFW = total accumulated fruit yield; LWC = final leaf water content; PLTW = total plant weight; FoW = foliage weight; RTSW = rootstock weight; RootW = root weight; Cl and Na = foliar concentrations of Cl⁻ and Na⁺, respectively.

[x]Candidate gene for: SOS1 and SOS2 (salt overly sensitive), NHX1 (Na⁺/H⁺ exchanger), CCC (cation chloride cotransporter), and Ethrec (ethylene receptor).

tolerance response in terms of fruit yield. For these reasons, a multiyear approach was used.

A general reduction of tree vegetative growth by ≈30% was observed under salinity conditions (Table 1). Similar effects have been reported in grafted citrus crops with reductions in plant height, canopy volume, and trunk diameter (García-Sánchez et al., 2006; Grieve et al., 2007). Our results showed that the rootstock greatly influenced fruit traits of the grafted cultivar (in agreement with the citrus bibliography), but no single vegetative trait could fully predict fruit yield. Heritability estimates of rootstock-mediated effects on fruit yield under salinity (Table 1) were low, 0.18 at most, below those reported for tomato (*Solanum lycopersicum*) rootstocks (0.3 in Estañ et al., 2009). In general, trait heritabilities were higher under control than under salinity conditions, which suggested that the

rootstock segregant population had larger genetic variability (i.e., more genes involved and/or larger effects on the traits) under control conditions than under salinity conditions. However, there were exceptions, because traits might be related to physiological mechanisms of salinity tolerance, including LDW, LDWrq, %DMA, LFWhj, LFWrq, LWC, and TrunkD. All these exceptional traits were related to vegetative growth and the osmotic stress response (Munns and Tester, 2008).

Citrus salt tolerance is related to the capacity to restrict Cl⁻ transport from root to leaves (Levy and Syvertsen, 2004; Maas, 1993; Ream and Furr, 1976). Heritability estimates of the leaf concentration for both toxic ions clearly decay under salinity, primarily as a result of non-genetic components. It is deduced from Table 1 that genetic variability (H²) to improve scion leaf [Na⁺] is available at the segregating rootstock population, but apparently not for [Cl⁻]. Therefore, is scion leaf [Cl⁻] a good criterion to select V × P rootstocks for salt tolerance? The [Cl⁻] is directly related to FN (0.69) and indirectly related to FW (–0.65) under control conditions (Table 2). Under salinity conditions, correlation coefficients decreased to 0.50 and –0.57 for FN and FW, respectively. Therefore, the value of leaf [Cl⁻] to predict salt tolerance in the V × P population is low. For candidate gene analyses, the CCC of the citrus rootstock is only associated with leaf [Cl⁻] under control conditions (Table 3); it does not appear useful to predict leaf [Cl⁻] under salinity in the V × P progeny. Chloride is an essential plant micronutrient that participates in osmoregulation, cell elongation, vacuolar turgor regulation, membrane potential stabilization, and pH regulation (White and Broadley, 2001). The double role (nutritious/toxic) of Cl⁻ in citrus plants might explain why different genes control leaf [Cl⁻] depending on the absence/presence of salinity. However, the lack of a significant association of CCC genotypes with leaf [Cl⁻] variations under salinity does not prove that CCC is not involved in salinity responses. The large non-genetic variability and/or a decay of its putative individual contribution under salinity conditions might explain the failure to detect it under salinity conditions.

Considering the scion leaf [Na⁺] as a good criterion to early select rootstocks for salt tolerance, *P. trifoliata* was reported as an efficient Na⁺ excluder at low salinities (Sykes, 2011; Walker, 1986), similar to some of its hybrids (Sykes, 1992). This is in agreement with the association of leaf [Na⁺] and AcTFW with NHX1 under control conditions (Table 3), in which high yield and low leaf [Na⁺] are related to the same 'Rubidoux' allele, suggesting a putative contribution of the citrus rootstock NHX1 to the variation of both scion traits. There is increasing evidence to show that NHX antiporters regulate the homeostasis of K⁺ and pH in intracellular membranes under normal and saline conditions (Barragán et al., 2012; Bassil et al., 2011; Leidi et al., 2010; Venema et al., 2003; Yamaguchi et al., 2013). The tonoplast vacuole NHX1 antiporter participates in *Arabidopsis thaliana* salt tolerance by compartmentalization of Na⁺ (Leidi et al., 2010; Munns and Tester, 2008). However, under salinity conditions, the association between AcTFW with NHX1 was not detected, and leaf [Na⁺] was significantly associated with 'Rubidoux' trifoliate orange alleles at SOS2 (Table 3).

The 'Rubidoux' alleles of the salt tolerance candidate gene SOS1 were consistently associated with fruit yield under control conditions (Table 3). As it has been recently reviewed by Yamaguchi et al. (2013), SOS1 appears to have several other functions that are not directly related to Na⁺ homeostasis but to pH homeostasis, both in the cytosol and the vacuole of root

cells. Noteworthy, SOS1 is also associated with root and rootstock weights (Table 3). The 'Volkamer' lemon alleles at both SOS1 and CCC were associated with LWC under salinity conditions. Therefore, maintaining the scion water content might be an important role of the citrus rootstock derived from 'Volkamer' lemon to manage excess Cl^- and Na^+ under moderate salinity, genetically connecting the physiological mechanisms of salt tolerance defined by Munns and Tester (2008). Among the exceptional traits whose heritability estimates increased under salinity conditions, LWC showed statistical evidence of differential rootstock behavior depending on the presence of salinity [significant $G \times E$ (Table 1)]. This result supports the hypothesis that salt tolerance in citrus depends to a great extent on water use (Moya et al., 2003; Syvertsen et al., 2010), although we found no significant genetic correlation between LWC and leaf $[Cl^-]$ under salinity nor control conditions. This salt tolerance strategy based on the rootstock's ability to maintain the scion water content was also found in tomato (Asins et al., 2010). Therefore, exploiting this tolerance mechanism might be useful for rootstock breeding in general.

Our results did not show a significant effect of salinity on mandarin juice pH (Table 1). Other citrus studies (reviewed by Navarro et al., 2010), with different experimental designs (one or two unrelated rootstocks) and different grafted cultivars, showed different salinity effects on juice pH, either increasing (Navarro et al., 2010) or decreasing (García-Sánchez et al., 2003) juice acidity.

Significant effects of salinity on other fruit quality traits were observed. Some traits such as JV and FD were reduced, whereas others were improved such as TSS and RT (Table 1). Higher TSS under salinity stress was previously observed for other grafted citrus cultivars (Boman, 2005; Dasberg et al., 1991; García-Sánchez et al., 2003, 2006; Navarro et al., 2010). Under salinity conditions, JV was directly correlated with FW, whereas TSS was directly correlated with FN and indirectly correlated with FW. Therefore, the TSS increase could be related to the reduction in JV (and FW) as a result of reduced water availability, as found previously for a Clementine mandarin (Navarro et al., 2010). The main components of variance influencing TSS were the rootstock genotype and the year ($P < 0.0001$; Table 1) and no significant interaction ($G \times E$, $G \times Y$, $E \times Y$, and $G \times E \times Y$) was found suggesting that all genotypes increase TSS similarly under salinity. Because this is not the case for JV, other than reduction of JV could contribute to the increment of TSS under salinity. The enhancement of sucrose hydrolysis, increasing the concentrations of hexose sugars, might be involved such as was proposed under moderate water deficit stress in Satsuma mandarin (Yakushiji et al., 1996; Yukushiji et al., 1998) and 'Valencia' sweet orange (Barry et al., 2004).

Given that mandarin juice volume (and FW) showed a significant $G \times E$ interaction, whereas TSS did not, it seems possible to select $V \times P$ rootstocks that would increase TSS and maintain JV under salinity conditions. As a consequence, these rootstocks would induce earlier fruit maturation for a given grafted cultivar under salinity conditions and expand the harvesting period of citrus cultivars, which is a major goal of the citrus industry worldwide.

In conclusion, our results on the inheritance of rootstock effects on salt tolerance in the progeny of 'Vokamer' lemon agrees the hypothesis that it depends to a great extent on water use (Moya et al., 2003; Syvertsen et al., 2010). Because only a 5% of $V \times P$ progeny induced higher fruit yield than the salt-tolerant parent, and the heritabilities of rootstock effects were low, selection assisted by associated markers might be useful to obtain rootstocks that confer salt tolerance to the grafted mandarin. Salt-tolerant candidate genes SOS1 and NHX1 were associated with fruit yield traits under control conditions (1.4 dS·m^{-1}), and SOS1 and CCC were associated with LWC under salinity conditions (4 dS·m^{-1}). Results from rootstock effects on fruit quality traits suggest that rootstocks inducing early fruit maturation can be selected within the $V \times P$ progeny to expand the harvesting calendar in citrus under moderate salinity conditions.

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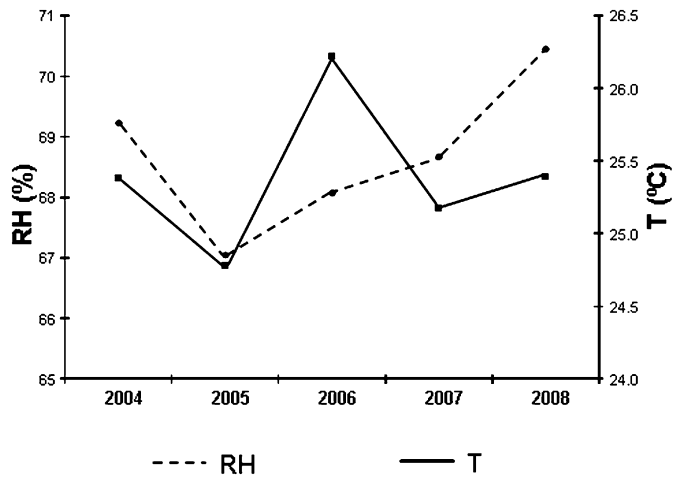
Supplemental Fig. 1. Undernet mean relative humidity (RH) and averaged temperature (T) recorded at Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain) meteorological station for the 5 yielding years during the salt treatment period.