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ORIGINAL PAPER

Genetic dissection of tomato rootstock effects on scion traits under moderate salinity

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Abstract

Key message **Rootstock HKT1 genotype affected fruit [Na^+] and non-commercial fruit yield; QTL analysis of rootstock-mediated scion nutrition is a powerful forward genetic approach to identify wild genes for rootstock breeding.**

Abstract The present study approaches the QTL dissection of rootstock effects on a commercial hybrid variety grafted on a population of RILs derived from *Solanum pimpinellifolium,* genotyped for 4370 segregating SNPs from the SolCAP tomato panel and grown under moderate salinity. Results are compared to those previously obtained under high salinity. The most likely functional candidate genes controlling the scion [Na^+] were rootstock *HKT1;1* and *HKT1;2* as it was previously reported for non-grafted genotypes. The higher fruit [Na^+] found when rootstock genotype was homozygote for *SpHKT1* supports the thesis that scion HKT1 is loading Na^+ into the phloem sap in leaves and unloading it in sink organs. A significant increment of small, mostly seedless, fruits was found associated with *SlHKT1* homozygous rootstocks. Just grafting increased the incidence of blossom end rot and delayed fruit maturation but there were rootstock RILs that increased commercial fruit yield under moderate salinity. The heritability and number of QTLs involved were lower and different than those found under high salinity. Four large contributing (>17 %) rootstock QTLs, controlling the leaf concentrations of B, K, Mg and Mo were detected whose 2 Mbp physical intervals contained B, K, Mg and Mo transporter-coding genes, respectively. Since a minimum of 3 QTLs (two of them coincident with leaf K and Ca QTLs) were also found governing rootstock-mediated soluble-solids content of the fruit under moderate salinity, grafting desirable crop varieties on stress-tolerant rootstocks tenders an opportunity to increase both salt tolerance and quality.

Introduction

More than 800 million hectares of land throughout the world are affected by salinity (FAO 2008), which can decrease yield and lead to increased poverty and reliance on imports (Witcombe et al. 2008). Tomato is one of the most important horticultural crops. In terms of human health, tomato fruit is a major component of daily meals in many countries and constitutes an important source of minerals, vitamins, and antioxidant compounds. However, the areas for tomato optimal growing conditions are becoming narrower around the world. Since salt tolerance, like tolerance to any abiotic stress, means adaptation, breeding for salt tolerance should take advantage of the evolution of *Solanum* species that occurred through adaptation to marginal environments. In this sense, *S. pimpinellifolium* L. has been frequently considered as possible donor of salt tolerance (Bolarin et al. 1991; Cuartero et al. 1992; Asins et al. 1993; Foolad and Lin 1997).

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Grafting is a biotechnological tool used since ancient times to improve the amount and uniformity of crop yield, and currently most fruit crops and many horticultural species are grown as scion–rootstock combinations. Although this strategy triples the work required by breeders (selection for rootstock, scion and their combination), main problems to breeding food crops for biotic and abiotic resistance can be circumvented when using rootstocks bred or selected to confer resistance (King et al. 2010). Improved nutrient absorption, salt stress tolerance and fruit quality are rootstock breeding objectives that would also benefit soil-less grafted tomato production in climate-controlled greenhouses in Europe and the US where extending the harvest season is a major goal (King et al. 2010). Besides, the genetic study of rootstock effects on a given variety is a valuable strategy to understand root functions (particularly, nutrient uptake and transport) since they are regulated by shoot through materials cycling between roots and shoots (Wang et al. 2006).

Estañ et al. (2009) showed that an efficient and profitable utilization of wild germplasm can be carried out through the improvement of rootstocks that confer salt tolerance in terms of fruit yield to the grafted variety. These authors found that salt tolerance rootstock effect was a heritable trait (H^2 near 0.3), governed by at least 8 QTLs. However, different QTLs were detected depending on the RIL population and/or the salinity level because the *S. cheesmaniae* RIL population was tested under moderate salinity (75 mM NaCl, CE = 8.6 dS m^{-1}) and the *S. pimpinellifolium* population, under high salinity (125 mM NaCl, 13.7 dS m^{-1}). Since salinity is variable in time and space under field conditions in marginal areas, breeding tomato rootstocks for salt tolerance through marker-assisted selection should take into account the differences in the QTLs controlling rootstock-mediated fruit yield under different levels of salinities.

Nowadays, analysis of QTL can also reach further research objectives by contributing to fill the gap between agronomic performance and the DNA sequences involved. With the advent of the complete tomato genome sequence by The Tomato Genome Consortium (2012), and the availability of a large panel of SNPs (SolCAP panel, http://solgenomics.net/), it is feasible to narrow the QTL interval where the gene(s) lies without the need for generating a physical map. The genome assembly also allows the rapid identification of candidate genes around the physical position of the SNP(s) with observed maximum LOD score. Thus, in a previous paper (Asins et al. 2013) we tried this approach to identify the tomato Na^+ transporters HKT1;1 and HKT1;2 as the most likely candidates for the major QTL controlling Na^+/K^+ homeostasis in tomato.

The objectives of this study were (1) to estimate the heritability of the rootstock effect on the fruit yield and quality of a commercial variety grafted on an *S. pimpinellifollium* RIL population grown under moderate salinity (2) to detect the QTLs involved, (3) to compare the results of this QTL analysis with those reported for the same population under high salinity (Estañ et al. 2009), (4) to investigate the genetic relationship of potential physiological components of salt tolerance conferred by the rootstock and searching for co-location of physiological and fruit yield QTLs, and (5) to test the efficiency of the present QTL analysis for the inference of candidate genes.

Materials and methods

Plant material, growth conditions and trait evaluation

A total of 130 F_{10} lines (P population) derived by single-seed descendent from the hybrid between a salt-sensitive genotype of *Solanum lycopersicum* var. Cerasiforme (formerly *L. esculentum*) and a salt-tolerant line from *S. pimpinellifolium* L. (formerly *L. pimpinellifolium*) (Monforte et al. 1997) were used for the study reported here.

The commercial tomato hybrid *Solanum lycopersicum* cv. Boludo (Bol) was used as scion, and plants from 124 lines of the P population were finally evaluated as rootstocks. Boludo (the scion) was also grafted onto roots derived from a different plant of the same genotype (Bol/Bol). Bol and Bol/Bol, non-grafted and self-grafted plants were included as controls. Bol/Bol plants were used to evaluate any physiological change that could be induced by the grafting process per se.

Grafting was performed when seedlings had developed 3–4 true leaves, seedlings were cut over the cotyledons, using the shoot as scion and the remaining plant part as rootstock. Grafts were made immediately after cutting the plants and grafting clips were used to adhere the graft union. After grafting, seedlings were grown as described in Estañ et al. (2009).

Five grafted plants per line and controls were transferred to a polycarbonate greenhouse, in Valencia, Spain (September 18th, 2012) at a density of 2.1 plants m^{-2} in an open soilless system using coco fiber as a substrate. The greenhouse had automatic roof ventilation and heating system (maintaining inside air temperature above 8 °C). The climate variables, in and out solar global radiation, inside photosynthetic active radiation, temperatures of the air, and air humidity were recorded by sensors connected to a data acquisition system. A latinised row–column design was proposed with 5 reps along the benches, 22 rows per rep and 6 columns (benches) across the reps. Extra repetitions were used to complete the 144 experimental units. A high-frequency fertirrigation system together with 4L/h drippers was used and handled to ensure homogeneity of the salinity of the roots of all plants in cultivation at the same time. To reach the salinity target level (75 mM NaCl)

10 days after the transplanting date, increasing amounts of NaCl were gradually added over a full-strength nutrient solution for tomato culture (in meq/L: NO_3^- 11.1; $H_2PO_4^-$ 1.3; SO_4^{2-} 1; NH_4^+ 0.7; K^+ 4.7; Ca^{2+} 3.5; Mg^{2+} 1; plus 0,025 g/L of an EDTA-microelements complex pH = 5.8, EC = 1.44 dS/m). The water for the nutrient solution was previously treated with reverse osmosis. Final electrical conductivity was 8.94 dS/m. The plants were cultivated to only one stem, eliminating all axillary buds.

The third (H3) and fifth (H5) Boludo leaves were harvested from 3 out of the 5 reps per RIL and controls for phenotyping after 46 days of salt treatment. Three evaluations of scion leaves at both H3 and H5 were carried out: leaf fresh weight (LFW, g); leaf dry weight (LDW, g) measured in samples dried at 80 °C for 3 days, and leaf water content (LWC, g) calculated as the difference between LFW and LDW. The difference between H5 and H3 for LDW and LWC was also considered and coded as dLDW and dLWC, respectively.

Each plant was evaluated during 8 weeks for fruit yield (number of ripe fruits, FN; their individual weight, FW and total fruit weight, TFW). If the weight of the fruit was <5 g (normally containing no seed), it was considered only for determining non-profitable fruit yield (FN < 5 and TFW < 5). Fruits larger than 5 g were used to estimate commercial fruit yield under moderate salinity (FN > 5, FW > 5 and TFW > 5). The variance of fruit weight per plant (varFW) was included as an additional trait. The number of fruits with blossom end rot (BER) and the number of days till harvesting the first ripe fruit (gDaysTFM) were also recorded.

Ripe fruits from the second and the third trusses of each plant (three plants per genotype) were evaluated for soluble-solids content (SSC2 and SSC3, respectively) and acidity. Fruit juice was obtained by squeezing of tomato through a cloth filter. This filtered juice was used to measure SSC, pH and citric acid content (Cit). Total soluble solids were measured as °Brix with a digital refractometer (PR-101α; Pallete, Atago). The citric content (%) and pH of fruit juice was measured in a 1:10 mL juice dilution with a digital pH meter (PH-Matic23; Crinson).

The type of scion/rootstock union was evaluated in all plants after 214 days of salt treatment using two classifications. Union1 corresponded to a three-type classification, where 1 means scion diameter smaller than rootstock diameter, 3 the opposite and 2, no diameter difference between rootstock and scion. Union2 concerned the presence, length and amount of roots arising from the union (from 0 to 4).

Two sets of 10 RILs each were selected by their HKT1 genotype (HKT1 sub-experiment). Within each HKT1 genotype, 10 RILs were chosen at random. One set was homozygous for the *S. lycopersicum* allele, *EE*; and the other set of 10, homozygous for the *S. pimpinellifolium* allele, *PP* (Asins et al. 2013). Three reps from each of these 20 RILs and from both controls (Bol and Bol/Bol) were further used for ionomic profile determination of the fruit juice (J) and the mesocarp (F), upper (TA) and lower stem (TB), and root (R) at the end of the experiment (20th May, 2013), after 234 days of saline treatment.

Tissue samples of F, H3, H5, TA, TB and R were fresh-weight determined, oven dried for 48 h at 80 °C, weighed (dry weight) and prepared for mineral analysis by digestion in a HNO_3:$HClO_4$ (2:1, v/v) solution. Inorganic solutes were determined in ppm by inductively coupled plasma spectrometry (ICP) (Ionomic Service; CEBAS–CSIC, Murcia, Spain). Cations in the fruit juice were determined by inductively coupled plasma spectrometry in ppm using a 1:10 mL dilution (Varian ICP 720-E, Scientific Instrumentation Service, Estación Experimental del Zaidín, CSIC, Granada, Spain). Third leaf Cl^- concentration (mg/L) was measured as described by Gilliam (1971) using a Sherwood chloride analyser 926.

Statistical analysis

A general factorial mixed model with genotypes (fixed), benches and reps (random) was used to asses the significance of each source of variation following the latinised row–column design. The Bayesian Information Criteria (BIC) was used to select the best model. Given that for most traits the model with genotypes and benches was the most parsimonious with the lowest BIC, it was used to estimate the adjusted mean traits per rootstock genotype for the QTL analysis and to study the grafting effects by comparing Bol vs. Bol/Bol adjusted means.

Pearson and Spearman (for type of scion/rootstock union) 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.

Broad-sense heritability (H^2) was calculated for traits measured in both populations assuming that the individuals from the ninth self-pollinated generation were nearly homozygous for all loci. Heritability was calculated as reported previously by Villalta et al. (2007), using the formula: $H^2 = V_g/(V_g + V_e)$ where V_g and V_e are the estimates of genotype and environmental variance, respectively, by REML (Restricted Maximum Likelihood). These estimates were obtained by a model with the same sources of variation as above but considering genotypes as random effects.

Differences between HKT1 rootstock genotypes for the HKT1 sub-experiment were analyzed by a mixed model with benches (random), HKT1 genotype (fixed), and RILs (random) nested within HKT1 genotype as factors.

Table 1 Adjusted means and standard errors for traits showing significant differences between non-grafted (Bol)- and self-grafted (Bol/Bol)-Boludo variety

Trait	Bol	Bol/Bol
BER	0.02 ± 0.24	0.72 ± 0.24
gDaysTFM	96.79 ± 8.59	136.88 ± 8.59
F_P	3,307.34 ± 195.40	3,962.48 ± 195.40
F_S	1,148.61 ± 98.68	1,514.33 ± 98.68
H3_Mo	0.477 ± 0.12	0.84 ± 0.12
H3_S	12,167.02 ± 1,036.31	16,222.74 ± 1,269.22
H5_S	32,667.476 ± 7,508.83	54,821.38 ± 7,508.83
H5_Zn	18.9 ± 22.39	110.7 ± 22.39
R_Ca	6,189.775 ± 1,321.76	9,836.98 ± 1,081.33
R_Tl	0.66 ± 1.59	4.98 ± 1.34

Traits: BER = frequency of fruits with blossom end rot; gDaysTFM = days till harvesting the first ripe fruit; fruit, root and leaf element concentrations are indicated by the element symbol after F_, R_ and H_, respectively. Third and fifth leaves are coded as H3 and H5

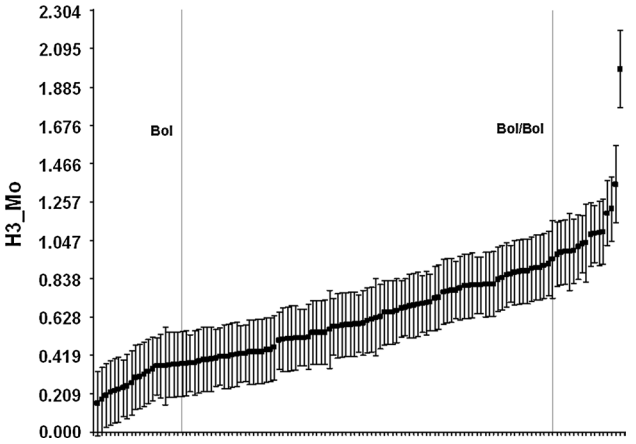

Fig. 1 Ordered adjusted means and standard errors for Mo leaf-3 concentration (H3_Mo) of Boludo using the lines of the P population as rootstock in comparison to the controls, non-grafted and self-grafted Boludo plants (Bol and Bol/Bol). *Vertical bars* indicate the relative position of these controls

Molecular markers and QTL analysis

One hundred and thirty P-RILs at F_{10} were genotyped for 7720 SNPs from the SolCAP tomato panel (Illumina BeadXhip WG-401-1004) using an external genotyping service (Fundación Investigación Clínico, Valencia, Spain). Linkage groups were set at LOD ≥ 17 using Joinmap 4 software for Windows (Van Ooijen 2006). A first genotype file including good quality segregating SNPs was used to know their distribution among linkage groups and to find out groups of SNPs for which all RILs showed the same genotype (groups of redundant markers). A second genotype file including non-redundant, segregating SNPs was used for the map construction of each chromosome using the same software.

QTL analyses were carried out using Interval Mapping (IM) and Multiple QTL Mapping (MQM) procedures in MapQTL® 6 (Van Ooijen 2009). Kruskal–Wallis procedure was also used to genetically analyze Union1 and Union2. A 5 % experiment-wise significance level was assessed by permutation tests. These LOD critical values ranged from 2.1 to 2.3 depending on the trait and chromosome.

Results

Significant differences between Bol and Bol/Bol, non-grafted and self-grafted plants were found for some traits (Table 1). Grafting delayed fruit maturation and increased BER, and fruits' and leaves' S concentrations. Mo concentration at third leaf also increased just by grafting (Fig. 1).

Heritabilities of rootstock effects (Table 2) were particularly large for H3_Na (0.86), H3_B (0.58), H3_Mg (0.49), H3_Mo (0.36), H3_Tl (0.35) and H3_Cd (0.33) but very low for total fruit yield. Nevertheless, some RILs conferred higher yield and fruit quality than the controls (Fig. 2).

The relationships among traits are visualized through the representation of the principal component analysis by the first two axes (27.1 %) (Fig. 3). Evaluations at third and fifth leaves appeared almost orthogonal in this representation. Commercial fruit yield (TFW > 5) was highly significant and directly correlated to FN > 5 (0.78), FW > 5 (0.30), TFW < 5 (0.31), varFW (0.43), LFW5 (0.28) and LWC5 (0.28) and inversely related to quality traits SSC2 (−0.28) and SSC3 (−0.31) (Table 3). No significant correlation was obtained between fruit yield and H3_Na or H3_Cl.

A total of 4,370 out of 7,720 SNPs conforming the SolCAP tomato panel were segregating in the P population (130 RILs), and 2,059 of them were genetically non-redundant. The number of non-redundant SNPs that was finally included in the linkage map of each chromosome (from chromosome 1–12) was 207, 198, 171, 175, 178, 165, 144, 117, 138, 97, 192 and 117, respectively. Thus, the linkage map of the P population contained 1899 non-redundant SolCAP SNPs and covered 1,326.37 cM of genetic length. An acceptable relationship between the genetic and physical positions of SNPs was found (supplementary figure S1) to use them for QTL analysis.

A total of 54 significant QTLs were detected for 24 Boludo traits, including two related to the scion/rootstock union (Table 4). Chromosomes 3 and 7 were particularly QTL rich. The contribution of some of them was large: H3_Na in chromosome 7 (68.2 %), H3_Mo in 10 (37.3 %), H3_Mg in 3 (20.8 %) and H3_B in 6 (20.5 %). Fruit yield QTLs were detected in chromosome 1 for large

Table 2 Broad-sense heritabilities for evaluated traits (H^2); sqrt (V_e) is the square root of the environmental variance; sqrt (V_g) is the square root of the genetic variance; # QTLs is the number of detected QTLs

Trait	sqrt (V_e)	sqrt (V_g)	H^2	#QTLs
BER	0.3991	0.0441	0.0121	0
SSC2	0.5958	0.2655	0.1657	4
SSC3	0.6711	0.3064	0.1725	4
Cit2	0.2693	0.0000	0.0000	0
Cit3	0.3093	0.0746	0.0550	1
dLDW	0.2983	0.0925	0.0877	0
gDaysTFM	13.2441	2.5054	0.0345	1
LDW3	0.2783	0.0819	0.0797	0
LDW5	0.1531	0.0351	0.0499	1
dLWC	2.1513	0.6212	0.0770	0
FN > 5	5.8744	0.7101	0.0144	0
FN < 5	6.4014	0.5180	0.0065	2
FW > 5	11.8911	0.0012	0.0000	0
FW < 5	1.5294	0.2944	0.0357	0
H3_Al	56.9602	14.4520	0.0605	0
H3_B	8.5879	10.1204	0.5814	6
H3_Ca	6,918.3692	4,631.9199	0.3095	2
H3_Cd	0.1095	0.0775	0.3337	3
H3_Cl	55.3668	24.1229	0.1595	1
H3_Cr	0.1730	0.0721	0.1480	0
H3_Cu	5.6769	1.0971	0.0360	0
H3_Fe	35.8077	12.3027	0.1056	0
H3_K	6,577.5301	3,821.8721	0.2524	2
H3_Li	0.8922	0.5259	0.2579	4
H3_Mg	808.8663	800.2734	0.4947	4
H3_Mn	29.6853	12.1259	0.1430	2
H3_Mo	0.2882	0.2167	0.3612	1
H3_Na	958.3751	2403.4226	0.8628	1
H3_P	1,306.4688	847.6431	0.2962	0
H3_Pb	0.3609	0.1963	0.2283	0
H3_S	17,689.7215	12,581.1111	0.3359	0
H3_Sr	36.6567	22.9766	0.2821	1
H3_Ti	1.6597	0.2489	0.0220	0
H3_Tl	5.4606	4.0044	0.3497	0
H3_Zn	33.0834	6.8478	0.0411	0
LFW3	2.2619	0.6084	0.0675	1
LFW5	1.2664	0.5117	0.1403	2
LWC3	1.9945	0.5453	0.0695	1
LWC5	1.1250	0.4750	0.1513	0
pH2	0.3143	0.0000	0.0000	0
pH3	0.1826	0.0000	0.0000	0
SD	1.8168	0.7131	0.1335	0
TFW > 5	307.7590	7.4264	0.0006	1
TFW < 5	33.0217	0.0115	0.0000	2
Union1	0.4663	0.2885	0.2768	5
Union2	0.5162	0.0938	0.0320	1
VarFW	489.5523	0.2363	0.0000	1

Traits whose heritability estimates are higher than 0.10 are in bold. Traits: BER = frequency of fruits with blossom end rot; SSC2 and SSC3 are soluble-solids contents of ripe fruits from the second and third trusses, respectively; Similarly, cit2 and cit3 correspond to their citric acid contents, and ph2 and ph3, to their pH; gDaysTFM = days till harvesting the first ripe fruit; the element concentration at the third leaf is indicated by the element symbol after H3_; FN, FW, TFW are fruit number, fruit weight and total fruit weight, respectively, followed by <5 if fruits weighted less than 5 g or >5 if they were heavier; SD = stem diameter; LFW5 and LDW5 are fresh and dried weights of the fifth leaf, respectively; LWC5 is its water content; dLDW and dLWC correspond to the difference between the third and the fifth leaves for the parameter; varFW = fruit weight variance

(commercial) fruits, and in chromosomes 3 and 6 for small fruits. Interestingly, at least three QTLs for soluble-solid content of the fruit as influenced by the rootstock genotype were detected under moderate salinity in chromosomes 3, 6 and 7. Several QTLs for the type of rootstock–scion union were detected by interval mapping but the position of the SNPs showing maximum significance should be better estimated using the non-parametric methodology of Kruskal–Wallis (Table 5).

Results from the HKT1 sub-experiment, showed significant differences between HKT1 rootstock genotypes for Na^+ concentrations of all tissues except for the root were K^+ concentration was significant instead (Table 6). Na^+ concentrations of all tissues of Boludo grafted on rootstocks homozygous for the *S. pimpinellifolium* HKT1 allele were larger than on rootstocks homozygous for the *S. lycopersicum* allele. Yield of fruits lighter than 5 g (TFW < 5) was significantly larger when the rootstock was homozygous for the *S. lycopersicum* allele than for the *S. pimpinellifolium* allele. Therefore, the HKT1 rootstock homozygote for the *S. pimpinellifolium* allele would be indirectly associated with agronomic salt tolerance given that it is associated with a lower non-commercial fruit yield.

Discussion

The effect of grafting

The first question regarding the benefits obtained using rootstocks is whether or not grafting (as a type of wounding) per se has any effect on the evaluated traits under

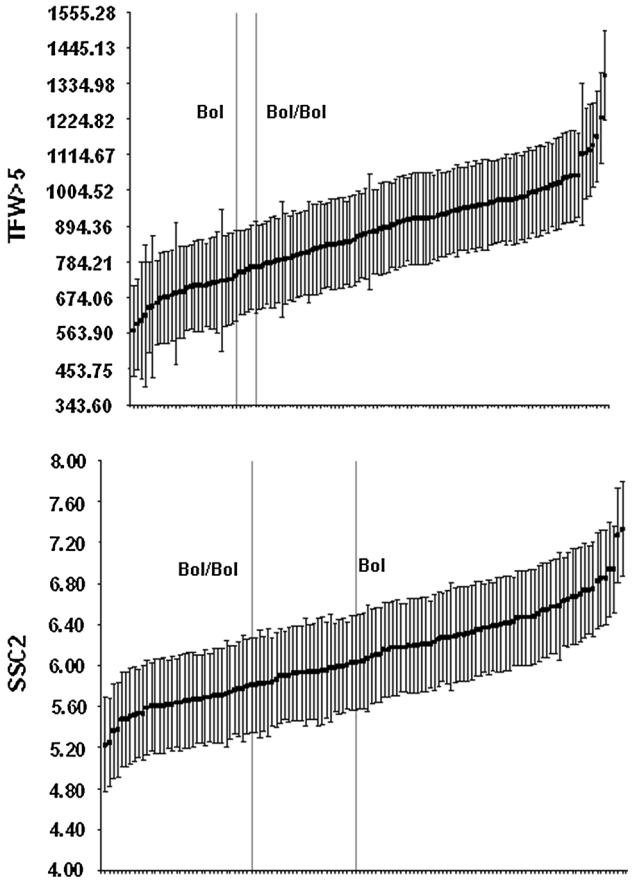

Fig. 2 Ordered adjusted means and standard errors for the fruit traits of Boludo: Total Fruit Weight (TFW) and Soluble-Solids Content (SSC) using the *lines* of the P population as rootstock in comparison to the controls, non-grafted and self-grafted Boludo plants (Bol and Bol/Bol). *Vertical bars* indicate the relative position of these controls

salinity. This question has been addressed by comparing non-grafted Boludo (Bol) to Boludo grafted on Boludo (Bol/Bol). There are several indications suggesting that just grafting could benefit resistance to salinity regardless the rootstock. As shown in Table 1 and Fig. 1, just grafting translated into an increment of certain nutrients in leaves and fruits (Mo and, mostly, S). Sulfate and molybdate share a high degree of similarity and it has been suggested that molybdate import and distribution are facilitated by sulfate transporters or related systems. In addition, the sulfur and molybdenum metabolisms are interconnected at several other stages such as Moco (molybdenum cofactor) biosynthesis and sulfite detoxification, so that both nutrients appear to interact closely at various levels in a common metabolic network (Bittner 2014, and references therein). Therefore, it seems possible to hypothesize that grafting affects their uptake, transport and/or storage. Thus, the invigorating effect of rootstocks in general might be due, at least in part, to a higher Mo translocation easiness by the grafting process and not to the rootstock genotype itself.

We have found also an increment of Ca^{2+} concentration in root just by grafting. Early and late changes in gene expression in shoot induced by root wounding were studied in *Arabidopsis* (Hasegawa et al. 2011) and, among early up-regulated root-to-shoot-responsive genes, some of them related to signal transduction are Ca^{2+} binding proteins. Then, following Scrase-Field and Knight (2003), the root of any grafted plant would have the Ca^{2+} "switch" permanently on. Besides, many of the root-to-shoot-responsive genes were associated with systemic production of jasmonic acid, OPDA (12-oxo-phytodienoic acid) and possibly ethylene. Therefore, the fact of grafting seems to prepare the plant to resist physiologically. Drawbacks of grafting are delayed fruit maturation and a higher incidence of BER, possibly due to lower fruit Ca^{2+} availability from the root since BER is a well-known Ca^{2+} deficiency symptom (Simon 1978). Although the heritabilities of BER and gDaysTFM were very low (Table 2), there were RILs showing no BER incidence, and one QTL was detected for gDaysTFM (Table 4), suggesting that rootstocks can be improved for this trait through selection in the P population.

The rootstock genotype for HKT1 genes affects fruit [Na^+] and non-commercial fruit yield

Asins et al. (2013) identified and mapped the location of two tomato *HKT1* genes encoding Na^+ transporters (HKT1;1 and HKT1;2) and studied their role as candidate genes for the major QTL involved in Na^+/K^+ homeostasis under high salinity. Now, we have shown that those *HKT1* genes are also positional candidates (only 35 Kb from the same SNP showing maximum LOD score) for the major rootstock-mediated QTL (68 %) controlling leaf Na^+ concentration (without any effect on K^+ concentration) of the grafted variety under moderate salinity. Similarly to what was reported previously using non-grafted plants, the *S. pimpinellifolium HKT1* allele, *SpHKT1;2*, coming from the wild salt-tolerant genotype in the rootstock, is associated with the highest Na^+ concentration of the aerial parts of the grafted variety (Tables 4, 6). These results fit the observation that the wild *ScHKT1;2* transcript level in roots was lower that *SlHKT1;2* in the experiment with NILs reported by those authors, suggesting that reduced expression of *ScHKT1;2* in roots translates into lower Na^+ retrieval from the xylem in roots and consequently more Na^+ is transported via the transpiration stream to the aerial part. We cannot discard that wild *S. pimpinellifolium* HKT1;2 had a lower affinity for Na^+ in comparison to SlHKT1;2 as Almeida et al. (2014) have reported recently for *S. pennellii* HKT1;2.

The results from the HKT1-rootstock sub-experiment showed significant difference for K^+ but not for Na^+ concentration in root (Table 6) as it was observed previously

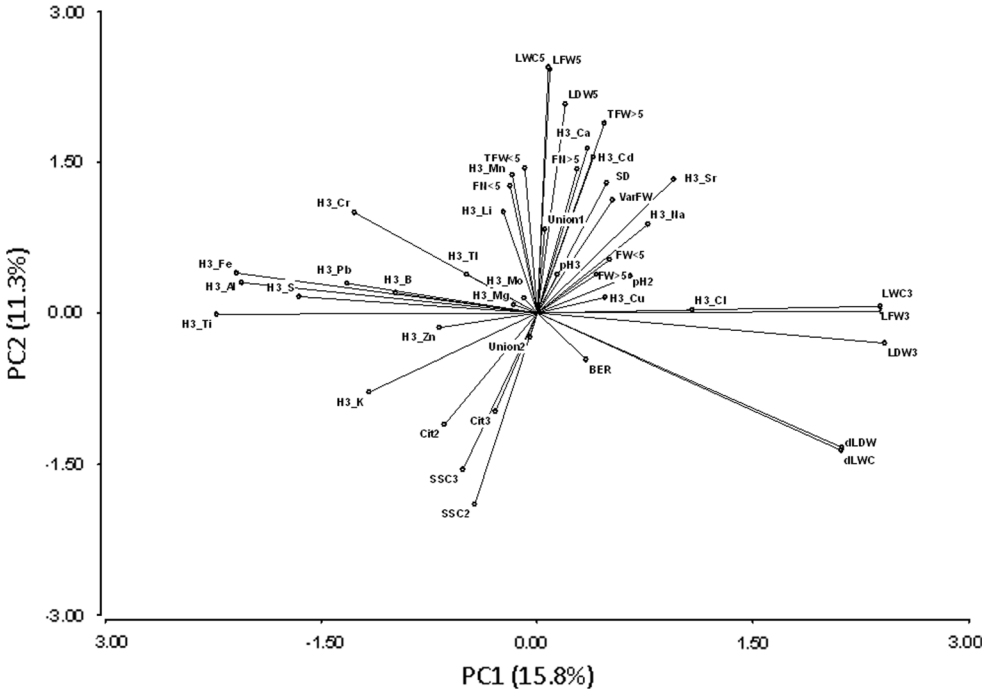

Fig. 3 Principal plane for the scion traits in the P population of rootstocks under moderate salinity

when comparing near-isogenic lines differing for HKT1 genotypes (Asins et al. 2013). The maximum difference between the HKT1 rootstock genotypes for Boludo Na^+ concentration corresponded to the oldest leaf, sampled after 46 days of salt treatment. Boludo fruits showed significant differences for Na^+ concentration at the juice and the mesocarp accordingly to the rootstock genotype. Therefore, the scion HKT1;1 and/or HKT1;2 must be involved in Na^+ loading into the phloem sap in leaves and unloading in sink organs, such as fruit and root, which would be in line with the hypothesis put forward by Berthomieu et al. (2003) that AtHKT1;1 plays an important role in Na^+ phloem loading. Besides, this root unloading (Na^+ transport from shoot to root) would explain the lack of significant differences between rootstock HKT1 genotypes for R_Na (Table 6). Nevertheless, the specific cellular/tissue location of tomato HKT1 transporters requires further study.

The concentrations of other elements appeared affected by the HKT1 rootstock genotype such as B in the fruit mesocarp. Regarding the relationship between rootstock HKT1 genotype and salt tolerance, no significant difference for vigor-related traits were detected but for yield of small fruits.

The tolerance to salinity depends on its level, moderate or high

Our results on fruit yield support the hypothesis that the genetic control of the rootstock-mediated salt tolerance depends on the level of salinity. First of all, a certain amount of small (seedless) fruits was yielded under moderate salinity. Second, only small-fruit QTLs detected in chromosomes 3 (98.4 cM) and 6 (48.0 cM) could be the same than those detected by Estañ et al. (2009) given that their position in the SNP map would be 95.5 and 39.9 cM, respectively. Besides, the increasing allele comes from the wild species in both cases. Correlations between fruit yield and vegetative traits also have shown several differences regarding the level of salinity. Under moderate salinity, fruit yield correlated with LWC5, while it correlated with both the LWC5 and LWC2 under high salinity (Asins et al. 2010). In fact, the physiology of the leaves at levels 3 and 5 under moderate salinity appeared as they were independent (see orthogonal positions of related traits in the PCA of Fig. 3). Contrary to the high-salinity experiment, we have not detected correlation with leaf Na^+ concentration here. Third, the TFW QTL detected for commercial fruits under moderate salinity in chromosome 1 was not detected under high salinity. Since both experiments were similar and uniform (fruit yield was evaluated at the same scion variety) it cannot be argued that crop management would be the reason of these differences (Kromdijk et al. 2014). Instead, all those results together support the hypothesis that plants (grafted tomato plants) respond to different salinity levels through different receptors (Munnik and Meijer 2001) or different signaling systems that might depend on the stress-induced primary event (Kacperska 2004). Thus, the final agronomic performance would depend on different QTLs according to the level of salinity. Under moderate salinity, the rootstock effect on salt tolerance is more closely related

Table 3 Pearson correlation coefficients of traits significantly correlated to yield

Trait	FW > 5	FN > 5	TFW > 5	TFW < 5	FN < 5
SSC2			**−0.28**	**−0.25**	−0.21
SSC3	**−0.26**		**−0.31**		
LDW5		0.21	−0.22		
FN < 5		0.26	**0.25**		
FW > 5		**−0.41**	**0.30**		
H3_Ca				0.19	0.20
H3_Cd		0.20	**0.24**		
H3_Cu		0.20	0.19		
H3_Mg		**−0.24**			
H3_Mn				0.22	0.22
H3_Sr					0.20
H3_Ti			−0.19		
H3_Tl		−0.20			
LFW5		**0.24**	**0.28**		
LWC5		**0.24**	**0.28**		
SD		0.22	**0.25**		
TFW > 5		**0.78**			
TFW < 5		**0.29**	**0.31**		**0.91**
VarFW	**0.41**		**0.43**		
Union1		**0.24**	0.23	**0.26**	**0.27**
Union1_Sp		**0.26**	**0.27**	**0.26**	0.23

Spearman correlation coefficient for Union1 (Union1_Sp) is also included. Coefficients in bold indicate $p < 0.01$. Traits: SSC2 and SSC3 are soluble-solids contents of ripe fruits from the second and third trusses, respectively; the element concentration at the third leaf is indicated by the element symbol after H3_; FN, FW, TFW are fruit number, fruit weight and total fruit weight, respectively, followed by <5 if fruits weighted less than 5 g or >5 if they were larger; SD = stem diameter; LFW5 and LDW5 are fresh and dried weights of the fifth leaf, respectively, while LWC5 is its water content; varFW = fruit weight variance

to maintaining water content at the shoot than for ion homeostasis, although Na^+ homeostasis might be involved in the production of non-commercial fruits. Non-commercial fruit yield was increased when rootstock genotypes for HKT1;1 and HKT1;2 were homozygotes for the *lycopersicum* allele, i.e., the allele responsible for avoiding Na^+ accumulation in all scion tissues, including fruits (Table 6). Therefore, Na^+ accumulation is not driving the reduction of fruit size through ovule abortion by salt stress (Sun et al. 2004). If it were so, it should happen for the S. *pimpinellifolium* HKT1 rootstocks. Besides, no significant QTL for small-fruit yield has been detected on chromosome 7 where HKT1 genes locate (Asins et al. 2013), discarding genetic linkage of QTLs as an explanation for the relationship between rootstock HKT1 genotype and TFW < 5. Alternatively, this relationship might be indirectly related to the effect of HKT1-rootstock genotype on the accumulation of other elements (Table 6) and would need future experiments to be tested.

Genetic effects of the rootstock on the tomato quality under moderate salinity

Numerous studies have shown that salinity increases the soluble-solid content of tomatoes (Cuartero and Fernández-Muñoz 1999; Dorais et al. 2001), even when using grafted plants (Fernandez-García et al. 2004; Estañ et al. 2008). However, Turhan et al. (2011) found this fruit quality trait was lower in the tomatoes of grafted plants than in the non-grafted ones. Here, we have shown the availability of genetic variability ($H^2 = 0.17$) in an RIL population derived from *S. pimpinellifolium* to increase the tomato total soluble solids of a grafted variety grown under moderate salinity. In fact, QTLs controlling this trait were detected in chromosomes 3, 6, 7 and possibly 9 (the only QTL where the wild allele would be increasing the trait). The small differences in the location of SNPs showing maximum LOD score for SSC2 and SSC3 (soluble-solid content of ripe fruits from the second and third trusses, respectively) could be explained by the sampling process itself (random errors). Full coincidence was obtained for SSC2 and SSC3 on chromosome 3. QTLs controlling soluble-solid and sugar contents of tomatoes from other non-grafted RIL populations were reported previously in chromosomes 9 (marker CT032) (Saliba-Colombani et al. 2001), 3 (marker TG214) (Saliba-Colombani et al. 2001, Ashrafi et al. 2012), 6 and 7 (marker TG252) (Ashrafi et al. 2012). Using the comparative map viewer tool (sol genomics network, http://solgenomics.net/) it was possible to compare the positions of the SNPs associated with the rootstock-mediated SSC QTLs (Tomato-Kazusa and SolCAP markers mapped to genome and TraitGenetics EXPEN2012) and the position of those reported previously for non-grafted plants (Tomato-EXPEN 2000) and no coincidence was found for those in chromosome 6 (43 cM apart), 9 (61 cM), 7 (12 cM), and 3 (88 cM). The QTL reported in chromosome 7 is the closest one but the increasing allele is different. Recently, Sauvage et al. (2014) have reported 9 peak SolCAP SNPs associated with SSC through GWA using a panel of 163 tomato accessions but none of them is coincident with the SSC peak SNPs of the present study. This fact suggests that rootstock-mediated SSC under moderate salinity is controlled by different genes from those governing SSC in non-grafted plants.

QTL analysis of rootstock effects as a powerful forward genetic approach to unveil wild genes with agronomic interest

Present QTL analysis, based on the SNPs from the SolCAP panel (http://solgenomics.net/), has been feasible without the need for generating a physical map for each parental line. In fact, the relationship between their physical

Table 4 List of SNPs (mostly SolCAP SNPs named by the number) where QTLs were detected using MQM procedure (5 % overall significance level)

Traits	Chr	cM	LOD	*EE*	*PP*	PEV	a	SNP	NSML
SSC2	P3	94.4	2.75	6.23	5.96	9.80	0.13	CL015369-0414	1
SSC2	P6	42.2	4.10	6.21	5.92	11.60	0.15	CL015317-0078	1
SSC2	P7	37.1	2.66	6.21	5.94	9.50	0.13	67896	1
SSC2	P9	83.5	2.25	5.98	6.22	8.10	−0.12	58234	1
SSC3	P3	94.4	3.62	6.59	6.24	12.70	0.18	CL015369-0414	1
SSC3	P6	51.4	5.16	6.57	6.19	14.40	0.19	27197	1
SSC3	P7	43.4	3.40	6.53	6.23	9.20	0.15	38698	3
SSC3	P7	85.7	3.11	6.53	6.23	8.40	0.15	53191	3
Cit3	P4	83.1	2.11	1.46	1.57	7.60	−0.05	47004	4
LDW5	P7	75.0	2.11	0.40	0.45	7.60	−0.03	53425	1
FN < 5	P11	83.7	3.14	7.13	5.17	10.80	0.98	56295	4
FN < 5	P3	98.4	4.00	5.14	7.28	13.90	−1.07	62008	3
gDaysTFM	P8	80.9	2.87	30.35	35.04	10.20	−2.34	65030	1
H3_B	P2	77.4	3.07	50.65	57.93	10.20	−3.64	50066	1
H3_B	P3	113.8	2.92	49.19	56.46	10.40	−3.64	34015	2
H3_B	P6	71.7	6.14	56.99	46.61	20.50	5.19	57681	9
H3_B	P7	101.1	4.30	48.40	57.07	14.90	−4.34	70700	2
H3_B	P8	90.8	2.13	50.26	56.75	7.70	−3.24	65262	1
H3_B	P9	109.6	4.69	48.84	58.13	16.10	−4.65	69787	2
H3_Ca	P3	53.1	2.21	52,433.20	49,214.10	6.80	1609.55	27246	1
H3_Ca	P3	94.4	4.88	48,380.00	53,267.40	15.90	−2443.71	CL015369-0414	1
H3_Cd	P3	103.0	2.36	0.22	0.28	8.40	−0.03	61820	1
H3_Cd	P6	39.9	3.30	0.22	0.29	11.60	−0.03	55921	1
H3_Cd	P9	47.2	2.55	0.28	0.22	9.10	0.03	54032	3
H3_Cl	P6	60.2	2.49	189.77	221.44	9.30	−15.84	42119	1
H3_K	P6	88.4	3.07	26,061.10	29,575.70	10.90	−1757.28	57093	1
H3_K	P7	43.4	5.64	29,919.60	25,339.90	17.50	2289.86	38698	3
H3_Li	P12	89.0	3.76	3.29	3.76	9.40	−0.23	32776	1
H3_Li	P4	80.5	2.23	3.22	3.65	8.00	−0.21	29243	3
H3_Li	P5	60.1	2.88	3.74	3.27	10.20	0.24	3155_3_592_b_3155_3_583	1
H3_Li	P7	100.5	2.40	3.33	3.73	7.20	−0.20	37054	1
H3_Mg	P12	88.3	4.60	5,137.96	5,755.26	10.70	−308.65	CL017120-0314	1
H3_Mg	P3	13.5	6.22	5,826.29	4,983.19	20.80	421.55	63356	1
H3_Mg	P5	60.1	4.28	5,744.66	5,107.24	11.70	318.71	23	3
H3_Mg	P8	104.6	3.32	5,107.46	5,745.38	11.70	−318.96	70177	5
H3_Mn	P12	53.7	2.47	113.53	125.79	8.80	−6.13	5794	3
H3_Mn	P3	92.6	2.54	114.05	126.36	9.10	−6.16	6230	3
H3_Mo	P10	79.7	12.45	0.82	0.48	37.30	0.17	60907	2
H3_Na	P7	40.0	30.58	5,172.02	9,613.42	68.20	−2220.70	57007	1
H3_Sr	P3	94.4	5.16	225.94	251.27	16.80	−12.67	CL015369-0414	1
LFW3	P5	26.0	2.15	6.90	6.11	7.70	0.39	CL017434-0125	1
LFW5	P11	94.2	2.17	4.05	3.55	7.80	0.25	56106	10
LFW5	P7	68.7	2.23	3.56	4.08	8.00	−0.26	53534	1
LWC3	P5	26.0	2.26	6.20	5.49	8.10	0.36	CL017434-0125	1
TFW < 5	P3	98.4	3.53	24.07	34.28	12.40	−5.10	62008	3
TFW < 5	P6	48.0	2.16	25.30	33.61	7.80	−4.15	1314	1
TFW > 5	P1	85.9	2.86	825.85	915.46	10.10	−44.81	15339	1
Union1	P11	28.8	4.14	2.18	2.48	14.40	−0.15	20956	1

Table 4 continued

Traits	Chr	cM	LOD	*EE*	*PP*	PEV	a	SNP	NSML
Union1	P2	22.4	2.65	2.53	2.26	9.50	0.14	10557	4
Union1	P7	94.1	2.99	2.20	2.45	10.60	−0.13	37096	1
Union1	P9	45.7	2.94	2.43	2.19	9.40	0.12	45078	1
Union1	P9	110.3	2.72	2.43	2.19	8.60	0.12	69835	1
Union2	P7	32.0	2.28	1.26	1.08	8.20	0.09	CL009201-0517	2
varFW	P11	62.6	2.56	1,069.96	936.19	9.10	66.88	6019	1

The map position (cM) of QTL peaks in the *Solanum* chromosomes (Chr) and the means for both homozygous genotypes, *EE* and *PP* are indicated. The estimated additive value is a and the percentage of explained variance, PEV. NSML is the number of SNPs associated with the maximum LOD score

Table 5 List of most significant SNPs (mostly SolCAP SNPs, named by the number) associated with Union1

Chr	cM	K*	Sig. L.	*EE*	*EP*	*PP*	SNP
9	91.8	23.7	0.0001	2.4	3.0	2.1	36845
11	28.8	21.8	0.0001	2.2	.	2.5	20973
7	94.1	14.1	0.001	2.2	2.8	2.4	37096
2	24.1	12.3	0.005	2.5	2.4	2.3	CL009018-1241

K is the Kruskal-Wallis statistic and Sig. L. its associated significant level. The adjusted means for the 3 genotypes, *EE*, *PP* and heterozygote, *EP*, when available, are indicated

Table 6 Adjusted means and standard errors (se) for traits significantly associated with the HKT1 rootstock genotype and R_Na

Trait	*p* values	*EE*	*se* (*EE*)	*PP*	*se* (*PP*)
F_B	0.0244	9.46	0.32	8.76	0.32
F_Na	0.0081	2,078.01	171.26	2,652.47	170.59
H3_Na	0.0000	5,939.50	683.55	10,729.88	668.07
H3_Pb	0.0145	0.49	0.05	0.31	0.05
H5_Na	0.0002	6,072.06	569.54	8,774.83	563.8
H5_Sr	0.0120	169.89	6.75	148.26	6.68
J_Na	0.0046	276.39	14.02	320.73	13.98
R_K	0.0008	17,906.27	2,026.53	24,356.16	2,026.83
R_Mn	0.0410	61.52	5.83	51.78	5.9
R_Mo	0.0480	1.53	0.18	1.08	0.18
R_Na	0.0706	24,214.11	1,374.97	20,631.86	1,374.97
R_Pb	0.0403	0.28	0.005	0.19	0.005
TA_Na	0.0145	19,627.49	1,778.76	24,702.87	1,802.19
TA_P	0.0110	11,456.70	1,214.89	13,552.58	1,211.13
TB_Li	0.0730	0.59	0.12	0.92	0.12
TB_Na	0.0143	20,593.31	1,921.53	24,294.89	1,904.77
TFW < 5	0.0066	31.69	6.78	14.95	6.69

The *SlHKT1* homozygous rootstock is *EE* and the *SpHKT1* homozygous rootstock, *PP*

and genetic positions (supplementary figure S1) was very similar to that reported by Sim et al. (2012) for populations EXPEN 2012 and, particularly, EXPIM 2012, derived from *S. pennellii* and *S. pimpinellifolium,* respectively. It also showed similarities with that reported by Pascual et al. (2014) for the MAGIC population whose total map length (2,156 cM) was the largest, followed by ours.

In general, there was good agreement between sensu lato-estimated heritabilities and number of QTLs that were detected in this experiment. There were some exceptions such as H3_Na and H3_Mo whose heritabilities were high (0.86 and 0.36, respectively) and only one QTL was detected although their contributions were large (68 and 37 %, respectively) (Table 4). More difficult to explain are the traits for which 1–2 QTLs were detected but their estimated heritabilities were <0.0001 (VarFW, TFW < 5) and should be considered with caution. In the case of qualitative traits, QTL location is better estimated by non-parametric methods. This seems the case of the QTLs for types of rootstock–scion union (Union1 on Table 4 through interval mapping, and through Kruskal–Wallis on Table 5). That one in chromosome 9 showed a peak at solcap_snp_sl_36845, precisely an SNP at gene Solyc09g089610.2.1 (ethylene receptor-like protein, ETR6). Ruzicka et al. (2007) proposed a model for ethylene-dependent root growth that accounts for auxin effects on root growth, and Aloni et al. (2008) proposed that the main cause for incompatibility is the occurrence of hormonal imbalance, primarily, of auxin and ethylene in the root system. Nevertheless, it is important to point out that none of the RILs resulted graft-incompatible with Boludo, so the undergrowth (Union1 class 1) and the overgrowth (Union1 class 3) of the scion cannot be considered here as an indication of

graft incompatibility, but of an invigorating rootstock effect since Union1 was significantly correlated with most (commercial and non-commercial) fruit yield traits (Table 3).

Some examples of correlations found between fruit yield and other traits on Table 3, can be explained just by linkage of QTLs controlling both traits. These could be the cases of QTLs in chromosome 11 for FN < 5, LFW5 and Union1; on 3 for FN < 5, TFW < 5, SSC2, H3_Ca, H3_Mn and H3_Sr; on 6 for TFW < 5 and SSC2 (Table 4). The negative correlation between fruit yield and soluble-solid content of the fruit juice was also found in citrus within a similar experiment on rootstock effects (Raga et al. 2014). These results fit the hypothesis by Kromdijk et al. (2014) that fruit composition is heavily influenced by fruit load and go further because at least part of this relationship is genetically supported by linked QTLs in chromosomes 3 and 6 of the tomato rootstock under moderate salinity. From the three fruit compositional traits influenced by the fruit load: water and solute accumulation, metabolic interconversions and the incorporation of solute into structural material discussed by Kromdijk et al. (2014), it seems reasonable to assign to the rootstock an important role for the first one, water and nutrient availability.

Our results on the genetic analysis of leaf nutrients content mediated by rootstock (Table 4) prove the hypothesis that the efficiency of nutrient assimilation could be improved by rootstock breeding. This objective would enhance crop yield under organic production (Farias et al. 2013) and nutrient stress (Savvas et al. 2010; King et al. 2010), increasing agriculture sustainability. Noteworthy, Savvas et al. (2011) reported that grafting onto three commercial tomato rootstocks, significantly reduced the leaf Mg concentration resulting in clear Mg-deficiency symptoms of salinized tomato. Our results provide information on QTL positions at four chromosomes that could be used to improve the scion Mg content under moderate salinity through a proper rootstock selection.

The tomato genome assembly also allows the rapid identification of candidate genes within a 2 Mbp interval around the physical position of the SNP(s) with maximum observed LOD score. Since the QTL peak may accurately indicate the genes or gene clusters responsible (Price 2006), we have used this approach to search for candidate genes (mostly transporter-coding genes), responsible for the rootstock QTLs controlling leaf content of some nutrients such as B, Ca, Cl, K, Mg, Mn and Mo (supplementary table S2). There were only 2 QTLs (H3_B and H3_Ca in chromosomes 9 and 3, respectively) for which no candidate could be envisaged. Very likely functional candidates (such as the Boron and Molybdate transporters for H3_B and H3_Mo, respectively) were found for 50 % (9 out of 18) of QTLs using the available information of annotated genes (http://solgenomics.net/) within each 2 Mbp interval and performing blast analysis to refine the results. Four of them corresponded to highly contributing rootstock QTLs for leaf Mo (37.3 %), B (20.5 %), Mg (20.8 %) and K (17.5 %). Candidate genes were also identified for minor QTLs like for leaf [Cl^-] in chromosome 6.

Noteworthy, two SSC QTLs co-located with Ca and K leaf concentration rootstock-mediated QTLs in chromosomes 3 (CL015369-0414) and 7 (Solcap_snp_sl_38698), respectively, providing a genetic support to phenotypic correlations. Is there any physiological connection behind it? It was surprising to find out that 2 Mbp around both SNPs, genes coding for proteins involved in fruit maturation (Steele et al. 1997; Rose et al. 1997) were also found: a glucan endo-1 3-beta-glucosidase (Solyc03g115200.2.1) and 5 expansins (Solyc03g115300.1.1, Solyc03g115310.1.1, Solyc03g115320.1.1, Solyc03g115340.1.1 and Solyc03g115350.1.1) for the former SNP, and a glucan endo-1 3-beta-glucosidase (Solyc03g017730.2.1) for the latter. Is there any joint root-to-shoot regulation at this genomic region?

In conclusion, a total of 37 QTLs have been mapped controlling important rootstock-mediated scion traits such as leaf concentration of nutrients, fruit yield, soluble-solids content of fruits and harvest time under moderate salinity that has allowed the search for candidate genes presumably involved. This will open the door to their validation by functional analysis and will facilitate marker-assisted selection in tomato rootstock breeding to improve fruit quality and the efficiency of nutrient uptake under salinity.

Author contribution statement Conceived: MJA; Experimental design: EAC, MJA, DR; Performed experiment: VR, DR, MJA; Analyzed data: EAC, MJA, AB; Wrote paper: MJA, AB, EAC.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The authors declare that the experiment complies with the current laws of Spain.

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