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# Genetic analysis of Na<sup>+</sup> and K<sup>+</sup> concentrations in leaf and stem as physiological components of salt tolerance in Tomato

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**Abstract** The sodium and potassium concentrations in leaf and stem have been genetically studied as physiological components of the vegetative and reproductive development in two populations of F<sub>8</sub> lines, derived from a salt sensitive genotype of *Solanum lycopersicum* cv. Cerasiforme, as female parent, and two salt tolerant lines, as male parents, from *S. pimpinellifolium*, the P population (142 lines), and *S. cheesmaniae*, the C population (116 lines). Genetic parameters of ten traits under salinity and five of them under control conditions were studied by ANOVA, correlation, principal component and QTL analysis to understand the global response of the plant. Two linkage maps including some tomato flowering time and salt tolerance candidate genes encoding for SISOS1, SISOS2, SISOS3, LeNHX1, LeNHX3, were used for the QTL detection. Thirteen and 20 QTLs were detected under salinity in the P and C populations, respectively, and four under control conditions. Highly significant and contributing QTLs

(over 40%) for the concentrations of Na<sup>+</sup> and K<sup>+</sup> in stems and leaves have been detected on chromosome 7 in both the populations. This is the only genomic position where the concentration QTLs for both the cations locate together. The proportion of QTLs significantly affected by salinity was larger in the P population (64.3%, including all QTLs detected under control) than in the C population (21.4%), where the estimated genetic component of variance was larger for most traits. A highly significant association between the leaf area and fruit yield under salinity was found only in the C population, which is supported by the location of QTLs for these traits in a common region of chromosome C1. As far as breeding for salt tolerance is concerned, only two sodium QTLs (*lnc1.1* and *lnc8.1*) map in genomic regions of C1 and C8 where fruit yield QTLs are also located but in both the cases the profitable allele corresponds to the salt sensitive, cultivated species. One of those QTLs, *lnc1.1* might involve *LeNHX3*.

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## Introduction

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 optimal growing conditions of tomato are becoming narrower around the world. World surface affected by salts is estimated as 800 million ha, out of them 437 being affected by sodicity (FAO 2005). This accumulation of salt in the soil causes deleterious effects and leads to a reduction in crop yield. Most crop plants including tomato (*S. lycopersicum* L.) have been described as moderately salt-tolerant (Maas and Hoffmann 1977), nevertheless, to date studies on salt tolerance of elite varieties have found

a limited variability on the extent to which they can withstand salinity (Cruz et al. 1990; Saranga et al. 1991; Pérez-Alfocea et al. 1996; Al-Karaki 2000; Agong et al. 2004). A major reason is that the usual breeding target trait corresponds to increase yield benefices only under optimal growing conditions, which has made elite cultivars bear only 10% of the total genetic variability amongst tomato species (Miller and Tanksley 1990). Since salt tolerance, such as tolerance to any abiotic stress, means adaptation, breeding for salt tolerance should take advantage of the evolution of *Solanum* species occurring through adaptation to marginal environments. In this sense, two tomato wild species have been considered as possible donors of salt tolerance: *S. pimpinellifolium* L. (Bolarin et al. 1991; Cuartero et al. 1992; Asins et al. 1993; Foolad and Lin 1997b) and *S. cheesmaniae* (L. Riley) Fosberg (Rush and Epstein 1976; Tal and Shannon 1983; Mahmoud et al. 1986; Asins et al. 1993).

Efforts on salt tolerance dissection using tomato experimental populations have been carried out taking into account traits such as germination and plant survival or vegetative growth at seedling stage (Foolad 1999, 2004) and fruit yield (Breto et al. 1993; Monforte et al. 1997a, b; Villalta et al. 2007). In the case of crop plants, it is ultimately the yield under specific field conditions that will determine whether or not a gene or combination of genes (or QTLs) is of technological importance. The challenge of abiotic stress is to bridge the gaps between agronomic, ecophysiological and basic research. Salt affects numerous plant processes at all levels of organization. At the very least, ion transport, selectivity, excretion, nutrition, and compartmentation are involved, together with growth, water use, and water use efficiency (Koyama et al. 2001). Therefore,  $\text{Na}^+$  and  $\text{K}^+$  uptake, balance and distribution within the cell and the plant have been considered of great interest for QTL analysis in other crops such as citrus (Tozlu et al. 1999), rice (Lin et al. 2004) and durum wheat (Lindsay et al. 2004). However, the relationship between the concentrations, or accumulations, of those cations and the plant yield has been less investigated. Here we report a comparative QTL analysis of these physiological components of salt tolerance, including some candidate genes encoding SOS and NHX proteins, using two tomato RI8 populations where QTLs for salt tolerance in terms of fruit yield were previously reported (Villalta et al. 2007) as reference for the discussion.

## Materials and methods

### Plant material, growth conditions and trait evaluation

Two populations of  $F_8$  lines were developed from a salt sensitive genotype of *Solanum lycopersicum* var. cerasiforme (formerly *Lycopersicon esculentum* Mill.) as the

female parent. Male parents were two salt tolerant lines from *S. pimpinellifolium* L. (formerly *L. pimpinellifolium*), for the P population and *S. cheesmaniae* (L. Riley) Fosberg (formerly *L. cheesmanii* L. Riley), for the C population. Both the populations were developed by single seed descent from 300 and 400 individual plants of the P and C  $F_2$  progenies, respectively (Monforte et al. 1997b) after six selfings, with no conscious selection at any generation, under greenhouse or screenhouse conditions. A total of 142  $F_8$  P lines and 116  $F_8$  C lines were used for the salinity tolerance experiments reported here.

The P and C populations were grown in a commercial polyethylene greenhouse in Malaga, Spain (36°45'N, 4°02'W), in the growing periods September–November and February–April, respectively. Seedlings were transferred from germination Petri dishes into 51-well plates, one plant per well with a mixture of litonite and soil (1:1) for growing under greenhouse conditions. Two-true-leaf seedlings were transplanted to 15-L pots containing an aerated Hoagland solution diluted with rainwater at 1:3 for the first 2 days and 1:2 afterwards ( $\text{KNO}_3$  6.59 mM,  $\text{NH}_4\text{H}_2\text{PO}_4$  1 mM,  $\text{MgSO}_4$  5.60 mM,  $\text{CaCl}_2$  5.00 mM, and micronutrients). Five days after transplanting, tomato plants were subjected to saline conditions for 5 weeks with 0 (control) or 100 mM NaCl (saline) (electrical conductivity 9.5 dS/L), reached gradually by adding 50 mM NaCl every two days.

For the C population, 2–8 plants per line-treatment combination were randomly cultured resulting in a total of 797 plants. As per the P population, 5–6 plants per combination were grown in four consecutive batches for a total of 1,681 plants. A total of ten quantitative traits were measured in both the populations under saline conditions: dry weight of leaves and stems, total leaf area,  $\text{K}^+$  and  $\text{Na}^+$  concentration in leaves and stems,  $\text{K}^+/\text{Na}^+$  ratio in leaves,  $\text{Na}^+$  transported to leaves and stems and  $\text{Na}^+$  leaf sensitivity. All traits except for those related with  $\text{Na}^+$  (five in total) were also measured under control conditions.

Vegetative dry weight was obtained at the end of the experiment by drying and weighting stems (DSW) (g) and leaves (DLW) (g) of the plants. Total leaf area (LA) ( $\text{dm}^2$ ) was determined by extending the leaves on a surface and projecting the shadows against illumination. The total area was then calculated digitally using a conventional image processing software.

Dry leaves and stems were bulked separately by line-treatment combination and three samples of these bulks were analyzed separately to determine the  $\text{Na}^+$  and  $\text{K}^+$  concentrations (mmole per Kg of dry weight) in leaves (LNC, LKC) and stems (SNC, SKC), and the ratio of  $\text{K}^+/\text{Na}^+$  in leaves (LKN).

The amount of total  $\text{Na}^+$  content in the aerial part of the plant (TN) was expressed as the percentage of the theoretical  $\text{Na}^+$  present in the volume of water absorbed by the

plant ( $DLW \times LNC + DSW \times SNC$  in relation to the theoretical  $Na^+$  absorbed by the plant). This parameter was considered as an indication of the  $Na^+$  distribution within the plant (leaves and stems vs. roots). The percentage of leaf area reduction ( $(LA_{control} - LA_{saline}) \times 100 / LA_{control}$ ) relative to the LNC was taken as an estimation of the sodium leaf sensitivity (NLS).

#### Molecular markers and candidate genes

Marker analyses and linkage maps used for the QTL analysis have been previously reported by Villalta et al. (2005). Some additional, flowering time candidate genes, *falsiflora* (LEFA\_550) and *Phytochrome B2* (PhyB2), were added to those maps (Villalta et al. 2007). Genes encoding the tomato plasma membrane  $Na^+/H^+$  antiporter, SOS1, and its regulatory proteins, SOS2 and SOS3 (R. Olías, Z. Eljkaoui, M. C. Marín-Manzano, J. Li., J. P. Donaire and A. Belver, manuscript in preparation), as well as the tomato endomembrane  $K^+$ ,  $Na^+/H^+$  antiporters, NHX1 and NHX3, considered as salt tolerance candidate genes, have been mapped for the QTL analysis. SOS encoding genes were found as mutations affecting the salt response during the vegetative growth in *Arabidopsis* (Liu and Zhu 1998; Zhu et al. 1998; Liu et al. 2000; Shi et al. 2000). NHX encoding genes were differentially expressed in *Arabidopsis thaliana* during the salt stress response (Yokoi et al. 2002). Primers for these genes were designed using their tomato cDNA sequences. In all cases except for *SOS1*, intron-flanking primers were designed. The position of introns in the genomic sequences were obtained for the *Arabidopsis* likely orthologues (gene IDs): *SOS1* (81472), *SOS2* (833502), *SOS3* (832494), *NHX1-4* (832773, 819665, 835640, 819811) through the NCBI gene tool (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>). The most likely position of the introns for the tomato cDNA sequences was obtained aligning the predicted tomato amino acid sequence with the *Arabidopsis* protein sequences using the OMIGA v2.0 software. The identity of the polymorphic amplification products was checked by sequence analysis. A total of 153 markers were genotyped for the P population and 124 markers for the C population using DNA pools of six plants per line, in both cases at  $F_7$  generation. The linkage analyses were calculated using Joinmap 3.0 software for Windows (Van Ooijen and Voorrips 2001). A minimum LOD of 3 was set as a threshold to create linkage groups using a recombination fraction of 0.5 for linkage analysis. Kosambi function (Kosambi 1944) was used to order markers, and estimate interval distances.

#### Statistical analysis

The genetic variance of each trait was estimated as the between-line component in both the populations, consider-

ing separately the control and salinity conditions and using the methodology described in Villalta et al. (2007).

The effects of the line (G), treatment (E) and  $G \times E$  interaction on each quantitative trait were analyzed using factorial two-way ANOVA. For the P population, the least squares estimates of the batch and batch by treatment interaction were used to adjust the values of the traits prior to the two-way ANOVA (data not shown).

Pearson's correlation coefficients were calculated for each trait combination in both the populations under control and saline conditions. Total fruit weight per plant (g) (TW), fruit number per plant (FN), average fruit weight (g) (FW) and the percentage of reduction of these three traits (pTW<sub>r</sub>, pFN<sub>r</sub> and pFW<sub>r</sub>) resulting from their difference between the control (NaCl 0 mM) and saline treatment (NaCl 150 mM) at  $F_7$  in the P and C populations (Villalta et al. 2007) were also included. Standardized data were used for principal component analysis (PCA) where the yield related traits studied by Villalta et al. (2007) were also considered for comparison purposes.

#### QTL analysis

Interval Mapping (IM) procedure by the Windows QTL cartographer software (Wang et al. 2006) was used to estimate the more likely position of QTLs in chromosomes, their additive effects and contributions and the presence of  $QTL \times E$  interaction. The analyses were carried out using a walk speed of 2 cM and ignoring lines with heterozygote plants in order to simplify the analysis (Villalta et al. 2007). LNC, SNC and LKN trait distribution slightly differed from normality in the P population. For this reason, logarithmic (LNC, SNC) and square root (LKN) transformations of the original variables were used for the detection of QTLs involved in these traits. Following previous results from permutation tests (Villalta et al. 2007), a QTL is reported if it is detected at a likelihood ratio test (LR) value larger than 8.29 using the approximation to a Chi-square distribution (equivalent to  $LOD > 1.8$  and  $P < 0.004$  in the P population).

## Results

#### Statistical analysis of traits

The correlation coefficients between vegetative, physiological and fruit yield traits that were significant under saline conditions are presented in Table 1. The vegetative traits (DLW, DSW and LA) were highly correlated between each other and two of them, DLW and LA, with LNC, SKC and the LKN ratio in both populations. Vegetative traits were also correlated with TN, which is negatively correlated with

**Table 1** Significant ( $P < 0.05$ ) correlation coefficients for traits studied in the P (upper triangle matrix) and C (lower triangle) populations under 100 mM NaCl treatment

Trait	DLW	DSW	LA	LNC	SNC	LKC	SKC	LKN	NLS	TN	FW	FN	
DLW	1	<b>0.80</b>	<b>0.75</b>		−0.28	<b>0.55</b>	<b>0.44</b>	0.31	0.27	<b>0.44</b>	0.30	−0.26	C
DSW	<b>0.70</b>	1	<b>0.65</b>		−0.25		0.26	0.20		<b>0.44</b>	0.25		
LA	<b>0.83</b>	<b>0.59</b>	1			<b>0.46</b>	<b>0.35</b>	0.26	0.26	0.33	<b>0.45</b>	−0.25	
LNC	−0.20		−0.23	1	<b>0.64</b>	<b>−0.51</b>	<b>−0.54</b>	<b>−0.86</b>	<b>−0.63</b>	<b>0.54</b>			
SNC				<b>0.68</b>	1	<b>−0.58</b>	<b>−0.44</b>	<b>−0.58</b>	−0.31	<b>0.42</b>			
LKC	<b>0.44</b>	<b>0.26</b>	<b>0.43</b>	<b>−0.39</b>		1	<b>0.84</b>	<b>0.72</b>	<b>0.41</b>		0.28	−0.21	
SKC	<b>0.44</b>	0.20	<b>0.58</b>	<b>−0.54</b>		<b>0.77</b>	1	<b>0.70</b>	<b>0.40</b>				
LKN	<b>0.42</b>		<b>0.47</b>	<b>−0.79</b>	<b>−0.39</b>	<b>0.82</b>	<b>0.83</b>	1	<b>0.71</b>	<b>−0.43</b>			
NLS	<b>−0.33</b>	<b>−0.39</b>	<b>−0.41</b>	<b>−0.36</b>	<b>−0.33</b>			0.27	1	<b>−0.33</b>			
TN	0.25	<b>0.42</b>	0.23	<b>0.71</b>	<b>0.69</b>			<b>−0.34</b>	<b>−0.40</b>	1			
FW				−0.18			0.19				1		
FWr					0.21						−	−	
FNr					−0.24					−0.20	−	−	
TWr					−0.18					−0.23	−	−	
P													

Significant correlations between physiological traits and yield traits FW, FN and TW and their percentages of reduction (pFWr, pFNr, pTwr) from control to saline conditions (150 mM NaCl) are also indicated. Values in bold indicate highly significant ( $P < 0.001$ ) correlations. Blanks: not significant. “−”: not measured

NLS. This trait was unexpectedly, negatively correlated with LNC in both the populations. Important differences were also observed between the populations. Fruit yield is not strongly associated with any physiological or vegetative trait under salinity except for the C population where the leaf area is highly correlated with mean fruit weight. Only in the C population, the potassium concentration (LKC and SKC) is also correlated with SNC and NLS.

The first two components of the principal component analysis explained about 35% of the total variance, in the four population-treatment combinations. Under control conditions, the first component is clearly explained by the yield traits (FN, FR, FS and TW) on the right, and the earliness traits (SF, NL, SH, FH) on the left [as reported by Villalta et al. (2007) ignoring vegetative and  $\text{Na}^+$  and  $\text{K}^+$  related traits]. However, under saline treatment, the first component is mainly defined by the sodium and potassium concentration traits while the fruit yield and earliness traits become major contributors to the second axis.

The estimated genetic variance ( $V_g$ ) of traits, as the between-line variance component, is presented in Table 2. This component seems to be higher under salinity than under control conditions in spite of the magnitude of the trait mean which is smaller under salinity (Table 3). The significance of the treatment effect (E), its direction from control to salinity condition ( $c \rightarrow s$ ) and the genotype by environment interaction ( $G \times E$ ) are also indicated in Table 2. Genotypic effect was always significant in both the populations. When treatment effect was studied, salinity

decreased by means of vegetative and potassium concentration traits in both the populations (Tables 2, 3).

#### Salt tolerance candidates and QTL analysis

The candidate genes involved in the SOS pathway *SISOS1*, *SISOS2*, and *SISOS3* are located in the chromosomes C1, 12 (P12 and C12) and P3, respectively. Candidate genes *LeNHX1* and *LeNHX3* map in chromosomes 6 (P6 and C6) and C1, respectively (Fig. 1). Only *LeNHX3* has been found significantly associated with  $\text{Na}^+$  concentration.

A total of 17 (4 under control and 13 under salinity) and 24 (4 under control and 20 under salinity) QTLs for vegetative traits,  $\text{Na}^+$  and  $\text{K}^+$  concentrations of stem and leaf and  $\text{Na}^+$  related traits are reported for the P and C populations, respectively (Table 4). The markers displaying the highest LR values of these QTLs within the linkage maps are shown in Fig. 1.

A genomic region in chromosome 7 has been found significantly associated with SKC, LKC, SNC and LNC under saline conditions in both the populations. In all the cases, the allele from the cultivated species (the *L* allele) is related to lower  $\text{Na}^+$  and higher  $\text{K}^+$  concentration in stem and leaves than the wild allele. The percentages of variance explained (indicated by  $R^2$  in Table 4) by the  $\text{Na}^+$  concentration QTLs are larger than those involving  $\text{K}^+$  in this region. QTLs for the sodium-derived traits LKN, NLS and TN were also detected in this same genomic region of chromosome 7. The *L* allele is related to lower values of trans-

**Table 2** MIVQUE variance components for P and C populations under 0 mM NaCl (C) and 100 mM NaCl (S) treatments (Tr)

Trait	Tr	P					C				
		Vg	Vs	E	c → s	G × E	Vg	Vs	E	c → s	G × E
LA	C	0.65	0.35	***	↓	***	0.55	0.45	***	↓	***
	S	0.61	0.39				0.78	0.22			
DLW	C	0.39	0.61	***	↓	***	0.42	0.58	***	↓	***
	S	0.46	0.54				0.67	0.33			
DSW	C	0.51	0.49	***	↓	***	0.47	0.53	***	↓	***
	S	0.56	0.44				0.60	0.40			
LNC	C	–	–	–	–	–	–	–	–	–	–
	S	0.69	0.31				0.72	0.28			
LKC	C	0.62	0.38	***	↓	***	0.70	0.30	***	↓	***
	S	0.72	0.28				0.85	0.15			
SNC	C	–	–	–	–	–	–	–	–	–	–
	S	0.63	0.37				0.79	0.21			
SKC	C	0.58	0.42	***	↓	***	0.70	0.30	***	↓	***
	S	0.59	0.41				0.81	0.19			
LKN	C	–	–	–	–	–	–	–	–	–	–
	S	0.71	0.29				0.78	0.22			
TN	C	–	–	–	–	–	–	–	–	–	–
	S	0.55	0.45				0.48	0.52			
NLS	C	–	–	–	–	–	–	–	–	–	–
	S	0.61	0.39				0.82	0.18			

Vg and Vs are the percentages of genetic and sampling variances, respectively. Significant two way ANOVA were also indicated for treatment effects (E) and genotype by environmental interaction (G × E). For significant treatment effect mean tendency (c → s) is indicated. “–”: not analyzed

**Table 3** Means and standard deviations of traits measured in the P and C populations under control (C) and 100 mM NaCl (S)

Trait	P		C	
	C	S	C	S
LA (dm <sup>2</sup> )	41.7 ± 14.6	24.6 ± 7.1	38.3 ± 14.1	12.0 ± 5.0
DLW (g)	23.8 ± 5.4	14.0 ± 2.8	12.9 ± 3.2	4.7 ± 1.7
DSW (g)	20.4 ± 5.0	8.8 ± 2.6	6.7 ± 2.1	1.8 ± 0.8
LNC (mmol/Kg)	–	784.8 ± 276.4	–	1,357.8 ± 472.4
SNC (mmol/Kg)	–	1,195.1 ± 419.1	–	1,791.4 ± 719.7
LKC (mmol/Kg)	839.6 ± 190.7	757.1 ± 183.4	1,346.2 ± 244.5	1,191.7 ± 287.0
SKC (mmol/Kg)	1,292.7 ± 372.6	1,188.8 ± 369.7	1,609.2 ± 223.0	1,242.7 ± 297.9
LKN	–	1.2 ± 0.6	–	1.1 ± 0.6
TN	–	1.8 ± 0.6	–	2.2 ± 0.9
NLS (%)	–	23.5 ± 26.4	–	10.0 ± 5.6

“–”: not measured

ported sodium (except for *tn5.1*), and higher values of LKN ratio and NLS. The contribution of *lnc7.1* is amongst the highest found in this study (0.34–0.43 depending on the population).

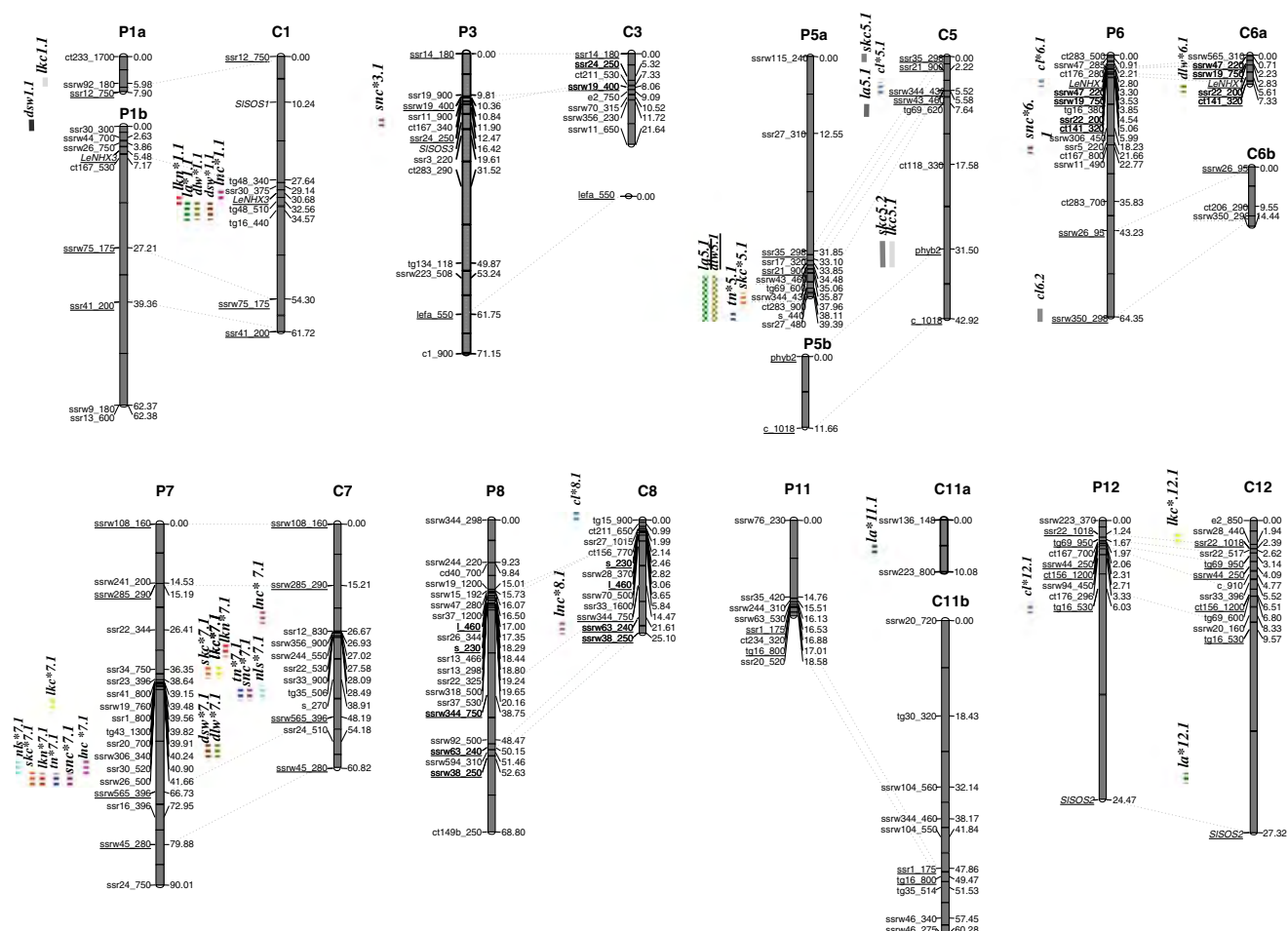
QTLs for SNC were also found in chromosomes P3 and P6. As the only exception, the wild allele at *snc3.1* is associated with less Na<sup>+</sup> concentration than the *L* allele. In the C population, other LNC QTLs were detected in chromosomes 1 and 8, but *L* alleles are associated with a reduction in the leaf Na<sup>+</sup> concentration.

More LKC QTLs, in addition to that on chromosome 7, were found in C12 and P1 under saline and control condi-

tions, respectively. Besides, three SKC QTLs were found in chromosome 5, one of them (*skc5.1*) is likely common between the populations, although its detection depends on the treatment. Two QTLs for vegetative traits, *la5.1* and *dsw1.1*, might be also orthologous between the populations, similar to the highly contributing Na<sup>+</sup> and K<sup>+</sup> QTLs on chromosome 7.

Salinity affects the detection of QTLs: 64.3% of QTLs in the P population and 21.4% in the C population. Only two QTLs might be considered the same in control and salinity conditions, *la5.1* and *dlw5.1* (in the P population) although salinity decreases the effect of the *L* allele on them





**Fig. 1** Linkage maps obtained for the P and the C populations. Common markers are connected by lines. Bars with the name of the QTLs (Table 4), are indicating markers that show significant association (maximum LR values) with the phenotypic variation of the vegetative

(Table 4). The major K<sup>+</sup> QTLs on chromosome 7 are highly affected by the salinity factor.

## Discussion

### Breeding for salt tolerance

Correlation analysis has shown that only a vegetative trait (LA) is highly significantly associated with fruit yield (the fruit weight component), only in the C population. The other significant associations with fruit yield involving Na<sup>+</sup> or K<sup>+</sup> traits are weak and inconsistent between the populations: [Na<sup>+</sup>] (SNC), in the case of the P population, and [K<sup>+</sup>] (LKC) in C. Supporting the association between FW and LA under salinity in the C population, there is a genomic region in C1 where QTLs for these traits and others (DLW, DSW, LNC and LKN) locate together (Fig. 1) being *L*, the allele from the cultivated species, the profitable

and physiological traits. Depending on the salinity condition under which the QTL is detected, the bar is continuous (control), discontinuous (salinity) or waved (both conditions)

one; i.e. here lower [Na<sup>+</sup>] is accompanied by a higher K<sup>+</sup>/Na<sup>+</sup> ratio, higher leaf vigor and larger fruits. Dry leaf weight was also found associated with average fruit weight under salinity (Table 1), which is supported by the location of QTLs for DLW and percentage of fruit weight reduction in C6, in addition to the cluster of QTLs in C1. Although Na<sup>+</sup> leaf concentration has not been found related to FW, *lnc8.1* is located tightly linked to *fw8.1* in C8, both under salinity conditions, again the *L* allele being the desirable one. From all these cases at C1, C6 and C8, wild allele is profitable only at C6 (where *LeNHX1* is located), in the sense that salinity greatly reduces FW of *LL* genotype at this position; in fact *fw6.1* (Villalta et al. 2007) is only detected under control conditions.

As expected, vegetative traits were all highly correlated and the co-localization of QTLs responsible for their variation on chromosomes P5 and P1 explains, at least in part, their associations. In the P population, QTLs for LA, DLW, SKC and TN are located in the same region of chromosome

**Table 4** List of QTLs detected in the P and C populations, indicating: the chromosome (Chr), the marker(s) showing the highest LR, the position in cM, the contribution to the total variance (R<sup>2</sup>) and the additive effect (a)

Pop	Trait	Chr	QTL	Tr	Marker	Position	LR	a	R <sup>2</sup>	PQTL × E	GIM	LL	PP	LP	P	L–
													CC	LC	C–	
P	LA	5	<i>la5.1</i>	C	SSRW43_460	34.49	12.82	366.75	0.09	0.04331	L–		38.09			45.46
			<i>la*5.1</i>	S	SSR35_298	31.86	18.65	217.24	0.13	NS	LL	27.25	22.63	22.90		
	DLW	5	<i>dlw5.1</i>	C	SSRW43_460	34.49	14.74	1.21	0.10	0.02227	L–		22.64			25.06
			<i>dlw*5.1</i>	S	SSR27_310	28.56	12.81	0.74	0.12	NS	L–		13.63			14.25
	DSW	1b	<i>dsw1.1</i>	C	SSR30_300	2.01	10.98	–1.18	0.08	0.04058	P–	19.26			21.56	
	LKC	1a	<i>lk1.1</i>	C	SSRW92_180	5.99	9.62	41.54	0.07	0.04694	LL	887.12	790.02	853.23		
		7	<i>lk*7.1</i>	S	SSRW19_760	39.49	18.61	56.26	0.13	0.00000	LL	807.90			695.00	
	SKC	5	<i>skc*5.1</i>	S	SSRW344_430	35.88	11.89	88.90	0.09	0.02506	LL	1,300.14	1,106.80	1,168.85		
		7	<i>skc*7.1</i>	S	SSRW26_500	41.67	11.70	86.65	0.08	0.00165	L–		1,087.26			1,263.27
	logLNC	7	<i>lnc*7.1</i>	S	SSR30_520	40.91	53.82	–0.08	0.34	NS	PP	676.64	939.43	734.55		
C	logSNC	3	<i>snc*3.1</i>	S	CT167_340	11.91	10.00	0.04	0.07	NS	LL	1,290.57			1,109.88	
		6	<i>snc*6.1</i>	S	CT167_800	21.67	9.44	–0.03	0.07	NS	P–	1,108.04			1,257.67	
	sqLKN	7	<i>snc*7.1</i>	S	SSRW26_500	41.67	47.72	–0.07	0.29	0.00044	PP		1,430.09			1,029.16
	NLS	7	<i>nl*7.1</i>	S	SSRW26_500	45.67	57.98	0.18	0.48	0.00003	L–		0.80			1.40
	TN	5	<i>tn*5.1</i>	S	SSR30_520	40.01	14.13	7.08	0.09	–	LL	28.70	14.22	27.48		
			<i>tn*7.1</i>	S	S_440	38.01	9.84	0.14	0.07	–	LL	1.95			1.67	
		7	<i>tn*7.1</i>	S	SSR30_520	40.01	36.80	–0.25	0.22	–	PP	1.55	2.11	1.59		
	LA	5	<i>la5.1</i>	C	TG69_620	11.01	7.84	3.77	0.10	NS	LL	41.32	35.05	38.17		
		1	<i>la*1.1</i>	S	TG48_510	32.01	10.36	1.63	0.10	NS	LL	14.03			10.73	
		11	<i>la*11.1</i>	S	SSRW136_148	6.01	9.40	1.65	0.11	NS	LL	13.10	10.44	12.44		
DLW		12	<i>la*12.1</i>	S	TG16_530	21.01	9.97	1.72	0.11	NS	LL	13.33	10.85	10.61		
		1	<i>dlw*1.1</i>	S	TG48_510	32.01	10.47	0.54	0.10	0.02300	LL	5.45			4.28	
		6	<i>dlw*6.1</i>	S	SSR22_200	5.01	8.86	0.50	0.08	NS	LL	5.34	4.26	4.49		
		7	<i>dlw*7.1</i>	S	SSR24_510	58.01	12.19	0.60	0.12	NS	L–		4.04			5.11
	DSW	1	<i>dsw*1.1</i>	S	TG48_510	32.01	10.15	0.26	0.09	NS	LL	2.15			1.63	
	LKC	5	<i>lk5.1</i>	C	<i>PHYB2</i>	31.01	20.05	110.08	0.20	NS	LL	1,445.22	1,233.82	1,328.12		
		7	<i>lk*7.1</i>	S	SSRW285_290	25.01	28.14	154.78	0.27	0.00010	LL	1,304.13	1,081.92	1,106.24		
		7	<i>lk*7.1</i>	S	SSR22_530	27.01	28.15	149.33	0.25	0.00010	LL	1,311.47			1,031.99	
		12	<i>lk*12.1</i>	S	SSR22_1018	1.01	8.40	81.88	0.08	NS	LL	1,253.09	1,116.70	1,221.76		
	SKC	5	<i>skc5.1</i>	C	SSR35_298	0.01	11.89	77.25	0.11	NS	L–		1,494.85			1,667.29
7		5	<i>skc5.2</i>	C	<i>PHYB2</i>	31.01	14.07	85.61	0.14	NS	LL	1,681.61	1,505.59	1,621.37		
		7	<i>skc*7.1</i>	S	SSR22_530	27.01	27.32	148.57	0.23	0.00001	LL	1,356.09			1,083.81	



Table 4 continued

Pop	Trait	Chr	QTL	Tr	Marker	Position	LR	a	R <sup>2</sup>	PQTL × E	GIM	LL	PP	LP	P	L–
LNC		<b>1</b>	<b><i>Inc*1.1</i></b>	S	<i>LeNHX3</i>	30.01	10.85	–150.74	0.10	–	CC	1,211.24	1,509.98	1,337.75		
		<b>1</b>	<b><i>Inc*1.1</i></b>	S	SSR30_375	29.01	11.62	–157.75	0.11	–	CC	1,226.38	1,514.08	1,272.56		
		7	<i>Inc*7.1</i>	S	SSRW285_290	23.01	44.82	–487.87	0.43	–	CC	1,147.53	1,570.25	1,534.14		
		7	<i>Inc*7.1</i>	S	TG35_506	30.01	51.03	–499.68	0.43	–	CC		1,653.83			1,149.07
SNC		8	<i>Inc*8.1</i>	S	SSRW63_240	23.01	12.04	–164.14	0.12	–	CC	1,224.50	1,533.45	1,354.41		
		7	<i>snc*7.1</i>	S	SSRW285_290	23.01	44.82	–487.87	0.40	–	CC	1,481.81	2,144.29	1,943.52		
		7	<i>snc*7.1</i>	S	TG35_506	30.01	51.03	–499.68	0.38	–	CC		2,361.79			1,463.71
		1	<i>lkt*1.1</i>	S	<i>LeNHX3</i>	30.01	9.08	0.18	0.09	–	LL	1.25	0.94	1.05		
NLS		7	<i>lkt*7.1</i>	S	SSRW356_900	26.01	36.24	0.35	0.30	–	LL	1.44	0.68	0.84		
		7	<i>nlst*7.1</i>	S	TG35_506	32.01	14.44	2.55	0.35	–	L–		7.91			11.60
TN		<b>7</b>	<b><i>tn*7.1</i></b>	S	SSRW285_290	25.01	28.07	–0.51	0.23	–	CC	1.85	2.56	2.41		
		<b>7</b>	<b><i>tn*7.1</i></b>	S	TG35_506	28.01	30.38	–0.53	0.24	–	CC		2.73			1.86

Significant *P* values from QTL × E analysis by interval mapping are also included. NS: non significant. An asterisk at the QTL name means significant only under saline treatment. Pairs of QTLs in bold might be just one QTL each. Genotype increasing the mean of the trait (GIM) is specified depending on the dominance or codominance nature of the marker

LL phenotypic mean of lines homozygous for the *S. lycopersicum* alleles, PP phenotypic mean of lines homozygous for the *S. pimpinellifolium* alleles, CC phenotypic mean of lines homozygous for the *S. cheesmaniae* alleles, LP and LC = phenotypic mean of heterozygote lines from P and C populations, respectively. L– phenotypic mean of lines with *L* being dominant, P– and C– phenotypic mean of lines when the wild allele is dominant in the P and C populations, respectively

5 according to the correlations found between  $K^+$  concentration and vegetative traits. The *L* allele at these QTLs increases SKC, LA and DLW, connecting  $K^+$  concentration and leaf vigor. There is also a flowering time QTL located at this region, *sf5.1* (Villalta et al. 2007). Therefore, the *L* allele here, not only enhances the vegetative development but also enlarges the period till flowering. Differently from the cluster of QTLs in C1, no  $Na^+$  concentration QTL is detected in this region but one for TN (*tn5.1*). The high TN percentage, over total expected  $Na^+$  in the plant, associated with the *LL* genotype at *tn5.1*, might be explained by a passive accumulation of  $Na^+$  in the aerial part of the plant because the *L* allele at *la5.1* and *dlw5.1* is associated with larger leaves. Therefore, as far as breeding for salt tolerance is concerned in terms of  $[Na^+]$  regulation (and fruit yield), the cluster of QTLs at C1 is of most interest. Concerning wild germplasm utilization, wild alleles are associated with less leaf and stem growth, and higher concentrations of  $Na^+$  and lower concentrations of  $K^+$  in stems and leaves under saline conditions in both the populations, except for *snc3.1* in the P population. This wild allele might be interesting for improving the  $K^+/Na^+$  balance. However, this is an exception; why is the  $Na^+$  inclusion strategy of these salt tolerant wild genotypes good for the plant but indifferent (or bad) for the fruit yield? Adaptation to salinity during evolution is related to plant survival. During vegetative growth, ABA-mediated adaptive responses are critical to plant survival during drought, salt and cold stress. From all the QTLs for vegetative traits detected here under salinity, *dlw1.1* on C1 shows a significant QTL  $\times$  E interaction (salinity-specific QTL) suggesting it might involve ABA responsive elements. Growth arrest (and therefore smaller fruits) can be considered as a possibility to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for a better recovery after stress relief (Bartels and Sunkar 2005).

#### Tomato salt tolerance candidate genes

In *Arabidopsis*, the SOS pathway controls  $Na^+$  and  $K^+$  homeostasis as well as  $Na^+$  long-distance transport under saline conditions (Zhu 2002). In this functional module participates SOS3, a myristoylated calcium binding protein that is thought to respond to salt-induced cytosolic  $Ca^{2+}$  elevations (Liu and Zhu 1998; Halfter et al. 2000; Ishitani et al. 2000). Activated SOS3 directly interacts with SOS2, a serine/threonine protein kinase, forming an activated SOS3/SOS2 complex (Halfter et al. 2000). One of the targets of this signaling pathway is SOS1, a  $Na^+/H^+$  antiporter localized to the plasma membrane (Shi et al. 2002) whose activity is regulated through phosphorylation by SOS3/SOS2 complex (Qiu et al. 2002).

In tomato, *SISOS2* locates at the same position as the salinity specific flowering time QTL *sf12.1* (Villalta et al.

2007) and at the vicinity of *la12.1* in the C population. The allele derived from the salt sensitive parent at *SISOS2* is associated with a reduction of the period till flowering and an increment of the leaf area in comparison to the wild allele, derived from the salt tolerant parent. Similar results concerning leaf development was found in *Arabidopsis* (Quesada et al. 2002) where *SOS2* was found to locate close to a QTL involved in vegetative trait variation and the salt tolerant genotype displayed a reduction of fresh weight. *SISOS3* locates near by *snc3.1* in the P population but the marker providing the highest LR value is 5 cM apart. Since this feature seems a good indication of the QTL position (Price 2006), *SISOS3* might be discarded as candidate gene involved in *snc3.1*.

Six NHX  $Na^+/H^+$  antiporters have been identified in *Arabidopsis* (Yokoi et al. 2002) and *AtNHX1* has been reported as a salt tolerance determinant (Apse et al. 1999; Gaxiola et al. 1999; Quintero et al. 2000). It displays a constitutive expression in shoots and roots and the protein locates at the tonoplast. *AtNHX1* is up regulated by salt stress at transcriptional level through ABA signaling (Yokoi et al. 2002) and at posttranslational level by the SOS pathway (Qiu et al. 2004). *LeNHX3* (highly similar to *AtNHX1-4*) locates very close to *lnc1.1* and *lkn1.1* in the C population, where it is polymorphic. Nevertheless, it is important to point out that the highest significantly associated marker with LNC at this genomic region is SSR30\_375 (Fig. 1). The wild allele at *lnc1.1* increases the mean trait. In *Arabidopsis*, salt tolerance conferred by NHX encoding genes has been related to  $Na^+$  inclusion. In this sense, *S. cheesmaniae* allele is related to higher levels of  $Na^+$  and lower  $K^+/Na^+$  ratio in leaves than the allele of the salt sensitive parental at this locus, however, the profitable allele in terms of FW at this genomic position corresponds to the cultivated species, whose vegetative growth under salinity is the largest. Future experiments of fine QTL mapping are focused on this region, nevertheless, from the agronomic point of view *Arabidopsis* might be a model species with low predictive value to improve tomato fruit yield under salinity using wild genetic resources.

No  $[Na^+]$  or  $[K^+]$  QTL has been found located at *LeNHX1*. However, a significant association between this gene and  $Cl^-$  concentration in young leaves was detected in a previous QTL study (Villalta et al. 2007) in the P population. The *P* allele of *LeNHX1* is associated with lower  $Cl^-$  concentration in young leaves of mature plants after 12 weeks of salt treatment (150 mM). It would be interesting to distinguish between tight linkage and pleiotropic effects here. In any case, Sottosanto et al. (2004) have shown in *Arabidopsis* that *AtNHX1* plays a significant role in intracellular vesicular trafficking, protein targeting and other cellular processes in the absence or presence of salt.

$\text{Na}^+$  and  $\text{K}^+$  concentrations. The cluster QTLs in Chromosome 7

In contrast to the monocots, where salt tolerance is typically associated with the ability to exclude sodium ( $\text{Na}^+$ ) from the photosynthetic tissues, dicotyledonous species show a large variation in the extent to which salt tolerance is associated with the tissular  $\text{Na}^+$  levels (Yeo et al. 1990; Tester and Davenport 2003). Our results show that, as it has been observed in other dicotyledonous species such as *Arabidopsis* (Tester and Davenport 2003), the salt tolerant phenotype displayed by *S. pimpinellifolium* and *S. chesmaniae* is not associated with lower values of sodium concentration in the leaf and the stem than those of the salt-partially sensitive *S. lycopersicon*.

$\text{Na}^+$  or  $\text{K}^+$  concentrations are larger in the stem than in the leaves under saline treatment in both the populations. In the case of  $\text{Na}^+$ , this would suggest the existence of mechanisms limiting its entry to the leaf (by accumulation in proximal parts of the stem) or favoring its retranslocation from leaves to stem. Protection of young leaves is one of the most important mechanisms for plant salt tolerance (Jeschke 1984). Higher  $\text{Na}^+$  accumulation in the xylem and greater  $\text{Na}^+$  retranslocation through the phloem was found in *S. pennellii* when compared with *S. lycopersicum* in a study using salt tolerant wild relatives (Pérez-Alfocea et al. 2000). These authors also indicated that the  $\text{K}^+/\text{Na}^+$  ratio in both, xylem and phloem was higher in *S. lycopersicum* than in *S. pennellii*.

Since  $\text{K}^+$  concentration is also higher in the stem than in the leaf, and under both control and saline conditions, this seems a general feature of the monovalent cation distribution within the plant even under salinity, where  $\text{K}^+$  concentrations decrease, especially in the C population. Are  $\text{Na}^+$  and  $\text{K}^+$  concentrations genetically related?

$\text{Na}^+$  and  $\text{K}^+$  concentration QTLs locate together only in the chromosome 7, where also leaf and stem concentration QTLs cluster under salinity in both the populations. Moreover, the only sodium leaf sensitivity QTL detected, *nls7.1* locates in this region too, providing an interesting interpretation. Here, lines with the *S. lycopersicum* allele(s) show not only lower  $\text{Na}^+$  and higher  $\text{K}^+$  concentrations in stems and leaves, but also a larger reduction of leaf area than lines with the allele from the wild relatives. This implies that in spite of a better  $\text{K}^+/\text{Na}^+$  ratio for the *L* allele at this genomic position, there is an associated larger reduction of the leaf area than for the wild allele; i.e. although the wild alleles increase  $\text{Na}^+$  concentration, their leaves tolerate it better.

From the two genomic positions, where transported sodium QTLs have been detected, one of them, *tn7.1*, locates here. Unlike *tn5.1*, no QTL for vegetative trait map exactly in the same position as *tn7.1* and the *L* allele is

associated with a lower TN mean, in agreement with a lower  $\text{Na}^+$  concentration for this allele at *Inc7.1* and *snc7.1*. Is  $\text{Na}^+$  being additionally removed from the aerial part of these plants? It seems so given that the allelic difference at *tn7.1* becomes even larger at 200 mM (data not shown). The joint interpretations of all these QTLs that cluster in chromosome 7 suggest that the gene(s) responsible for them govern an active mechanism of  $\text{Na}^+/\text{K}^+$  regulation (Koyama et al. 2001). In this case, the  $\text{Na}^+$  regulation seems to be of most interest. Two reasons are argued: first, that from all SKC and LKC QTLs, none of them is detected under both control and salinity conditions. Thus, most of the  $\text{K}^+$  QTLs (all in the P population) present significant QTL  $\times$  E interactions, especially those major ones located in chromosome 7. Second, in the P population, where  $\text{Na}^+$  concentration was also measured under control conditions (data not shown), QTLs for LNC, SNC and LKN were detected in the same region of the chromosome 7. Consequently, at least in the P population, the gene(s) at these QTLs seems to control constitutively (i.e. in absence of salinity) the sodium concentration of the aerial part of the plant. When salinity becomes high (and  $\text{K}^+$ , limiting), allelic differences at this position also become relevant regarding  $\text{K}^+$  concentration. Reinforcing this hypothesis, our data show that the contributions to the total variance of *snc7.1* or *Inc7.1*, specially in the C population, were indeed among the highest (43% for *Inc7.1*) compared to those found for the salt tolerance in terms of fruit yield (Villalta et al. 2007). Lin et al. (2004) also found two major QTLs with very large effect on shoot  $\text{Na}^+$  concentration (*qSNC-7*, 48.5%) and shoot  $\text{K}^+$  concentration (*qSKC-1*, 40.1%) in rice, and one (*Nax1*, 38%) in durum wheat (Lindsay et al. 2004). These QTLs being so important for the tomato plant, why are not they highly associated with fruit yield? Instead of fruit yield, this cluster of QTLs might be related to plant survival. Pointing at this hypothesis, Lin et al (2004) found that *qSNC-7* was approximately at the same map position in chromosome 7 of rice as *qSDS-7*, a QTL for survival days of seedlings (140 mM NaCl).

Identification of a sodium transporter as responsible for a major salt tolerance QTL controlling  $\text{K}^+$  shoot content in rice has been recently reported (Ren et al. 2005). Similarly, two independent *Arabidopsis thaliana* natural variants of *AtHKT1* have been shown to be responsible in a great extent of elevated  $\text{Na}^+$  content in shoots (Rus et al. 2006). In the present study, a major QTL controlling  $\text{Na}^+$  (and  $\text{K}^+$  under salinity) concentration in aerial part of the plant has been identified in chromosome 7 but none of the  $\text{Na}^+$  transporters or regulatory proteins tested in this study locates at this genomic region. More candidate genes and future fine mapping studies are being focused on this region of chromosome 7.

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