Plant Physiology Preview. Published on December 11, 2017, as DOI:10.1104/pp.17.01605

Running title:

SlCBL10 ensures plant development under salinity

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Title:

The SlCBL10 calcineurin B-like protein ensures plant growth under salt stress by regulating Na+ and Ca2+ homeostasis

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One sentence summary:

Tomato CALCINEURIN B-LIKE PROTEIN 10 (SlCBL10) ensures plant growth by regulating proper distribution of Na+ and Ca2+ in the shoot apical meristem and developing organs under salt stress.

Author contributions:

I.E., B.P., A.O-A., F.A.P., S.D., B.G-S., F.J.Y-L., C.C. and J.B. performed the experiments; A.A., F.B.F., F.B. and T.A. collaborated in the design of the experiments and data analyses; J.S., W.V., C.B., M.C.B., V.M. and R.L. conceived the project and research plans, and supervised the experiments; I.E. and R.L. wrote the article with contributions of all the authors.

Funding information:

This study was supported by grants from the Plant KBBE Program (EUI2009-04074), the Spanish Ministerio de Economia y Competitividad (AGL2012-40150 and AGL2015-64991-C3-1-R/2-R/3-R), as well as the French National Research Agency ENDOREPIGEN project. A.O-A. was supported by a PhD fellowship from the Ministerio de Economia y Competitividad (BIO2009-11484).

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ABSTRACT

Characterization of a new tomato T-DNA mutant allowed for the isolation of the *CALCINEURIN B-LIKE PROTEIN 10* (*SlCBL10*) gene whose lack of function was responsible for the severe alterations observed in the shoot apex and reproductive organs under salinity conditions. Physiological studies proved that *SlCBL10* gene is required to maintain a proper low Na^+/Ca^{2+} ratio in growing tissues allowing tomato growth under salt stress. Expression analysis of the main responsible genes for Na^+ compartmentalization [i.e. *Na^+/H^+ EXCHANGERs* (*LeNHX3* and *LeNHX4*), *SALT OVERLY SENSITIVE* (*SlSOS1* and *SlSOS2*), *HIGH-AFFINITY K+ TRANSPORTER 1;2* (*SlHKT1;2*), *H^+-pyrophosphatase AVP1* (*SlAVP1*) and V-ATPase (*SlVHA-A1*)] supported a reduced capacity to accumulate Na^+ in *Slcbl10* mutant leaves, which resulted in a lower uploading of Na^+ from xylem, allowing the toxic ion to reach apex and flowers. Likewise, the tomato *CATION EXCHANGER 1* (*SlCAX1*) and *TWO-PORE CHANNEL 1* (*SlTPC1*), key genes for Ca^{2+} fluxes to the vacuole, showed abnormal expression in *Slcbl10* plants indicating an impaired Ca^{2+} release from vacuole. Additionally, complementation assay revealed that *SlCBL10* is a true orthologue of the *Arabidopsis CBL10* gene supporting that the essential function of CBL10 is conserved in *Arabidopsis* and tomato. Together, the findings obtained in this study provide new insights into the function of *SlCBL10* in salt stress tolerance. Thus, it is proposed that SlCBL10 mediates salt tolerance by regulating Na^+ and Ca^{2+} fluxes in the vacuole, cooperating with the vacuolar cation channel *SlTPC1* and the two vacuolar H^+-pumps, *SlAVP1* and *SlVHA-A1*, which in turn are revealed as potential targets of *SlCBL10*.

Keywords: Salinity, *Solanum lycopersicum*, CBL10, TPC1 channel, vacuolar H^+-pumps, Na^+ and Ca^{2+} homeostasis.

INTRODUCTION

The development of crop plants tolerant to abiotic stress is crucial to meet the growing food demand through sustainable agriculture. Along their life cycle, plants need to balance development and adaptive responses to unfavourable conditions, salinity being one of the most severe factors limiting the productivity of agricultural crops (Flowers et al., 2010; Shabala, 2013). Significant advances have been made in the study of genes involved in salt stress tolerance and ion homeostasis (Maathuis, 2014), especially in the model species *Arabidopsis thaliana*, whereas knowledge about species of agronomic interest such as tomato remains scarce. Salt tolerance is determined by the ability of the plant to regulate Na^+ transport rate from the root to the shoot through the xylem and by the capacity to accumulate Na^+ ion into the vacuoles of the adult leaves and stem, which allows the plants to protect young developing tissues from Na^+ toxicity (Shabala, 2013; Maathuis, 2014). Na^+ efflux is mediated by the plasma membrane Na^+/H^+ antiporter SALT OVERLY SENSITIVE 1 (SOS1; Hasegawa, 2013), whereas HIGH-AFFINITY K+ TRANSPORTER (HKT) proteins, particularly those belonging to class I (Platten et al., 2006), are critical determinants of Na^+ unloading from xylem vessels to other cells in the stele (Hasegawa, 2013). In tomato, two HKT1-like isoforms have been identified, *SlHKT1;1* and *SlHKT1;2*, which underlie a major tomato QTL for Na^+/K^+ homeostasis (Asins et al., 2013). Mainly, *SlHKT1;2* has been involved in the regulation of Na^+ movement from root to shoot through xylem and therefore, in the Na^+ concentration in leaves under saline conditions (Almeida et al., 2014; Asins et al., 2015). Compartmentalization in the vacuole of Na^+ ions is an effective mechanism to avoid the toxic effects of Na^+ in the cytoplasm (Maathuis, 2014). The transport of Na^+ from the cytoplasm into the vacuole occurs via tonoplast Na^+/H^+ EXCHANGERs (NHXs). Four NHX isoforms have been identified in tomato; among them, *LeNHX3* and *LeNHX4* show the strongest induction upon salinity (Galvez et al., 2012). In addition, *LeNHX3* has been associated with a QTL for Na^+ concentration in leaves (Villalta et al., 2008). In *Arabidopsis*, the Na^+ compartmentalization process mediated by vacuolar Na^+/H^+ antiporters is driven by the electrochemical gradient of protons across the tonoplast generated by the vacuolar H^+ pumps, H^+- pyrophosphatase (H^+-PPase) AVP1 and V-ATPase (Maeshima, 2000, Hasegawa, 2013). Two full-length cDNA clones (*SlVHA-A1* and *SlVHA-A2*) coding for two isoforms of the V-

ATPase catalytic subunit (V-ATPases A1 and A2) have been isolated in tomato. In response to salinity, the abundance of the *SlVHA-A1* transcript in leaves was nearly doubled with respect to control conditions, while *SlVHA-A2* did not change and was mostly expressed in roots (Bageshwar et al., 2005).

Salt tolerance in plants also required a proper balance of Ca^{2+} and Na^{+} ions (Manaa et al., 2013). Thus, it has been well documented that Ca^{2+} has a direct inhibitory effect on Na^{+} entry into the cell by decreasing Na^{+} influx through non-selective cation channels and acting as a counter-cation inside storage organelles (Shabala et al., 2005). However, a Ca^{2+} deficit situation can occur in plants growing under salinity, since the elevated concentration of Na^{+} hinders Ca^{2+} uptake by roots (Zhai et al., 2015). The large central vacuole of a typical mature cell is by far the largest intracellular Ca^{2+} storage in plants, therefore, the mobilization of Ca^{2+} vacuolar reservoirs by the plant in this unfavourable situation is crucial to maintain the growth of young tissues (Bonales-Alatorre et al. 2013). A steady state level of vacuolar Ca^{2+} depends on the balance between active Ca^{2+} import to vacuoles and vacuolar channels mediating Ca^{2+}-induced Ca^{2+} release (Conn et al., 2011). CATION EXCHANGER (CAX) are ion transporters located on the tonoplast membrane (Hirschi, 1999; Manohar et al., 2011) and several studies in *A. thaliana* have suggested they play a critical role in plant adaptation to certain stresses such as salinity (Cheng et al., 2003; Park et al., 2005). These antiporters also use the driving force of the proton gradient generated by the vacuolar pumps (V-ATPase and AVP1) to accumulate Na^{+} into the vacuole against its electrochemical gradient. In addition, the *TWO-PORE CHANNEL 1* (*TPC1*) gene encodes for most prominent cation Slow Vacuolar channel which represents the major cation conductance of the largest organelle in most plant cells (Kintzer and Stroud, 2016), mainly Ca^{2+}, but also other ions such as K^{+} and Na^{+} (Hedrich and Marten, 2011). In *Arabidopsis*, it has been proven that *TPC1* mediates a voltage-activated Ca^{2+} influx in leaf cells (Furuichi et al., 2001), contributing to the cytosolic calcium elevation and therefore to stress signalling (Hedrich and Marten, 2011; Choi et al., 2014).

CALCINEURIN B-LIKE PROTEIN 10 (CBL10), the last CBL family member to be identified so far, has also been involved in the regulation of salt stress response in *A. thaliana* (Kim et al., 2007; Quan et al., 2007). CBLs are EF-hand Ca^{2+} protein sensors and upon Ca^{2+} binding, they undergo conformational changes to

associate with a group of CBL-INTERACTING PROTEIN KINASES (CIPKs) (for review see Kolukisaoglu et al., 2004; Luan, 2008; Kim, 2013). Different combinations of CBLs and CIPKs complexes may generate temporal and spatial specificity in Ca^{2+} signalling, integrating various stimuli to determine cellular responses (Batistic et al., 2010). Previous studies have determined that CBL10 interacts and recruits CIPK24 (SOS2) towards the tonoplast, speculating that the CBL10-CIPK24 complex might phosphorylate and activate a tonoplast Na^+ channel or transporter yet unknown in order to transport cytosolic Na^+ into the vacuole (Kim et al., 2007; Quan et al., 2007; Waadt et al., 2008; Lin et al., 2009). Moreover, a recent study has also demonstrated that *A. thaliana* CBL10 is critical for reproductive development under salt stress, although this function occurs independently from SOS2 interaction (Monihan et al., 2016). Likewise, Kang and Nam (2016) have provided an additional explanation for the positive role of CBL10 in salt tolerance by regulating sensitivity to brassinosteroids. Following the initial discovery of CBL10 in *A. thaliana*, a homologue has been reported in *Populus* and attributed similar functions (Tang et al., 2014). A CBL10 homologue has also been reported in tomato and its function in pathogen response within the reactive oxygen species signaling pathway has been demonstrated (de la Torre et al., 2013). However, the role for *SlCBL10* in the regulation of abiotic stress responses in tomato remains unexplored. Furthermore, the relationship of *CBL10* gene with other genes involved in the regulation of ion homeostasis (*SOS1, HKT1s, AVP1, VHA-A1* and *TPC1*) has neither been established so far in any species. This study reports the identification of the tomato *Slcbl10* knock-out mutant which exhibited very high salt-sensitivity. The functional characterization of this mutant revealed a new role of the *SlCBL10* gene in the salt tolerance of tomato by balancing Na^+ and Ca^{2+} homeostasis.

RESULTS

Isolation of the *pms916* salt hypersensitive T-DNA mutant and molecular cloning of the tagged gene

An *in vitro* phenotypic screening to identify mutants with altered salt stress responses was performed in a tomato T-DNA mutant collection by growing T2 segregating families in a basal culture medium (SCM)

supplemented with 100 mM NaCl (for details, see Materials and Methods). As a result, the *pms916* (*protecting meristem from salt stress 916*) mutant was isolated, which exhibited abnormal thickening of the hypocotyl, severe inhibition of vegetative development and a collapse of the apical meristem after 20 days of salt treatment (20 DST; Fig. 1A). A similar mutant phenotype was observed in three additional repeated assays (Experiments E1, E2 and E3 in Supplemental Table S1) performed using identical salt stress conditions. Subsequently, the salt-sensitivity of the *pms916* mutant was corroborated *in vivo* by growing T2 plants under greenhouse conditions (Experiments E4 and E5 in Supplemental Table S1). In all *in vitro* and *in vivo* experiments, genetic analysis indicated that *pms916* mutation was inherited as monogenic and recessive (Supplemental Table S1).

Southern blot analysis showed that the original T1 plant carrying the *pms916* mutation harboured three T-DNA copies (Fig. 1B). To identify lines with a single T-DNA insertion, T3 progenies were used for kanamycin sensitivity test. The results showed a 3:1 (tolerant:sensitive) segregation for kanamycin response in four of the ten progenies evaluated, indicating a single-locus insertion of the T-DNA. These four T3 progenies were grown *in vitro* under identical salt conditions as described above (SCM + 100 mM NaCl). From each T3 progeny, a single plant showing a *pms916* mutant phenotype was analysed by Southern blotting. The results indicated that two T-DNA insertions were present in all mutant plants (Fig. 1B). Genomic regions flanking T-DNA insertions were cloned by anchor-PCR, and the sequencing analysis revealed that the two T-DNA copies were inserted in a head-to-tail tandem orientation (Fig. 1C). Hence, both T-DNA copies were inherited as a single locus, which agreed with the results of the kanamycin sensitivity test. The T-DNA tandem insertion was located on chromosome 8 of the tomato genome, within the *CALCINEURIN B-LIKE PROTEIN 10* (*SlCBL10*) gene (*Solyc08g065330*). The inserted fragment caused a deletion of 1,836 bp, between 1,634 bp upstream and 202 bp downstream of the translation start codon of the *SlCBL10* gene (Fig. 1C), preventing, in all likelihood, the translation of a functional protein.

A PCR co-segregation analysis was then carried out to determine whether the T-DNA insertion correlated with the mutant phenotype. For this purpose, the *SlCBL10* genotype of 25 T2 individuals was determined using G-F, G-R, and T2-R primer combinations (Fig. 1, C and D, and Supplemental Table S2). Among the 18 wild-

type (WT) individuals, 14 plants were heterozygous and 4 homozygous for the WT allele, while the remaining 7 mutant plants carried the mutant allele in the homozygous state (Fig. 1D). These results strongly supported that the disruption of the *SlCBL10* gene by the T-DNA insertion was responsible for the *pms916* mutant phenotype and therefore, the tomato *pms916* insertional mutant was renamed as *Slcbl10*.

Phenotypic characterization of *Slcbl10* mutant plants

In order to more deeply characterize the *Slcbl10* mutant phenotype, T3 homozygous and azygous lines for the *Slcbl10* mutation, both belonging to the same T2 family, were grown in a hydroponic system as described in the Materials and Methods section. In the absence of salt stress, *Slcbl10* mutant plants grown normally with the only exception of a slight chlorosis at the margins of some young leaflets at the moment when plants have developed a few number of leaves. The sensitive phenotype was accentuated as mutant plants grew, showing bulging and thickening of the leaflets forming the shoot apex (Supplemental Fig. S1).

Short-term hydroponic salt treatment (HST) assays showed that salinity caused severe damages in the aerial part of *Slcbl10* mutant plants, in both young and adult plants (for further details Materials and Methods section). Vegetative growth of *Slcbl10* homozygous plants was arrested at young stages (HSTy assay) and they showed swelling and curved appearance of leaves, chlorosis at edge of leaflet and apical collapse (Fig. 2A). Salt treatment also induced growing abnormalities in *Slcbl10* adult plants (HSTa assay). Thus, after 2 DST, mainly young apical tissues became burnt and wilted, and subsequently mutant plants stopped growing as a consequence of apical collapse just after 6 DST (Fig. 2B). However, it was surprising that leaves and stems at basal positions on *Slcbl10* mutant plants did not show evident symptoms of salt sensitivity. Instead; their external appearances were similar to that of WT plants after 6 DTS (Fig. 2B), indicating that in a first instance, loss of *SlCBL10* function mainly affects shoot apex and growing tissues although finally the whole plant is affected and dies from apical collapse.

To corroborate that the salt hypersensitivity phenotype of the *Slcbl10* mutant was due to the loss of *SlCBL10* gene function, two clonal replicates of 14 independent RNAi *SlCBL10* lines and 13 independent regenerants,

these latter obtained under the same conditions except for the use of the RNAi gene construct (control plants), were grown and characterized under hydroponic salt treatment (HSTa assay conditions). All RNAi *SlCBL10* lines displayed salt stress sensitivity, showing an altered phenotype almost equal to that of mutant plants (Fig. 2C), including decreased fresh weight of shoots and roots (Fig. 2D). These results confirmed that the T-DNA mutation affecting the *SlCBL10* gene was responsible for the salt-hypersensitivity initially observed in the *pms916* mutant.

Functional complementation of the *Arabidopsis cbl10* mutant line

In order to test whether the *SlCBL10* gene is an orthologue of the *Arabidopsis CBl10*, the entire ORF corresponding to the putative *SlCBL10* was cloned under the control of a 35S promoter. This construct was used to transform and test a functional complementation of the *Arabidopsis cbl10* knock-out T-DNA line (Quan et al., 2007). Salt tolerance of three independent transgenic lines overexpressing *SlCBL10* in a *cbl10* genetic background was compared to that of wild-type (Col-0) and the *cbl10* mutant plants (Fig. 3A). Whilst growth in 100 mM NaCl is more severely compromised in the *cbl10* mutant than in WT plants, heterologous expression of *SlCBL10* in this mutant genetic background restores growth to WT levels (Fig. 3B). Previous studies have shown that despite being hypersensitive to salinity, the *Arabidopsis cbl10* mutant accumulated less Na^+ after high salt exposure (Kim et al., 2007). Accordingly, results here obtained demonstrated that the expression of *SlCBL10* restored Na^+ accumulation to WT levels in *cbl10* mutant plants (Fig. 3C). In conclusion, *SlCBL10* was able to reiterate *CBL10* function in *Arabidopsis* indicating that *SlCBL10* is a true orthologue of the *CBL10* gene.

The *SlCBL10* gene is differentially expressed in tomato tissues, and is induced by salt stress

Changes of *SlCBL10* expression induced by salt stress were further analysed in adult WT plants grown in a hydroponic system (HSTa assay conditions). Salinity induced an increase of *SlCBL10* expression in all tissues analysed (Fig. 4A). In shoot, the highest expression levels were detected in upper adult leaves (3-fold increase),

followed by young leaves and stems (2-fold increase). The lowest *SlCBL10* expression was found in roots, although a significant increase was also detected in salt-treated plants.

The temporal effect of salt stress on *SlCBL10* expression was analysed in the first developed leaf of WT plants from the same experiment mentioned above (Fig. 4B). Transcript levels of *SlCBL10* were significantly induced by salt treatment after 12 h, reaching the maximum levels between 24 and 48 h. Later, *SlCBL10* expression decreased to the basal levels found in the absence of salt stress after 60 h of treatment. At 6 DST, a further increase of *SlCBL10* expression was observed, therefore suggesting that two phases of induction may occur during exposure to salt stress.

Na^+ distribution pattern is altered in *Slcbl10* mutant plants

To ascertain whether the salt sensitivity phenotype was associated with changes in Na^+ distribution patterns induced by salt stress, ion contents were analysed in several adult plant tissues from the HSTa assay (Fig. 5A). Results showed that although Na^+ uptake at whole level was lower in *Slcbl10* mutant than in WT plants (25% and 20% lower in mutant after 2 DST and 6 DST, respectively), the Na^+ distribution pattern along the mutant plant was completely altered. In WT plants, Na^+ is preferentially accumulated in roots, and later in adult leaves and stems to prevent Na^+ from reaching toxic levels in young developing tissues. In fact, the lowest Na^+ accumulation was found in the shoot apex of WT plants in salt conditions. Contrarily, the *Slcbl10* mutant did not retain Na^+ properly in roots or in adult leaves or stems as 30% less Na^+ is detected in these tissues, and therefore similar Na^+ contents were found along the mutant plants. The incapacity of the *Slcbl10* mutant to retain Na^+ in adult vegetative tissues allows the ion to reach the apex in higher concentration, 70% higher than in WT, after 6 DST.

An opposite tendency was observed for K^+ distribution. Thus, during salt treatment, the K^+ content was higher in roots, upper adult leaves and stems of mutant plants than in WT plants, while a similar K^+ content was detected in the apex (Supplemental Fig. S2). This fact, promoted that the Na^+/K^+ ratio was lower in roots and upper adult leaves of the *Slcbl10* mutant, but higher in the apex due to a higher Na^+ accumulation in these

255 tissues (Fig. 5B). Together these results strongly evidence that salt stress induces significant alterations in Na^+
256 and K^+ homeostasis of *Slcbl10* mutant plants.

257 Transcript levels of main genes involved in Na^+ homeostasis were analysed in the first developed leaf and
258 roots (Fig. 5C). With this purpose, expression of genes responsible for the uptake and long-distance Na^+
259 transport in tomato, *SlSOS1* and *SlHKT1s*, (Olias et al., 2009; Asins et al., 2013), as well as genes involved in
260 Na^+ accumulation into vacuole, *LeNHX3, LeNHX4* (Galvez et al., 2012), *SlAVP1* and *SlVHA-A1* genes (Gaxiola
261 et al., 2007) were determined. In addition, expression of *SlSOS2* gene, which has been related to both long-
262 distance Na^+ transport and Na^+ accumulation process (Huertas et al., 2013; Olias et al., 2009), was also
263 analysed. Although no differences in the expression levels of these genes were detected between *Slcbl10* mutant
264 and WT plants when they were grown under control conditions, some significant changes were found when
265 plants grew under salt stress, mainly affecting leaf tissue (Fig. 5C). Salinity induced up-regulation of all
266 analysed genes involved in Na^+ compartmentalization, i.e. *LeNHX3*, *LeNHX4*, *SlSOS2*, *SlAVP1* and *SlVHA-A1*,
267 in WT but not in mutant leaves where only *LeNHX3* and *SlVHA-A1* were up-regulated. Furthermore, the
268 *SlHTK1;2* gene was significantly down-regulated in leaves of mutant plants. In roots, the only remarkable
269 difference was the induction by salinity of *SlSOS1* expression in mutant but not in WT plants, which indicated
270 that Na^+ extrusion from root to external medium was increased when *SlCBL10* expression is knocked-out
271 (Supplemental Fig. S3).

272

273 **Disruption of *SlCBL10* gene affects Ca^{2+} homeostasis under salt stress**

274 Another singular feature of *Slcbl10* mutant extracted from ion analysis was an altered Ca^{2+} distribution pattern
275 under salinity (Fig. 6A). Thus, while the hydroponic salt treatment (HSTa assay) caused a significant reduction
276 of Ca^{2+} content in both stems and upper adult leaves of WT plants after 6 DST, no significant changes of Ca^{2+}
277 content were observed in *Slcbl10* mutant plants. Consequently, Ca^{2+} content was 80% and 60% higher in stem
278 and upper adult leaves, respectively, in *Slcbl10* than in WT plants.

279 The higher Ca^{2+} levels detected in upper adult leaves and stem of *Slcbl10* mutant plants could be due to Ca^{2+}
280 retention or to an increased Ca^{2+} transport from roots to shoots during salt treatment. In order to check these two
281 possibilities reciprocal grafting experiments were performed between WT and *Slcbl10* plants, and Ca^{2+} contents
282 were measured in grafted plants grown under hydroponic salt treatment (HSTa assay conditions; Fig. 6B). Level
283 of Ca^{2+} was significantly higher in leaves of grafted plants using *Slcbl10* as scion independently of the rootstock
284 background, while a similar decrease of Ca^{2+} content was found in leaves when WT scion was grafted on either
285 WT or mutant rootstock. In addition, no significant differences of Ca^{2+} content were found in roots of the
286 different combinations. Therefore, it is possible to conclude that the higher Ca^{2+} content found in *Slcbl10*
287 mutant organs was not due to a higher Ca^{2+} transport from root to shoot but, most likely, to retention in leaves
288 of Ca^{2+} stores, which did not diminish under salinity.

289 Under calcium deficit conditions, such as those promoted by salinity (White and Broadley, 2003), plants
290 need to mobilize their calcium reservoirs to ensure fruit development and yield. Therefore, possible effects on
291 fruit yield promoted by Ca^{2+} reservoirs improperly retained in leaves and stems of *Slcbl110* plants were
292 analysed in a long term salt treatment assay in a greenhouse (GST assay conditions; see Materials and
293 Methods). Fruit yield and blossom end rot (BER) incidence, the latter being a well- known symptom of Ca^{2+}
294 deficiency disorders in tomato fruit (de Freitas et al., 2014; Zhai et al., 2015), were determined in adult plants
295 after 50 DST (Fig. 6C). A considerably decrease of fruit production and higher BER incidence (7-fold) was
296 detected in *Slcbl10* plants with respect to WT). In addition, other phenotypic alterations that coincided with
297 those promoted by Ca^{2+} deficiency were observed in long term salt-treated *Slcbl10* and RNAi *SlCBL10* plants,
298 among others, reduced growth of the apical meristem, small leaves with evident chlorosis symptoms at their leaf
299 edges and thickened petioles and stems (Supplemental Fig. S4 and Fig. S5). Taken together, these results
300 indicated that under salinity conditions, Ca^{2+} reservoirs are less available in *Slcbl10* plants than in WT, thus
301 limiting their proper development and productivity.

302 To corroborate that the low Ca^{2+} availability is responsible for the growth restriction of *Slcbl10* mutant in salt
303 stress conditions, an *in vitro* assay was carried out using 1 mM suboptimal Ca^{2+} concentration. Under these

304 conditions, the *Slcbl10* mutant showed higher sensitivity to a Ca^{2+} deficiency condition (Supplemental Fig. S6,
305 A and B). The first Ca^{2+} deficiency symptom observed was a collapse of the subapical shoot region resulting in
306 a constriction necrosis below the shoot tip, followed by the apical meristem senescence. Light microscopic
307 analysis also revealed significant alterations affecting SAM morphology and ground cells of *Slcbl10* mutant
308 plants (Supplemental Fig. S6, C and D). In addition, swollen cells were observed in the submeristematic region
309 immediately below the collapsed zone leading to thickened stems (Supplemental Fig. S6E) whose appearance
310 strongly resembled that observed in *Slcbl10* young plants grown under salt conditions (Fig. 2). These results
311 strongly support that tomato plants lacking *SlCBL10* are not able to balance their development under Ca^{2+}
312 deficient conditions.

313

314 **Expression pattern of key genes involved in vacuolar fluxes of Ca^{2+}**

315 To ascertain how *SlCBL10* might be involved in the mobilization of Ca^{2+} stores, and taken into account that the
316 vacuole is by far the largest intracellular Ca^{2+} store in mature cells (Peiter, 2011), the expression profile of key
317 genes involved in Ca^{2+} fluxes were analysed. Concretely, transcript accumulation of *CAX1*, *AVP1* and *VHA-A1*
318 genes, all required for Ca^{2+} compartmentalization into the vacuole (Pittman, et al., 2009), and *TPC1* involved in
319 Ca^{2+} release from vacuole (Herdrich and Marten, 2011), were measured in upper adult leaves of *Slcbl10* mutant
320 and WT plants grown under HSTa assay conditions. Given the essential role of Ca^{2+} as a second messenger of
321 signalling stress, gene expression analyses were performed at early and later steps of salt treatment (Fig. 7). In
322 WT plants, salinity induced a down-regulation of *SlCAX1*, while *SlTPC1*, the main responsible channel for the
323 release of Ca^{2+} from vacuole, was up-regulated. The lowest levels of *SlCAX1* transcripts were detected after 48h
324 of salt treatment which remained low until the end of treatment. *SlTPC1* registered two maximum levels of
325 expression, one soon after 48 hours and the other later, at the end of salt treatment. In *Slcbl10* mutant plants,
326 expression of *SlTPC1* was not induced by salinity, but it was down-regulated (65% reduction) after 48 hours of
327 salt exposure. Moreover, *SlCAX1* expression was more pronounced and earlier repressed in *Slcbl10* mutant than
328 in WT plants. Indeed, after 60 hours of salt exposure, the level of *SlCAX1* transcripts was almost null in the

329 mutant (98% of inhibition). The expression pattern of these genes in WT indicated that salinity induced an
330 efflux of Ca^{2+} from vacuole, while in mutants this efflux may be impaired by the strong down-regulation of
331 *TPC1*.

332 As regards the vacuolar proton-pumps coded by *SlAVP1* and *SlVHA-A1* genes, salinity induced a similar
333 profile expression to that described for *SlTPC1* in wild-type plants, as a peak of induction was detected at 48
334 hours of salt treatment (Fig. 7). However, the level of transcripts of both *SlAVP1* and *SlVHA-A1* genes were
335 significantly lower in *Slcbl10* mutant plants than in WT. It is interesting to highlight that the expression patterns
336 of *SlTPC1* and the two vacuolar proton pumps, *SlAVP1* and *SlVHA-A1* followed a very similar temporal pattern
337 expression, suggesting that they may cooperate to regulate proper Ca^{2+} flux in the vacuole under salt stress in
338 tomato.

339

340 **_SlCBL10_ gene is needed to maintain a proper ratio Na^+/Ca^{2+} in flowers and apex under salinity**

341 It is known that under salinity conditions, the maintenance of a proper Na^+/Ca^{2+} low ratio in growing tissues,
342 such as shoot apex and flowers, is essential to maintain plant growth (Manaa et al., 2013). Thus, in order to
343 ascertain when the lack of *SlCBL10* gene prevents the maintenance of a proper Na^+/Ca^{2+} ratio that ensures the
344 growth in tissues such as apex and flowers under salinity, Na^+/Ca^{2+} ratio was assessed in WT, *Slcbl10* mutant
345 and RNAi *SlCBL10* plants. After 6 DST Na^+ and Ca^{2+} contents were analysed in upper adult leaf, apex and
346 flowers (first flower truss immediately below 1st fully developed leaf). In RNAi *SlCBL10* and *Slcbl10* mutant
347 plants, a higher Ca^{2+} content but a lower Na^+ content were registered in upper adult leaves as compared to WT
348 (Fig. 8, A and B). Contrarily, in apex and flowers, Na^+ levels increased and Ca^{2+} levels decreased both in
349 *Slcbl10* mutant and in RNAi plants (Fig. 8, A and B), causing the Na^+/Ca^{2+} ratios in these tissues to be
350 improperly high (Fig. 8C), which might be the ultimate responsible factor for the observed alterations in
351 mutants cultivated under salinity.

352

DISCUSSION

This study reports the identification and functional characterization of the tomato *Slcbl10* knock-out mutant identified from a screening of a T-DNA tomato mutant population (Pineda et al., 2012; Pérez-Martín et al., 2017). Through an anchor-PCR approach, *SlCBl10* was identified as the tagged gene responsible for the salt hypersensitive mutant phenotype. Molecular complementation experiments proved that *SlCBL10* is orthologous to the *A. thaliana CBL10* gene, and encodes a calcium sensor CALCINEURIN B-LIKE PROTEIN 10. Although *SlCBL10* was previously involved in a signaling pathway mediating tomato plant immunity (de la Torre et al., 2013), its role in regulating abiotic stress has not been studied so far in this model species.

SlCBL10 protects growing tissues from salt stress by Na^+ retention in adult tissues

Results here reported indicate that the salt sensitivity phenotype of *Slcbl10* mutant plants (i.e. growth inhibition, hypocotyl thickening and apical necrosis) was similar both under *in vitro* and *in vivo* stress conditions and that such phenotype was also corroborated in RNAi *SlCBL10* lines. In all cases, the lack of the *SlCBL10* finally drove plants to die from apical collapse (Fig. 2). In addition, expression analysis showed that *SlCBL10* was up-regulated in WT plants cultivated under salinity conditions, the highest expression level being detected in upper adult leaves close to the shoot apex. Together, these results indicate that transcriptional activity of *SlCBL10* plays a key role in the adaptive response of tomato plants to salt stress by protecting shoot apical meristem and growing tissues from physiological damages caused by salinity.

Previous studies have shown that CBL10 is also involved in salt response in *A. thaliana* (Kim et al., 2007; Quan et al., 2007) and *Populus* (Tang et al., 2014). Indeed, *Arabidopsis cbl10* mutant plants accumulated lower Na^+ and a higher K^+ content as a consequence of salt treatment, being the first salt-hypersensitive mutant with a lower Na^+/K^+ ratio reported so far (Kim et al., 2007). Here it has been showed that tomato *Slcbl10* mutant also accumulates lower Na^+ and higher K^+ than wild-type plants; in addition, functional complementation of the *Arabidopsis cbl10* mutant by *SlCBL10* leads to the recovery of Na^+ levels. Under these premises, it is necessary

377 to explain how plants accumulating less Na^+, as are the cases for *Slcbl10* and *cbl10* mutants, showed a salt-
378 hypersensitive phenotype when the opposite would be expected. A precise dissection of Na^+ relative content in
379 young *versus* adult leaves as well as in leaves *versus* stems in tomato allowed for the elucidation of the role
380 played by *SlCBL10* in Na^+ homeostasis. It is known that the mechanism of salinity tolerance in tomato involves
381 a preferential Na^+ accumulation in adult leaves and stems, which prevents Na^+ from reaching the shoot apex
382 (Cuartero et al., 2010). Our results proved that this physiological mechanism was totally altered in *Slcbl10*
383 mutant, which was not able to retain Na^+ in adult tissues. This alteration leads to a 7-fold increase in Na^+
384 content that reaches the shoot apex, which results in an inadequate high Na^+/K^+ ratio in growing tissues.
385 Changes in Na^+/K^+ ratios are consistent with the physiological damages detected in the apical part of mutant
386 plants and strongly support a functional role of the *SlCBL10* gene in protecting shoot apex and developing
387 tissues from salt stress conditions.

388

389 **_SlCBL10_ function is required for Na^+ compartmentalization into vacuole**

390 In the cytoplasm, inappropriate Na^+ levels cause important metabolic alterations as this ion inhibits enzyme
391 activity (Maathuis, 2009); for that reason, cytoplasmic Na^+ content have to be kept at low level by exporting
392 Na^+ into the vacuole (Albaladejo et al., 2017). Results from expression analysis revealed that the decreased
393 capacity of *Slcbl10* plants to retain Na^+ in adult leaf was associated with a significant lower salt-induced
394 expression of genes involved in Na^+ compartmentalization into vacuole, such as *LeNHX4, SlAVP1, SlVHA-A1*,
395 and *SlSOS2*. Moreover, in the leaves of *Slcbl10* plants it was observed both a reduced expression of *SlHKT1;2*,
396 the main responsible for uploading Na^+ from the xylem into the cells (Asins et al., 2013; 2015), as well as an
397 increase of *SlSOS1* gene expression, responsible for Na^+ extrusion from leaf cells to xylem (Olias et al., 2009).
398 Hence, expression analysis support the hypothesis that Na^+ compartmentalization into vacuoles as well as Na^+
399 upload from xylem into cells were severely inhibited in *Slcbl10* mutant plants, while Na^+ extrusion from leaf
400 cells to xylem was favoured (Fig. 9). Such physiological changes allow the toxic ion to reach the apex and
401 flowers promoting their collapse and the subsequent death of the plants. Therefore, results indicate that

402 *SlCBL10* is needed for regulating Na^+ homeostasis through the activity of genes involved in the
403 compartmentalization of Na^+ into vacuole.

404 In *Arabidopsis*, the CBL10-SOS2 complex has been proposed as a positive regulator of a still unknown
405 vacuolar protein triggering Na^+ compartmentalization into vacuoles (Kim et al., 2007; Waadt et al., 2008).
406 Additionally, it has been determined that SOS2 regulates AtNHX1 antiporter activity (Qiu et al., 2004) and
407 directly activates the vacuolar H^+ pump V-ATPase (Batelli et al., 2007). However, a previous study in *Populus*
408 suggested that CBL10 is not directly related to the function of NHX proteins (Tang et al., 2014), which points at
409 the vacuolar H^+-pumps as the potential candidates for protein targets of CBL10 (Fig. 9). In tomato, the lack of
410 *SlCBL10* function was associated with a repression of *SlSOS2*, and the constitutive expression of *SlCBL10*
411 rescued the phenotype of the *Arabidopsis cbl10* mutant, indicating that the molecular mechanism underlying
412 Na^+ compartmentalization into vacuole mediated by *SlCBL10* may be shared between *Arabidopsis* and tomato.

413

414 **_SlCBL10_ promotes Ca^{2+} mobilization and availability under salt stress conditions**

415 Ca^{2+} deficit usually occurs in plants growing in salinized soils, since elevated Na^+ concentrations hinder Ca^{2+}
416 uptake by roots (Zhai et al., 2015), which usually induces a reduction of Ca^{2+} content in upper leaves and stem.
417 Decreased Ca^{2+} levels were detected in WT but not in *Slcbl10* mutant plants when they grew under salinity
418 conditions (Fig. 6). Using reciprocal grafting between WT and the *Slcbl10* mutant, it was also proved that the
419 higher Ca^{2+} levels detected in *Slcbl10* mutant leaves resulted from Ca^{2+} retention in these tissues rather than
420 from a higher Ca^{2+} transport from the root to the shoot during salt treatment. Likewise, given that Ca^{2+} stores in
421 apical leaves and stem could be exchanged and mobilized to other tissues (apical meristem, flower and fruit)
422 according to the physiological Ca^{2+} needs of plants (White and Broadley, 2003; Dayod et al., 2010), the
423 retention of Ca^{2+} in *Slcbl10* upper leaves could cause an inefficient supply of Ca^{2+} to other demanding tissues,
424 such as flowers and shoot apex, thus contributing to their collapse under salinity. Several specific abnormalities
425 have been reported due to Ca^{2+} deficit, mainly reduced growth of apical meristems, chlorotic leaves, tissue

softening and high BER incidence in fruits (Robertson, 2013; Uozumi et al., 2012; de Freitas et al. 2014). Therefore, abnormal Ca^{2+} retention detected in upper adult leaves may also be responsible for the almost null production of fruits and high BER incidence detected in *Slcbl10* plants characterized under salinity conditions.

The fact that the *Slcbl10* mutant was not able to balance its development in a Ca^{2+} deficient medium (1 mM Ca^{2+}) also indicates that *SlCBL10* is required for an appropriate Ca^{2+} partitioning in tomato plants. Results showed that under suboptimal Ca^{2+} concentration, *SlCBL10* promotes the adequate expansion and division of pith cells below the shoot apical meristem. Indeed, the truncation of *SlCBL10* resulted in the collapse of subapical cells, and finally caused the death of the shoot apical meristem; said symptoms have been previously described as resulting from a suboptimal concentration of calcium reaching growing tissues (Busse, 2008). Therefore, under salt stress conditions, *SlCBL10* gene function makes it possible that Ca^{2+} reservoirs can be mobilized, a feature which is essential to regulate plant growth and survival of developing tissues.

SlCBL10 and *SlTPC1* cooperate in the proper Ca^{2+} release

The vacuole is by far the largest intracellular Ca^{2+} store in mature cells (Peiter, 2011), and constitutes the main Ca^{2+} reservoirs from which Ca^{2+} is exchanged and mobilized according to the physiological Ca^{2+} needs of plant (White and Broadley, 2003; Dayod et al., 2010). Expression profile of key vacuolar genes involved in Ca^{2+} homeostasis suggest that the mechanism required to originate Ca^{2+} fluxes in vacuole was severely affected when *Slcbl10* plants grew in salt stress conditions (Fig. 9). Thus, salinity did not induce the expression of *SlAVP1* and *SlVHA-A1* genes, which implies that the proton gradient, which is necessary to energize the Ca^{2+} transport towards the vacuole through CAX1 antiporters (Manohar et al., 2011), was impaired by the lack of *SlCBL10* gene function. Also, the stronger inhibition of *SlCAX1* observed in *Slcbl10* mutant plants could contribute to altering the proton gradient. Indeed, an indirect feedback mechanism has been proposed between CAX transporters (CAX1, CAX2, CAX3) and H^+-pump V-ATPase in *Arabidopsis*, which would generate H^+ flux across the tonoplast (Cheng et al., 2003). Since TPC1 has been reported as the main responsible factor for

promoting Ca^{2+} efflux from vacuole to cytoplasm in leaf cells (Furuichi et al., 2001), the lower H^+ pump into the vacuole of *Slcbl10* plants might result in an inefficient opening of the SlTPC1 channel since a low luminal pH is required to regulate the aperture of this channel (Kintzer and Stroud, 2016). Moreover, the fact that the expression of *SlTPC1* was not induced by salinity in *Slcbl10* mutant, as occurred in WT plants, together with a probable loss of efficiency for opening of SlTPC1 channel suggest that in mutant plants, the Ca^{2+} release from vacuole induced by salinity is disturbed, which in turn could cause a defective calcium mediated salt-stress signalling and therefore, salt sensitivity (Choi et al., 2014). Indeed, it has been proven that TPC1 is involved in the generation of Ca^{2+} cytoplasmic concentration elevation waves directed to stress signalling purposes (Evans et al., 2016). Based on this study's results, it seems possible to suggest that SlCBl10 plays a role in Ca^{2+}-mediated stress signalling through direct or indirect TPC1 channel regulation (Fig. 9). Direct regulation of TPC1 by CBL10 would imply that TPC1 is a target for the CBL10-CIPK24 complex to be phosphorylated and activated. In support of that hypothesis, it has been proposed that the TPC1 channel is regulated by phosphorylation (Kintzer and Stroud, 2016). Indirect regulation of TPC1 by CBL10 could be mediated by the acidification of vacuole through the regulation of vacuolar H^+-pumps, as a low pH is required to open the channel (Kintzer and Stroud, 2016). In such a way, SlAVP1 or SlV-ATPase would be the potential target for SlCBL10, which would agree with the mechanism discussed for Na^+ compartmentalization (Fig. 9).

The role of *SlCBL10* in Ca^{2+} releases from vacuole could also contribute to adaptation mechanism to salinity, allowing mobilization of Ca^{2+} vacuolar stores in leaf cells towards fast-growing tissues in order to compensate for the lower Ca^{2+} uptake by root under salinity. The hypothesis of a double function of SlCBL10, although associated with the same mechanism (regulation of Ca^{2+} fluxes in vacuole), is supported by the profile expression registered in WT tomato leaves during salt treatment, in which two induction phases of expression were detected. The first induction took place during short-time periods (after 24 h of salt treatment) which could be involved in the signaling of the salt stress process. Later, a second increase of expression could be attributed to an increasing Ca^{2+} demand by fast-growing tissues as a consequence of Ca^{2+} deficiency caused by salinity

(Fig. 9). The same expression profile was recorded by *SlTPC1*, *SlAVP1* and *SlVHA-A1* which reinforces the idea of a functional relationship between these genes.

CONCLUSIONS

This study has proved that the *SlCBL10* gene function is required to maintain a proper Na^+/Ca^{2+} ratio in growing tissues allowing plant growth under salt stress conditions. Although the functional role of CBL10 in controlling Na^+ homeostasis has been previously demonstrated in *Arabidopsis* (Kim et al., 2007), the regulation of Ca^{2+} homeostasis by CBL10 has not been proposed until now. Nevertheless, Monihan et al., (2016) determined that CBL10 is critical for reproductive development under salt stress conditions and detected a higher Na^+ and lower Ca^{2+} accumulation in *Arabidopsis* flowers. Such results together with the functional complementation of the *Arabidopsis cbl10* mutant phenotype by *SlCBL10* strongly support that *SlCBL10* is a true orthologue of the *Arabidopsis CBL10* and its function is conserved. Thus, it is proposed that the mechanism by which SlCBL10 participates in salt tolerance mechanism is directly related to the regulation of Na^+ and Ca^{2+} fluxes in the vacuole of leaf cells, through the activation of a tonoplast target, being the cation channel SlTPC1 and the two vacuolar H^+-pumps, SlAVP1 and SlV-ATPase the potential targets of SlCBL10 (Fig. 9). Accordingly, under salinity conditions, CBL10 confers to adult leaves the capacity to retain Na+ avoiding toxic ion accumulation in young developing tissues as well as facilitates activation of Ca2+ release from vacuoles in leaves counterbalancing Ca2+ deficiency caused by salt stress.

MATERIALS AND METHODS

Screening and identification of *pms916* (*Slcbl10*) tomato mutant

The tomato (*Solanum lycopersicum* L.) cv Moneymaker was used to generate a collection of T-DNA mutants by means of the enhancer trap vector pD991 (Atarés et al., 2011; Pineda et al., 2012; Campos et al., 2016; Pérez-Martín et al., 2017). The *in vitro* screening of 1200 T2 families of tomato T-DNA lines (10-12 plants per

498 family) grown in basal culture medium (SCM) supplemented with 100 mM NaCl led to the detection of a
499 mutant initially named *pms916* (*protecting meristem from salt stress 916*) due to its hypersensitive phenotype to
500 salt stress. To estimate the number of inserts bearing a functional *NPTII* marker gene, a segregation analysis of
501 T2 progeny in kanamycin-containing medium (KCM) consisting of Murashige and Skoog (MS) salts
502 (Murashige and Skoog, 1962), sucrose (10 g l^{-1}) and kanamycin 100 (mg l^{-1}) was carried out. The identification
503 of the insert responsible for the mutation and the co-segregation analysis between the insert and the mutant
504 phenotype were performed by segregation analysis with T2 and T3 progenies in both kanamycin-containing
505 medium (KCM) and NaCl-containing medium (SCM). To corroborate the *in vitro* salt sensitivity phenotype of
506 the *pms916* mutant, two new experiments were conducted under *in vivo* conditions. In both experiments, pre-
507 germinated seeds of the T2 segregating progeny were sown into pots containing coconut fiber and grown under
508 controlled climatic conditions: 26 ± 3 °C day/18 ± 1 °C night and extra lighting provided by wide-spectrum
509 tubes (450 µmol s^{-1} m^{-2}; Gro-lux, Sylvania, Germany) to expose plants to 16 h day length. To assess salt
510 hypersensitivity, T2 plants were irrigated with half-strength Hoagland solution (Hoagland and Arnon, 1950).
511 Salt treatment (100 mM NaCl) was initiated when the plants had developed two true leaves.

512 The number of T-DNA copies was determined by Southern blot hybridization experiments. Genomic DNA
513 was isolated from young leaves as described by Dellaporta et al. (1983). Ten µg of genomic DNA were digested
514 with *Eco*RI and *Hind*III endonucleases, electrophoresed in 0.8% agarose gel and blotted onto Hybond N+
515 membranes (GE Healthcare - Piscataway, NJ) as described by Ausubel et al. (1993). Hybridization was
516 performed with a chimeric probe, fusing the complete coding sequence of the *NEOMYCIN*
517 *PHOSPHOTRANSFERASE II* (*NPTII*) gene to 811 pb of coding sequence from the endogenous tomato
518 *FALSIFLORA* (*FA*) gene, which was employed as hybridization positive control (Yuste-Lisbona et al., 2016).

519

520 **Cloning of T-DNA flanking sequences and PCR genotyping**

521 The sequences flanking T-DNA were isolated by anchor-PCR according to the procedure previously established
522 by Pérez-Martín et al. (2017). The sequences of primers used are listed in Supplemental Table S2. The cloned
523 sequences were compared with SGN Database (http://solgenomics.net/) to assign the T-DNA insertion site on
524 tomato genome.

525 Co-segregation analysis of the T-DNA insertion site with the mutant phenotype in the T2 progeny was
526 checked by PCR using i) the specific genomic forward (G-F) and reverse (G-R) primers to amplify the WT
527 allele (without T-DNA insertion) and ii) one specific genomic primer (G-F) and the specific T-DNA border
528 primer (T2-R) to amplify the mutant allele (carrying the T-DNA insertion). Primers were designed based on
529 sequence information available from SGN Database (http://solgenomics.net/). The sequences of genotyping
530 primers used are listed in Supplemental Table S2. Amplification of the genotyping primers was performed in a
531 30 µl volume using 25 ng of total DNA, 50 ng of each primer, 0.25 mM dNTPs, 2.5 mM MgCl2, and 1 U of
532 REDTaq DNA polymerase (SIGMA-Aldrich) in 1X Taq buffer. DNA was amplified under the following
533 thermal cycling conditions: 94ºC for 5 min, followed by 35 cycles at 94ºC for 30 s, 60ºC for 30 s, and 72ºC for
534 2 min, and a final extension of 5 min at 72ºC. PCR products were analysed in 1% agarose gels in SB buffer (10
535 mM sodium boric acid) and visualized with ethidium bromide.

536

537 **Generation of transgenic tomato lines**

538 In order to generate *SlCBL10* silencing lines, a RNA interference (RNAi) approach was followed. With this
539 aim, a 123-bp fragment of the *SlCBL10* cDNA was amplified using the SlCBL10-RNAiF and SlCBL10-RNAiR
540 primers (Supplemental Table S2), and the PCR product was cloned in sense and antisense orientation separated
541 by intronic sequences into the pKANNIBAL vector (Wesley et al., 2001) to generate a pKANNIBAL-SlCBL10
542 plasmid. The resulting plasmid was digested with *Not*I, and the entire construct was cloned into the binary
543 vector pART27 (Gleave, 1992). In all cases, the binary plasmids generated were electroporated into
544 *Agrobacterium tumefaciens* LBA 4404 strain for further use in genetic transformation experiments.

Agrobacterium-mediated transformation was performed following the protocol described by Gisbert et al. (2000). Fourteen independent diploid transgenic lines silencing *SlCBL10* were generated in tomato. The *SlCBL10* expression level was measured by RT-qPCR as described below. Regenerant plants (control plants) were also obtained under the same conditions except for the use of the RNAi gene construct.

Arabidopsis thaliana transformation and complementation test

The *Arabidopsis cbl10* mutant line (SALK_056042) was kindly donated by Professor Karen Schumaker (University of Arizona). To generate transgenic lines over-expressing *SlCBL10*, a 774 bp fragment was cloned spanning the entire ORF of *SlCBL10* (*Solyc08g065330*) in the vector pK7WG2D.1. The resulting construct was electroporated into *A. tumefaciens* GV3101 and transformed into *cbl10* mutant by floral dip method (Clough and Bent, 1998).

Seeds were surface-sterilized in 100% hypochlorite sodium for 5 minutes, washed five times in sterilized water and sown in petri dishes containing 0.5X MS medium supplemented with 1% sucrose and 0.9% agar. Seeds were stratified for 2 days at 4 °C before growth at 22 °C under long-day photoperiod (16h/8h light/dark). Five-day-old seedlings were transferred to MS medium supplemented with 100 mM NaCl. Na^+ contents were determined by atomic emission spectrophotometry.

Treatment assays

Tomato WT (cv. Moneymaker), T3 homozygous and azygous plants for the *Slcbl10* mutation, RNAi *SlCBL10* and regenerant plants were used for the phenotypic and physiological characterization of the *Slcbl10* mutant and the functional analysis of the *SlCBL10* gene. Seeds were surface-sterilized briefly with 20% (v/v) commercial bleach for 15 min, and then washed with sterilized water four times and suspended in sterile water at 4ºC for 72 h. Germination was performed in darkness, in a 8:3 (v/v) mixture of peat:perlite, at 28°C temperature and 90% relative humidity. Seedlings were then maintained in a controlled-environment chamber (8 h/16 h day/night

cycle at 345 µmol m^{-2} s^{-1} light, 23-25 ºC, 50 -60% relative humidity) until they reached the desired developmental stage for each experimental assay. During this period, plants were irrigated daily with half-strength Hoagland solution (Hoagland and Arnon, 1950).

Hydroponic salt treatment assay (HST) and grafting experiments

Short-term HST assays were performed in a controlled-environment chamber (conditions above described). Tomato plants were grown hydroponically in an aerated half-strength Hoagland solution. Two types of salt treatments were performed depending on the development stage of plants: i) young plants at cotyledon stage were treated at 50 mM NaCl for 24 hours and then at 100 mM for 10 additional days (HSTy assay conditions), and ii) adult plants at the 5^{th} fully developed leaf stage were treated at 100 mM NaCl for 24 hours and then at 200 mM for 5 additional days (HSTa assay conditions). In HSTa assays, shoot and root fresh weights were taken prior to salt treatment and after 2 and 6 days of salt treatment (DST). Ions content was analysed in root, stem (taken at the 1^{st}-2^{nd} leaves insertion), upper adult leaf (1^{st} fully developed leaf) and shoot apex (leaf primordial and apical meristem) at 0 DST and after 6 DST. Expression induction by salinity of interesting genes was determined in shoot apex, young leaf (not fully developed leaf), upper adult leaf, stem and root of plants after 2 DST. Additionally, gene expressions were also determined in upper adult leaf after 12, 24, 30, 60 and 144 hours of salt stress. All samples were previously frozen in LN_2 and kept at -80ºC until further gene expression analysis. For each genotype, three biological replicates constituted by five plants each were analysed. Two independent assays were carried out in the same experimental hydroponic conditions described above.

An additional HSTa assay was performed using reciprocal grafting between WT and *Slcbl10* mutant plants as well as autografting with WT and *Slcbl10* mutant plants in which Ca^{2+} content was analysed in root and upper adult leaf of grafted plants at 0 DST and after 6 DST.

Greenhouse salt treatment assay (GST)

A long-term salt experiment was conducted in a greenhouse of South-eastern Spain with adult plants. At the 7th-8th fully developed leaf stage, WT and *Slcbl10* plants were transferred from the controlled culture chamber to a polyethylene greenhouse and grown on cocoa peat, using a drip irrigation system, as previously described (García-Abellán et al., 2014). The fertirrigation solution was prepared in 2000-liter tanks with local irrigation water (Electrical Conductivity (EC) = 0.9 dS m^{-1}). Before salt treatment, plants were grown under those conditions for 21 additional days until 10 leaves were fully developed. Then, fifteen plants per genotype were salt-treated (100 mM NaCl), keeping fifteen additional plants per genotype growing in the absence of salt. Salt treatment was performed by adding 100 mM NaCl to the tanks under 30/15 ºC day/night temperatures, 40% relative humidity and 500 µmol m^{-2} s^{-1} of natural light irradiance. After 50 days, fruits of each plant were counted (number of fruits), weighed (reproductive biomass) and the BER incidence calculated (percentage of fruit with BER symptoms).

Calcium deficiency in vitro assay

Pregerminated WT and *Slcb110* mutant seeds were grown on MS medium supplemented with a suboptimal calcium concentration of 1 mM using calcium chloride as a source. Culture media contained 3 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol, and 0.9% agar. The pH was adjusted to 5.6 ± 0.02. Media was autoclaved at 121ºC for 20 min before use. Plants were cultured in 20 x 150 mm glass capped tubes containing 10 mL of media. Culture tubes were placed under 8 h/16 h day/night cycle light at 76 µmol m^{-2} s^{-1} photosynthetic photon flux density from cool white fluorescent lamps measured at the top of the culture tubes. The temperature was maintained at 23 ± 2 ºC. Fifty plants per genotype were examined after 20 and 35 days of *in vitro* culture. After 20 days of culture in a suboptimal calcium concentration medium, five shoot tips were taken from each genotype and fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 3 h at 4º C. Afterwards, samples were washed thrice with phosphate buffer, and then incubated with 1% osmium tetroxide in the same buffer for 2 h. Subsequently, three washes with phosphate buffer were performed. Fixed tissues were dehydrated in a graded series of ethanol (35, 50, 70, 96 and 100%), and then infiltrated with a propylene oxide and JB4 resin mixture. After that, they were immersed in JB4 resin overnight at 4º C and finally

transferred to flat embedding molds filled with JB4 resin that polymerized at 68º C for 24 h. Polymerized blocks were sectioned (0.5–0.7 mm thick) with a Leica EM UC6 ultramicrotome (Leica Mikrosysteme, Vienna, Austria). The sections were stained for 5 min at 60º C in 1% (w/v) toluidine blue and rinsed with de-ionized water. Finally, stained sections were observed under light microscopy and digital images were obtained.

Ion content analysis

Concentration of Na^+, K^+ and Ca^{2+} was measured in plant material dried for 48 h at 80°C, milled to powder and digested in a concentrated HNO_3:$HClO_4$ (2:1 v/v) solution. Na^+, K^+ and Ca^{2+} were analysed by inductively coupled plasma spectrometry (ICP) (Ionomic Service of CEBAS-CSIC, Murcia, Spain).

Gene expression analysis

Different vegetable tissues previously frozen in LN_2 and stored at -80ºC were analysed by RT-qPCR. Total RNA was isolated using a RNeasy kit (Qiagen); contaminating DNA was removed with RNAse-free DNase (DNA-free kit, Ambion) and RNA quality was assessed by electrophoresis on a denaturing agarose gel. Total RNA was quantified in a GeneQuant II spectrophotometer (Pharmacia Biotech) and 5 µg were used for cDNA synthesis with the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). RT-qPCR was performed using 1 µl of undiluted cDNA mixed with iQSyBr Green Supermix (BioRad), and 0.45 µM of forward and reverse primers using assay conditions as previously described (Asins et al., 2013). All reactions were performed in triplicate. The presence of a single band on an agarose gel electrophoresis and of a single peak in the melting temperature curve confirmed the specificity of RT-qPCR amplification. Relative expression data were calculated as described by Asins et al. (2013) using the tomato elongation factor 1α (*LeEF1α*, acc. AB061263) as housekeeping gene. The expression level was calculated from $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), using the expression level of each gene from non-treated untransformed tissue as the calibrator sample. Data were statistically analysed using the SPSS 13.0 software package. All data are given as mean ± SE of three biological

replicates of five plants each. Significant differences among means were analysed by Student's *t* and ANOVA tests ($P < 0.05$).

Specifically, it was evaluated the expression level of *SlCBL10* (*Solyc08g065330*), *SlSOS1* (*Solyc01g005020*), *SlSOS2* (*Solyc12g009570*), *SlHKT1;1* (*Solyc07g014690*), *SlHKT1;2* (*Solyc07g014680*), *LeNHX3* (*Solyc01g067710*), *LeNHX4* (*Solyc01g098190*), *SlAVP1* (*Solyc06g068240*), *SlVHA-A1* (*Solyc12g055800*) and *SlTPC1* (*Solyc07g053970*) genes. Regarding tomato CAX homologues, two genes homologous to *Arabidopsis CAX1* and *CAX3*, i.e. *Solyc09g005260* (83.9% and 79.1% similarity to *CAX3* and *CAX1*, respectively) and *Solyc06g006110* (79.7% and 77.1% similarity to *CAX3* and *CAX1*, respectively) were identified. Out of the two CAX genes analysed, only *Solyc06g006110* was responsive to salinity, and therefore it was named as *SlCAX1* and used for further analysis. Sequences of evaluated genes are available in SGN Database (ITAG 2.5; http://solgenomics.net/). All primers used for RT-qPCR are listed in Supplemental Table S2.

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Table S1. Genetic analysis of the T2 progeny of *pms916* mutant.

Supplemental Table S2. Primers used for standard and RT-qPCR analyses.

Supplemental Figure S1. Phenotype of T3 azygous (WT phenotype, left) and homozygous (*Slcbl10* mutant phenotype, right) plants for the *Slcbl10* mutation grown in absence of salt stress.

Supplemental Figure S2. The lack of *SlCBL10* alters K^+ content in tomato plant under salt stress.

Supplemental Figure S3. Na^+ extrusion is increased in *Slcbl10* under salinity.

Supplemental Figure S4. Phenotype of *Slcbl10* tomato mutant plants salt-treated for a long time in a greenhouse.

Supplemental Figure S5. Phenotype of RNAi *SlCBL10* plants salt-treated for a long time in a greenhouse.

664 **Supplemental Figure S6.** Effects of calcium deficiency on shoot apex development.

665

666 **ACKNOWLEDGMENTS:**

667 This study was supported by grants from the Plant KBBE Program (EUI2009-04074), the Spanish Ministerio de
668 Economia y Competitividad (AGL2012-40150 and AGL2015-64991), and Seneca Foundation (18973/JLI/13),
669 as well as the French National Research Agency ENDOREPIGEN project. A.O-A. was supported by a PhD
670 fellowship from the Spanish Ministerio de Economia y Competitividad (BIO2009-11484).

671

672 **FIGURE LEGENDS**

673 **Figure 1.** Molecular cloning of *pms916* salt hypersensitive T-DNA mutant. (A) Phenotype of *pms916* mutant
674 plants grown *in vitro* after 20 days of 100 mM NaCl salt treatment (DST). (B) Southern blot analysis using a
675 chimeric probe which includes the complete coding sequence of the *NEOMICIN PHOSPHOTRANSFERASE II*
676 *(NPTII)* gene fused to 811 pb of the coding sequence of *FALSIFLORA (FA)* gene (used as hybridization positive
677 control). (C) Genomic organization of the *SlCBL10* gene and the two T-DNA copies inserted in a head-to-tail
678 tandem orientation in the *pms916* mutant. The tandem T-DNA insertion resulted in a 1,836 bp deletion between
679 −1,634 and 202 bp in the *SlCBL10* gene. Number 1 indicates the translation start site, and 8,766 indicates the
680 last nucleotide of the coding region. Exons are depicted as black boxes; the lines between boxes are introns. The
681 dotted lines indicate the position where the insertion is located. The grey arrows indicate the primers used for
682 genotyping the T2 population. G-F and G-R: specific genomic forward and reverse primers, respectively, used
683 to amplify the wild-type allele (without T-DNA insertion). G-F and T2-R: specific genomic forward and
684 specific T-DNA border primers, respectively, used to amplify the mutant allele (carrying the T-DNA insertion).
685 (D) Genotyping of T2 individuals. All T2 plants homozygous for the mutant allele (1, 5, 7, 8, 12, 13, and 15)
686 displayed *pms916* mutant phenotype, while T2 plants heterozygous (2, 3, 4, 6, 9, 10, 11, 14, 16, 17, 18, 20, 23,
687 and 24) and homozygous for the Wild-type allele (19, 21, 22, and 25) showed wild-type phenotype.

688

689 **Figure 2.** *SlCBL10* protects the tomato shoot apex and young tissues from salt stress conditions. (A) WT and
690 *Slcbl10* mutant plants grown in a hydroponic system and salt-treated at cotyledon development stage (HSTy
691 assay). Framed in red a *Slcbl10* mutant plant severely damaged by salinity, particularly in the shot apex (arrow).
692 (B) Phenotype of adult WT and *Slcbl10* mutant plants grown in a hydroponic system and salt-treated at the 5th
693 fully developed leaf stage (HSTa assay). Note that shoot apex and young tissues of mutant plants are severely
694 affected by salinity while adult leaves display a similar appearance to WT (pointed with red arrows). (C)
695 Phenotype of RNAi *SlCBL10* and control plants subjected to hydroponic salt treatment (HSTa assay
696 conditions). Note that RNAi *SlCBL10* plant phenocopies the mutant phenotype under saline conditions. (D)
697 Changes in shoot and root fresh weights of WT, *Slcbl10* mutant and RNAi *SlCBL10* plants during salt
698 treatment. Values are the mean ± SE of two independent assays, each with three biological replicates. Asterisks
699 indicate significant differences (Student's *t*-test, $P < 0.05$).

700

701 **Figure 3.** Ectopic *SlCBL10* expression restores salt tolerance in the *Arabidopsis cbl10* mutant. Five-day-old
702 seedlings of WT (Col-0), *cbl10* mutant and three transgenic lines (L3-5) overexpressing *SlCBL10* in an
703 *Arabidopsis cbl10* mutant genetic background were transferred to MS supplemented with 100 mM NaCl. (A)
704 Representative plants 10 days after transfer. (B) Fresh weight per seedling. Black bars, without NaCl treatment;
705 hatched bars 100 mM NaCl treatment. (C) Sodium accumulation in plants of the indicated genotypes after 10
706 days.

707

708 **Figure 4.** Expression pattern of *SlCBL10* gene in WT plants under salt stress conditions. WT plants grown in a
709 hydroponic system and salt-treated at the 5th fully developed leaf stage (HSTa assay). (A) Levels of *SlCBL10*
710 transcripts were quantified by RT-qPCR in apex, young leaf, upper adult leaf (1st fully developed leaf), stem (1st
711 internode) and root of WT plants developed in absence of salt (0 DST) and after 2 days of salt treatment (2

712 DST). (B) Time-course analysis of *SlCBL10* gene expression during 6 days of salt treatment (144 h) was
713 analysed in upper adult leaf. Values are the mean ± SE of two independent assays, each with three biological
714 replicates. Different lowercase letters indicate significant differences determined by ANOVA (P <0.05).

715

716 **Figure 5.** The salt-hypersensitivity phenotype of *Slcbl10* mutant is associated with an altered Na^+ long distance
717 distribution promoted by an impaired capacity to compartmentalize Na^+ into leaf vacuole. WT and *Slcbl10*
718 mutant plants were cultivated in a hydroponic system under salt conditions (HSTa assay). (A) Na^+ content and
719 (B) Na^+/K^+ ratio were analysed in apex, upper adult leaves (1^{st} fully developed leaf), stem (1^{st} internode) and
720 root after 2 and 6 days of salt treatment (DST). (C) Relative expression of key genes involved in long-distance
721 Na^+ distribution (*SlSOS2, SlSOS1* and *SlHKT1;2*) and in Na^+ compartmentalization into the vacuole (S*lNHX3,*
722 *SlNHX4, SlAV1, SlVHA-A1*) was analysed in upper adult leaves at 0 DST and after 2 DST. Values are the mean
723 ± SE of two independent assays, each with three biological replicates. Asterisks indicate significant differences
724 (Student's *t*-test, $P < 0.05$).

725

726 **Figure 6.** *SlCBL10* disruption promoted the retention of Ca^{2+} in leaf and stem under salinity conditions. (A) WT
727 and *Slcbl10* mutant plants grown in a hydroponic system under salt conditions (HSTa assay). Ca^{2+} content was
728 analysed in stem (1^{st} internode) and in the 1^{st} developed leaf prior to salt treatment (0 DST) and after 2 and 6
729 DST. (B) Grafted plants between WT and *Slcbl10* mutant were subjected to hydroponic salt treatment (HSTa
730 assay conditions). Ca^{2+} content was analysed in the 1^{st} developed leaf and in root at 0 DST and 6 DST. (C) Fruit
731 yield, fruit number and BER incidence in WT and mutant plants at 0 DST and after 50 DST. Values are the
732 mean ± SE of three biological replicates of five plants each. Asterisks indicate significant differences between
733 WT and *Slcbl10* mutant (Student's *t*-test, $P < 0.05$). Different lowercase letters indicate significant differences
734 in each tissue (root or leaf) determined by ANOVA ($P < 0.05$).

735

Figure 7. *SlCBL10* disruption alters the influx and efflux of Ca^{2+} in vacuole. WT and *Slcbl10* mutant plants grown in a hydroponic system under salt conditions (HSTa assay). Relative gene expression of *SlCAX1*, *SlTPC1*, *SlAVP1* and *SlVHA-A1* was recorded in upper adult leaf of WT and *Slcbl10* mutant during 6 days of salt stress. Values are the mean ± SE of two independent assays, each with three biological replicates. Asterisks indicate significant differences (Student's *t*-test, $P < 0.05$).

Figure 8. *SlCBL10* gene is involved in maintaining a suitably low Na^+/Ca^{2+} ratio in tomato apex and flower under salinity conditions. WT, *Slcbl10* mutant and RNAi *SlCBL10* plants grown in a hydroponic system and salt-treated at the 5^{th} fully developed leaf stage (HSTa assay). Na^+ (A) and Ca^{2+} contents (B) were analysed in upper adult leaf, apex and flower after 6 days of salt treatment (DST), and then the Na^+/Ca^{2+} ratio was calculated (C). Values are the mean ± SE of two independent assays, each with three biological replicates. Different lowercase letters represent significant differences ($P > 0.05$) calculated by ANOVA. (D) Representative images of WT and *Slcbl10* mutant flowers after 10 DST.

Figure 9. Hypothetical model of the genetic and physiological mechanism proposed to explain the functional role of *SlCBL10* gene in regulating Na+ and Ca2+ homeostasis under salt stress conditions. On the right side, it is indicated the activity of Na+ antiporters (SOS1, NHK and KKT), Ca2+ antiporter (CAX), the vacuolar pumps (AVP1 and V-ATPase) and the cation vacuolar channel TPC1, all of them involved in maintaining Na+/Ca+2+ balance through the vacuolar transport. On the left side, the mechanism would be impaired due to the lack of SlCBL10 (see Discussion section for details). Red and green colours mean down-regulated and up-regulated genes under salt stress, respectively, while grey colour represents absence of gene expression changes in salinity conditions. Different lightness of red and green colours indicate different levels of gene expression in *Slcbl10* mutant (left) respect to WT plants (right) grown in salt conditions, where darker hues represent higher induction

759 (green) or inhibition (red). The names of the proteins correspond to those of *Arabidopsis thaliana*, although
760 results provided in this work indicate that, in general terms, this mechanism could be conserved in tomato.

761

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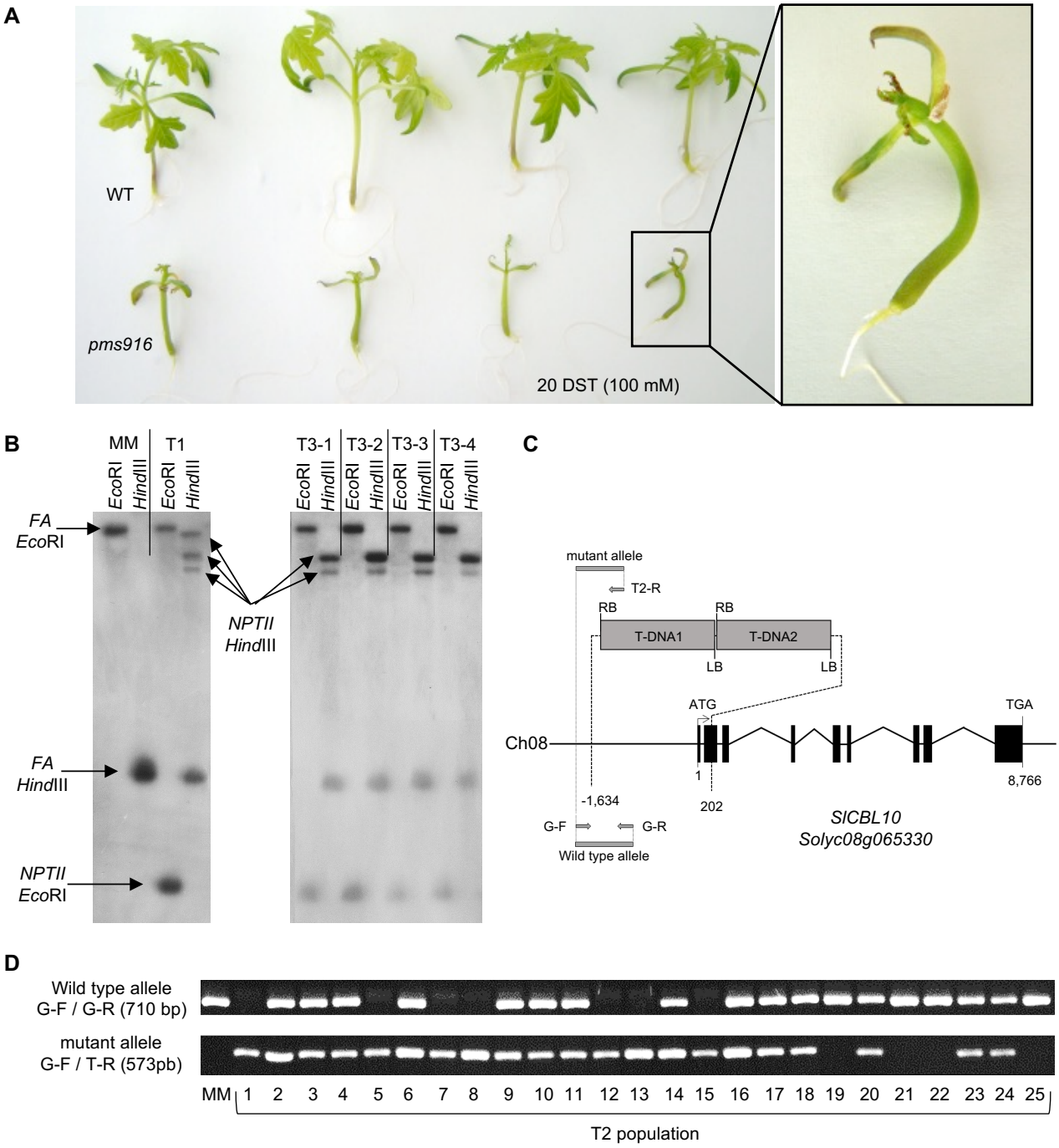

Figure 1. Molecular cloning of *pms916* salt hypersensitive T-DNA mutant. (A) Phenotype of *pms916* mutant plants grown *in vitro* after 20 days of 100 mM NaCl salt treatment (DST). (B) Southern blot analysis using a chimeric probe which includes the complete coding sequence of the *NEOMICIN PHOSPHOTRANSFERASE II (NPTII)* gene fused to 811 pb of the coding sequence of *FALSIFLORA (FA)* gene (used as hybridization positive control). (C) Genomic organization of the *SlCBL10* gene and the two T-DNA copies inserted in a head-to-tail tandem orientation in the *pms916* mutant. The tandem T-DNA insertion resulted in a 1,836 bp deletion between −1,634 and 202 bp in the *SlCBL10* gene. Number 1 indicates the translation start site, and 8,766 indicates the last nucleotide of the coding region. Exons are depicted as black boxes; the lines between boxes are introns. The dotted lines indicate the position where the insertion is located. The grey arrows indicate the primers used for genotyping the T2 population. G-F and G-R: specific genomic forward and reverse primers, respectively, used to amplify the wild-type allele (without T-DNA insertion). G-F and T2-R: specific genomic forward and specific T-DNA border primers, respectively, used to amplify the mutant allele (carrying the T-DNA insertion). (D) Genotyping of T2 individuals. All T2 plants homozygous for the mutant allele (1, 5, 7, 8, 12, 13, and 15) displayed *pms916* mutant phenotype, while T2 plants heterozygous (2, 3, 4, 6, 9, 10, 11, 14, 16, 17, 18, 20, 23, and 24) and homozygous for the Wild-type allele (19, 21, 22, and 25) showed wild-type phenotype.

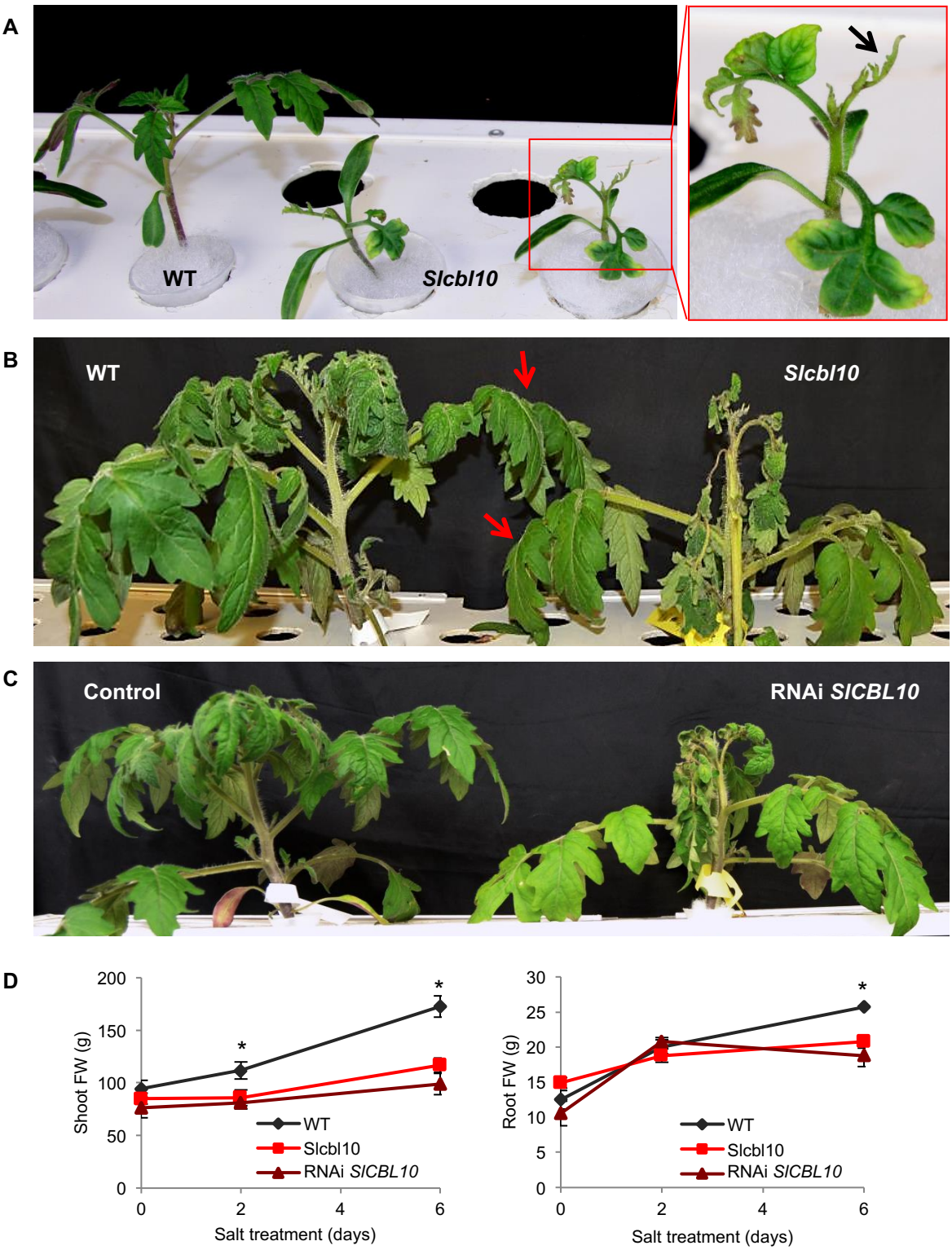

Figure 2. *SlCBL10* protects the tomato shoot apex and young tissues from salt stress conditions. (A) WT and *Slcbl10* mutant plants grown in a hydroponic system and salt-treated at cotyledon development stage (HSTy assay). Framed in red a *Slcbl10* mutant plant severely damaged by salinity, particularly in the shot apex (arrow). (B) Phenotype of adult WT and *Slcbl10* mutant plants grown in a hydroponic system and salt-treated at the 5th fully developed leaf stage (HSTa assay). Note that shoot apex and young tissues of mutant plants are severely affected by salinity while adult leaves display a similar appearance to WT (pointed with red arrows). (C) Phenotype of RNAi *SlCBL10* and control plants subjected to hydroponic salt treatment (HSTa assay conditions). Note that RNAi *SlCBL10* plant phenocopies the mutant phenotype under saline conditions. (D) Changes in shoot and root fresh weights of WT, *Slcbl10* mutant and RNAi *SlCBL10* plants during salt treatment. Values are the mean ± SE of two independent assays, each with three biological replicates. Asterisks indicate significant differences (Student's *t*-test, $P < 0.05$).

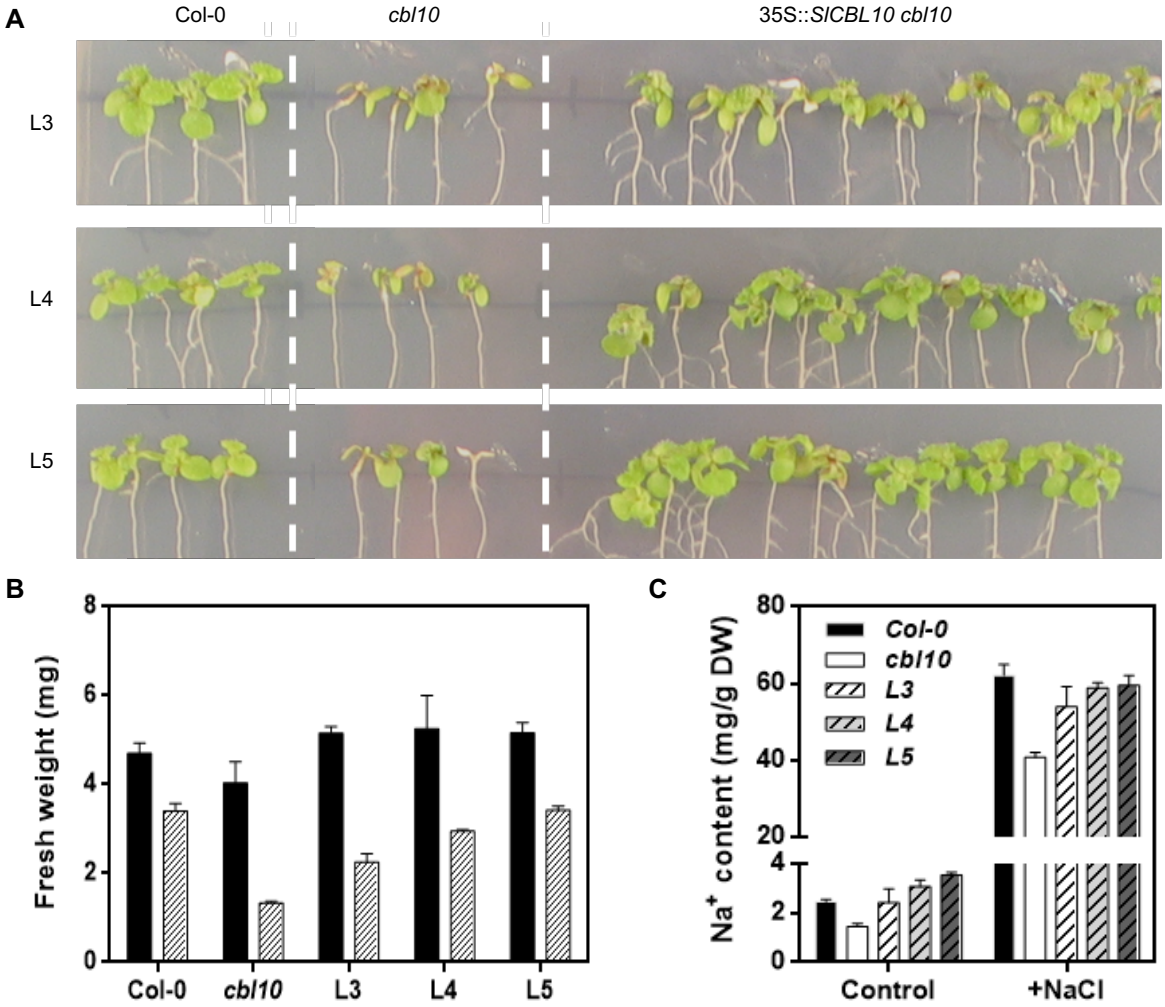

Figure 3. Ectopic *SlCBL10* expression restores salt tolerance in the *Arabidopsis cbl10* mutant. Five-day-old seedlings of WT (Col-0), *cbl10* mutant and three transgenic lines (L3-5) overexpressing *SlCBL10* in an *Arabidopsis cbl10* mutant genetic background were transferred to MS supplemented with 100 mM NaCl. (A) Representative plants 10 days after transfer. (B) Fresh weight per seedling. Black bars, without NaCl treatment; hatched bars 100 mM NaCl treatment. (C) Sodium accumulation in plants of the indicated genotypes after 10 days.

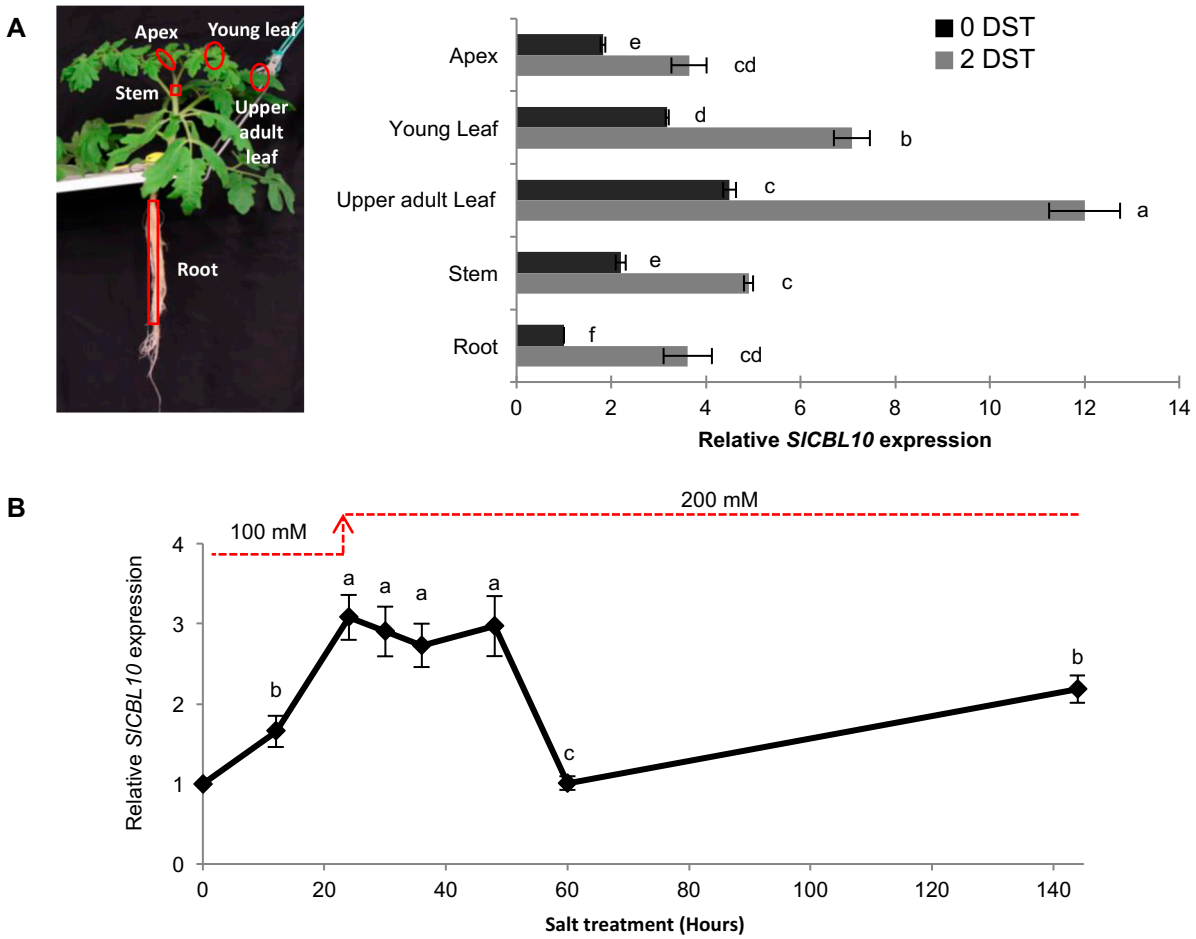

Figure 4. Expression pattern of *SlCBL10* gene in WT plants under salt stress conditions. WT plants grown in a hydroponic system and salt-treated at the 5th fully developed leaf stage (HSTa assay). (A) Levels of *SlCBL10* transcripts were quantified by RT-qPCR in apex, young leaf, upper adult leaf (1st fully developed leaf), stem (1st internode) and root of WT plants developed in absence of salt (0 DST) and after 2 days of salt treatment (2 DST). (B) Time-course analysis of *SlCBL10* gene expression during 6 days of salt treatment (144 h) was analysed in upper adult leaf. Values are the mean ± SE of two independent assays, each with three biological replicates. Different lowercase letters indicate significant differences determined by ANOVA (P <0.05).

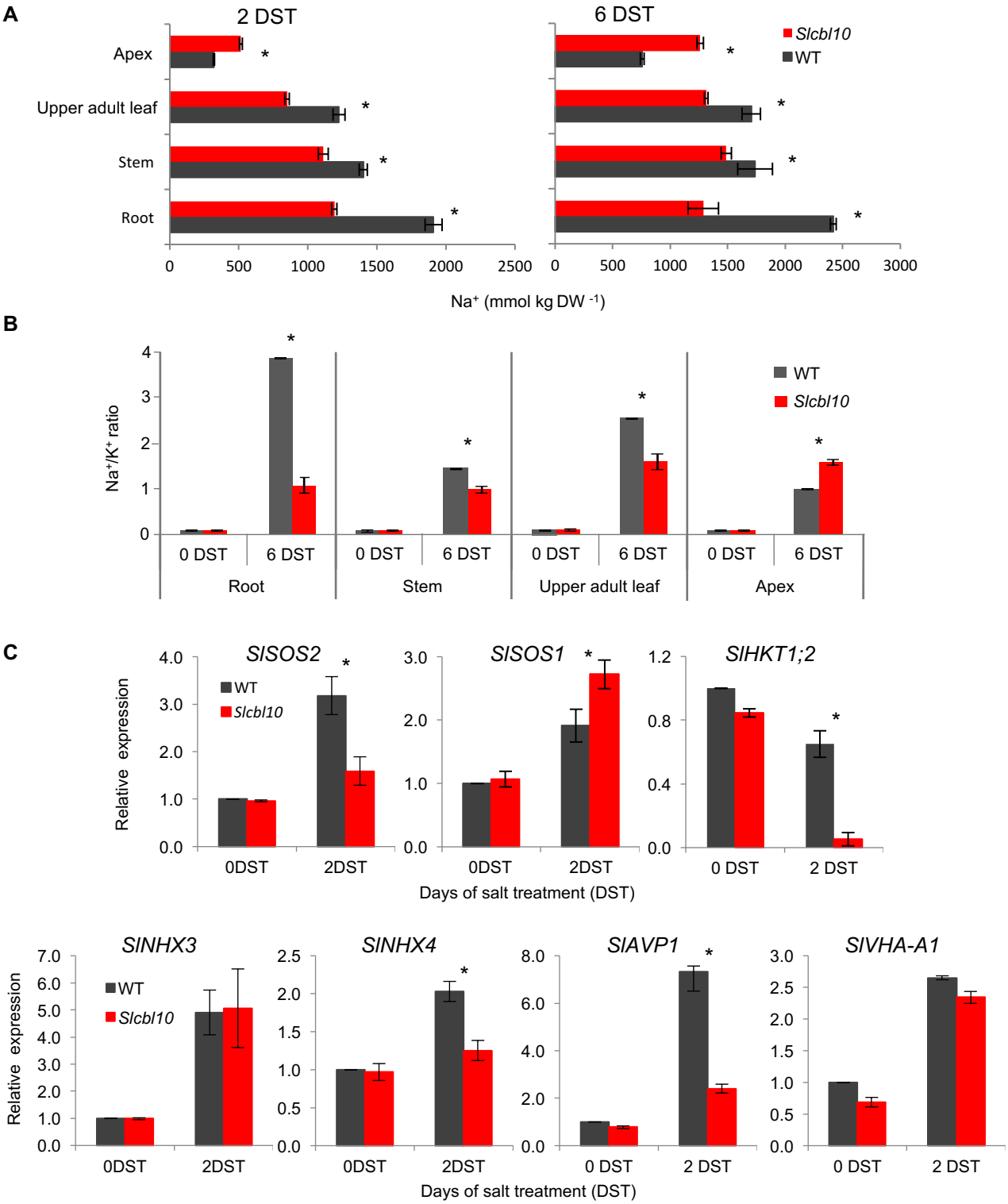

Figure 5. The salt-hypersensitivity phenotype of *Slcbl10* mutant is associated with an altered Na^+ long distance distribution promoted by an impaired capacity to compartmentalize Na^+ into leaf vacuole. WT and *Slcbl10* mutant plants were cultivated in a hydroponic system under salt conditions (HSTa assay). (A) Na^+ content and (B) Na^+/K^+ ratio were analysed in apex, upper adult leaves (1st fully developed leaf), stem (1st internode) and root after 2 and 6 days of salt treatment (DST). (C) Relative expression of key genes involved in long-distance Na^+ distribution (*SlSOS2, SlSOS1* and *SlHKT1;2*) and in Na^+ compartmentalization into the vacuole (*SlNHX3, SlNHX4, SlAV1, SlVHA-A1*) was analysed in upper adult leaves at 0 DST and after 2 DST. Values are the mean ± SE of two independent assays, each with three biological replicates. Asterisks indicate significant differences (Student's *t*-test, $P < 0.05$).

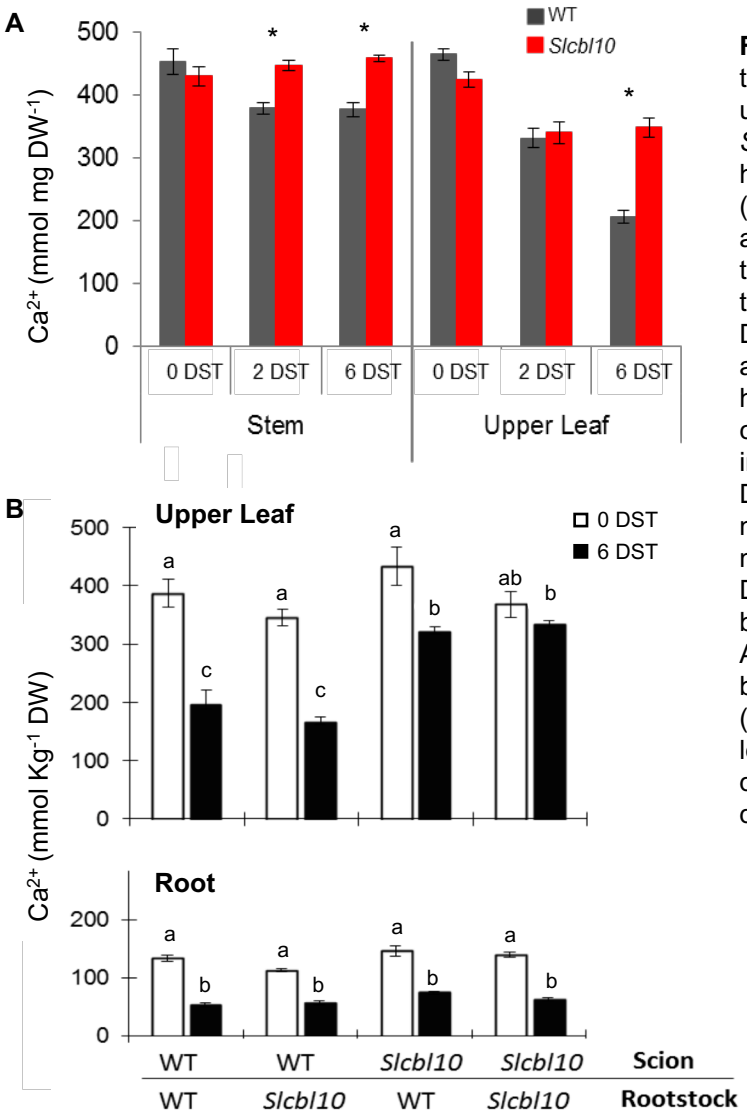

Figure 6. *SlCBL10* disruption promoted the retention of Ca^{2+} in leaf and stem under salinity conditions. (A) WT and *Slcbl10* mutant plants grown in a hydroponic system under salt conditions (HSTa assay). Ca^{2+} content was analysed in stem (1st internode) and in the 1st developed leaf prior to salt treatment (0 DST) and after 2 and 6 DST. (B) Grafted plants between WT and *Slcbl10* mutant were subjected to hydroponic salt treatment (HSTa assay conditions). Ca^{2+} content was analysed in the 1st developed leaf and in root at 0 DST and 6 DST. (C) Fruit yield, fruit number and BER incidence in WT and mutant plants at 0 DST and after 50 DST. Values are the mean ± SE of three biological replicates of five plants each. Asterisks indicate significant differences between WT and *Slcbl10* mutant (Student's *t*-test, $P < 0.05$). Different lowercase letters indicate significant differences in each tissue (root or leaf) determined by ANOVA ($P < 0.05$).

C

		Fruit yield (g)	Fruit number	BER (%)
0 DST	WT	1057.0 ±132.4	43.4 ± 3.3	1.1 ± 0.3
	Slcbl10	399.1 ± 103.5 *	26.2 ± 4.1 *	10.2 ± 0.2 *
50 DST	WT	376.6 ± 55.2	40.2 ± 5.2	3.0 ± 0.4
	Slcbl10	9.9 ± 4.0 *	6.4 ± 1.8 *	70.1 ± 3.3 *

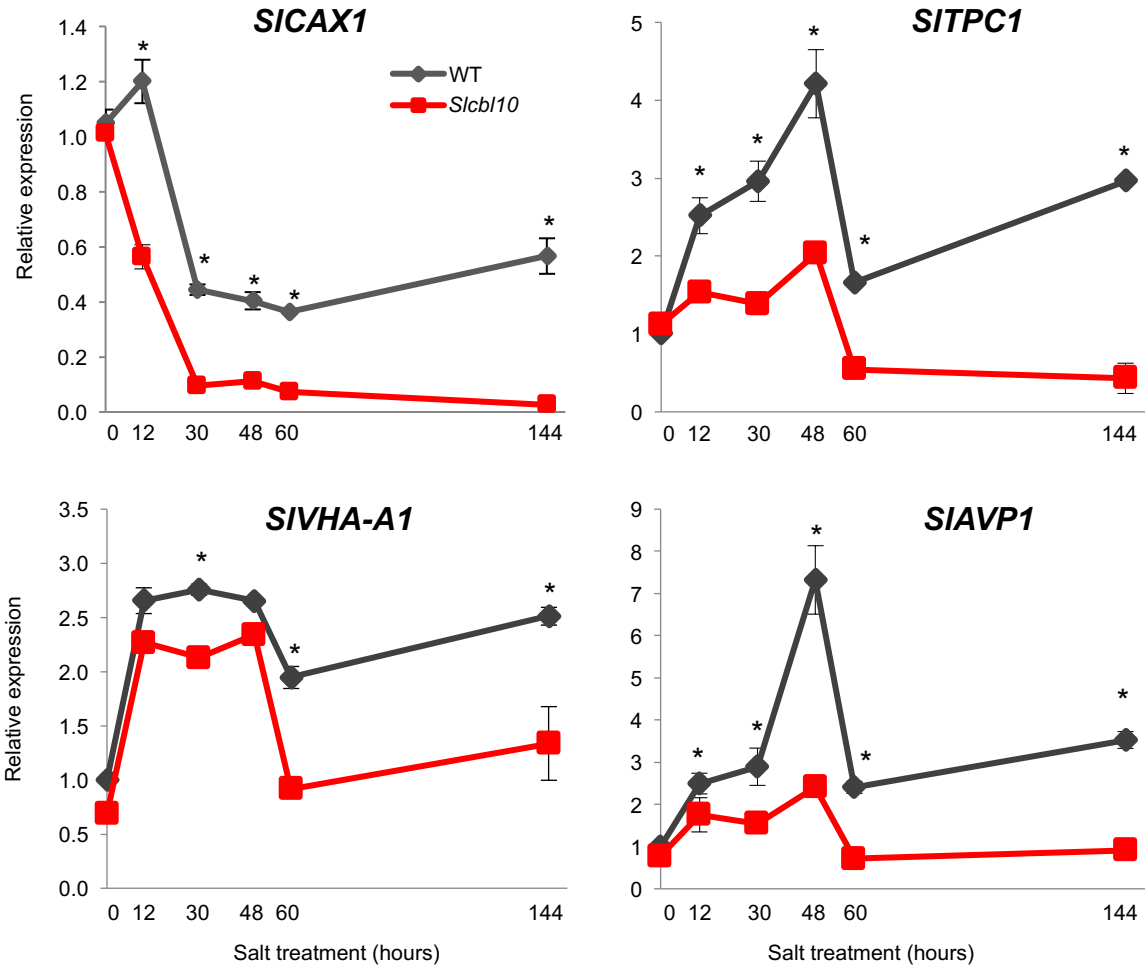

Figure 7. *SlCBL10* disruption alters the influx and efflux of Ca^{2+} in vacuole. WT and *Slcbl10* mutant plants grown in a hydroponic system under salt conditions (HSTa assay). Relative gene expression of *SlCAX1, SlTPC1, SlAVP1* and *SlVHA-A1* was recorded in upper adult leaf of WT and *Slcbl10* mutant during 6 days of salt stress. Values are the mean ± SE of two independent assays, each with three biological replicates. Asterisks indicate significant differences (Student's *t*-test, $P < 0.05$).

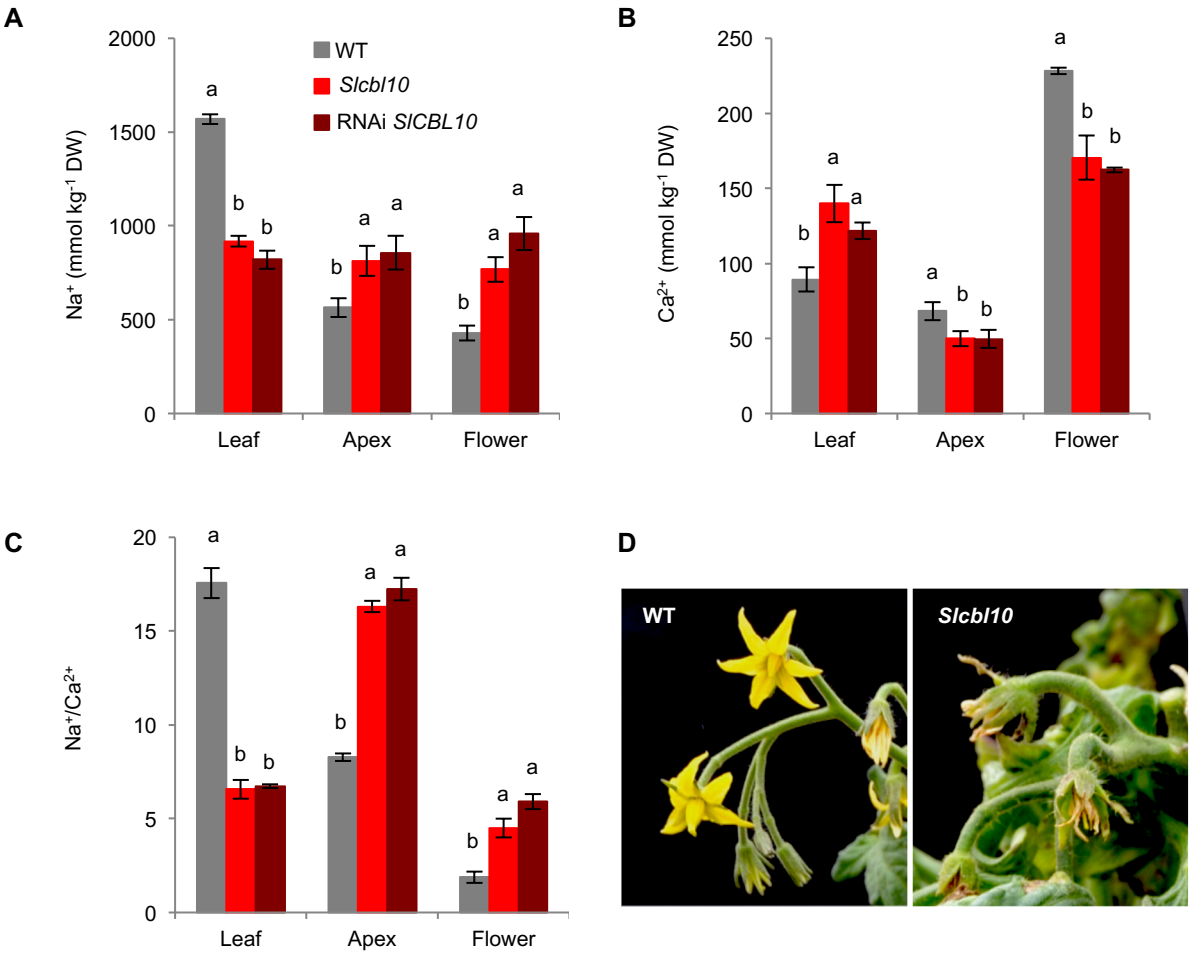

Figure 8. *SlCBL10* gene is involved in maintaining a suitably low Na^+/Ca^{2+} ratio in tomato apex and flower under salinity conditions. WT, *Slcbl10* mutant and RNAi *SlCBL10* plants grown in a hydroponic system and salt-treated at the 5th fully developed leaf stage (HSTa assay). Na^+ (A) and Ca^{2+} contents (B) were analysed in upper adult leaf, apex and flower after 6 days of salt treatment (DST), and then the Na^+/Ca^{2+} ratio was calculated (C). Values are the mean ± SE of two independent assays, each with three biological replicates. Different lowercase letters represent significant differences (P > 0.05) calculated by ANOVA. (D) Representative images of WT and *Slcbl10* mutant flowers after 10 DST.

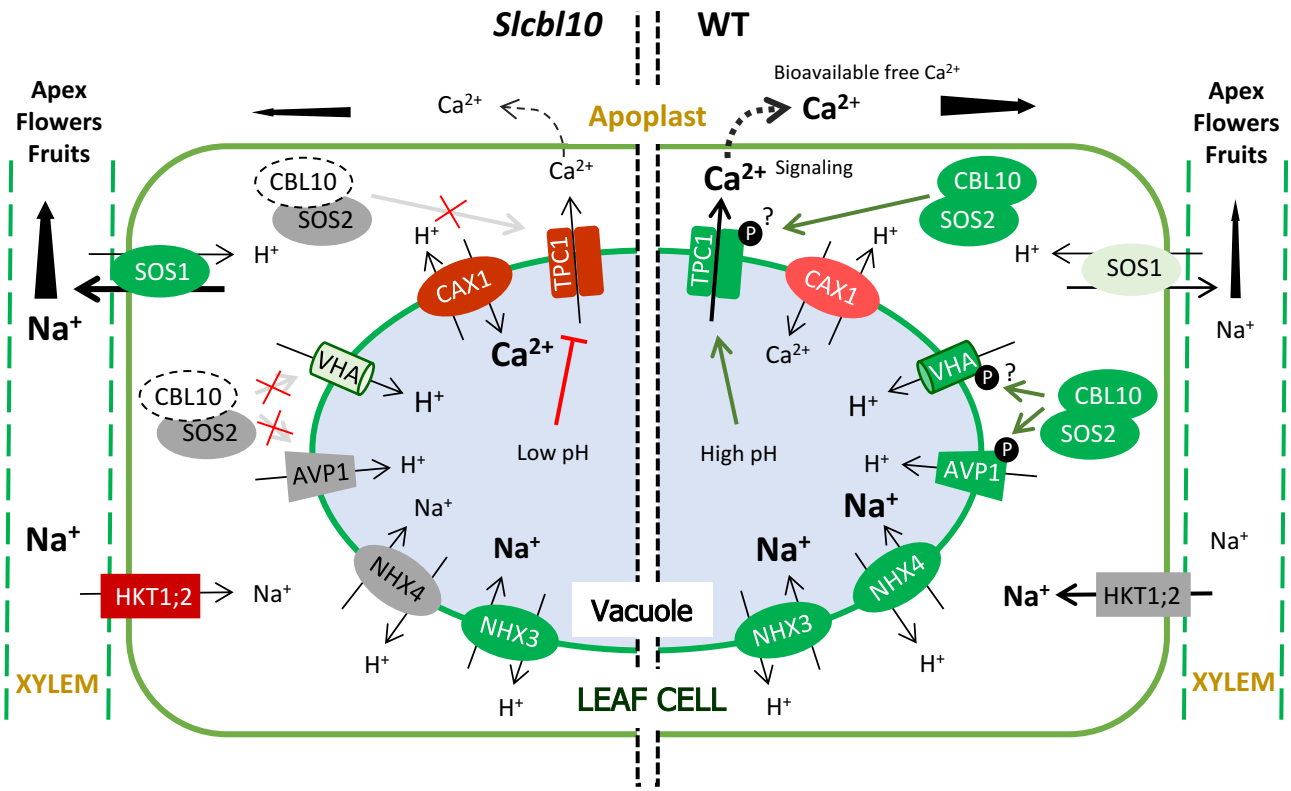

Figure 9. Hypothetical model of the genetic and physiological mechanism proposed to explain the functional role of *SlCBL10* gene in regulating Na+ and Ca2+ homeostasis under salt stress conditions. On the right side, it is indicated the activity of Na+ antiporters (SOS1, NHK and KKT), Ca2+ antiporter (CAX), the vacuolar pumps (AVP1 and V-ATPase) and the cation vacuolar channel TPC1, all of them involved in maintaining Na+/Ca+2+ balance through the vacuolar transport. On the left side, the mechanism would be impaired due to the lack of SlCBL10 (see Discussion section for details). Red and green colours mean down-regulated and up-regulated genes under salt stress, respectively, while grey colour represents absence of gene expression changes in salinity conditions. Different lightness of red and green colours indicate different levels of gene expression in *Slcbl10* mutant (left) respect to WT plants (right) grown in salt conditions, where darker hues represent higher induction (green) or inhibition (red). The names of the proteins correspond to those of *Arabidopsis thaliana*, although results provided in this work indicate that, in general terms, this mechanism could be conserved in tomato.

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