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The tomato mutant *ars1* (*altered response to salt stress 1*) identifies an R1-type MYB transcription factor involved in stomatal closure under salt acclimation

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SUMMARY

A screening under salt stress conditions of a T-DNA mutant collection of tomato (*Solanum lycopersicum* L.) led to the identification of the *altered response to salt stress 1* (*ars1*) mutant, which showed a salt-sensitive phenotype. Genetic analysis of the *ars1* mutation revealed that a single T-DNA insertion in the *ARS1* gene was responsible of the mutant phenotype. *ARS1* coded for an R1-MYB type transcription factor and its expression was induced by salinity in leaves. The mutant reduced fruit yield under salt acclimation while in the absence of stress the disruption of *ARS1* did not affect this agronomic trait. The stomatal behaviour of *ars1* mutant leaves induced higher Na^+ accumulation via the transpiration stream, as the decreases of stomatal conductance and transpiration rate induced by salt stress were markedly lower in the mutant plants. Moreover, the mutation affected stomatal closure in a response mediated by ABA. The characterization of tomato transgenic lines silencing and overexpressing *ARS1* corroborate the role of the gene in regulating the water loss via transpiration under salinity. Together, our results show that *ARS1* tomato gene contributes to reduce transpirational water loss under salt stress. Finally, this gene could be interesting for tomato molecular breeding, since its manipulation could lead to improved stress tolerance without yield penalty under optimal culture conditions.

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INTRODUCTION

Abiotic stress, especially salinity and drought, is responsible for reduced crop growth and the cause of important economic losses in agricultural production. Therefore, in worldwide agriculture the development of new crop varieties able to maintain yield production while facing abiotic stresses is a critical issue. Tomato is one of the most important succulent fruit bearing species in agriculture but additionally it has become a model species in plant research (Bergougnoux, 2014; Ichihashi and Sinha, 2014; Ranjan *et al.,* 2012; Schwarz *et al.,* 2014). Despite the economic relevance of tomato, the mechanisms that govern responses to abiotic stresses in this horticultural species are not well characterized, and only a very small number of genes playing key roles in tomato tolerance to salinity and drought have so far been identified (Asins *et al.*, 2013; Atarés *et al.*, 2011; Pineda *et al.*, 2012). Salinity causes not only ion imbalance but also water deficiency, similarly to drought stress does and both effects may persist during longer periods of salt stress (Munns and Tester, 2008; Muñoz-Mayor *et al.,* 2012). In relation to the Na^+ toxic component of salt stress, the plant salt tolerance is mainly due to its ability to regulate the Na^+ transport rate from root to the shoot over time, as the time-dependent regulation of the rate of Na^+ transport to the shoot appears to be critical for plant salinity tolerance (Maathuis, 2014; Shabala, 2013).

During the plant response and acclimation to abiotic stress, important changes in biochemistry and physiology take place and many genes are activated, leading to accumulation of numerous proteins involved in abiotic stress tolerance. In recent years, the use of transcription factors in the genetic engineering of crop plants has emerged as a powerful approach to enhance tolerance against abiotic stresses (Lindemose *et al.,* 2013; Nakashima *et al.,* 2014). Among these transcription factors, members of the

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MYB family have been characterized for their regulatory role in the plant response to abiotic stress, particularly in *Arabidopsis* and rice, although MYB proteins from other plants have also been demonstrated to be involved in abiotic stress response regulation (Chen et *al.*, 2014; Dubos et *al.*, 2010; Li *et al.,* 2015). Plant MYB proteins can be classified into three major subfamilies according to the number of imperfect repeats (50-53 amino acids), also called SANT domains, which are the DNA-binding domains, present in the sequence; the R1-MYB-related group (one single SANT domain), the R2R3-type group (two SANT domains), the R1R2R3-MYB group (three SANT domains), and a minor subfamily of MYB genes carrying four SANT domains (Chen *et al.,* 2006; Dubos *et al.,* 2010). Most of the MYB family genes involved in response to diverse abiotic stress belong to the R2R3-type group (Du *et al.*, 2012; Feller *et al.*, 2011). Thus, several studies reveal that different MYB genes with two SANT domains play a positive role in plant tolerance to abiotic stress (He *et al.*, 2012; Jung *et al.*, 2008; Liang *et al.*, 2005; Lippold *et al.*, 2009; Yang *et al.*, 2012; Zhang *et al.*, 2014). In contrast to R2R3-MYB, few studies on the functional roles played by the other MYB-related genes in abiotic stress response have been reported (Chen *et al.*, 2014).

Insertional mutagenesis is an effective genomics tool that allows the identification and functional analysis of genes implicated in different biological processes. The mutated gene remains tagged by the inserted element (transposon or T-DNA), which greatly facilitates its genomic localization and posterior cloning. Insertional mutant collections have proven to be highly efficient molecular tools for both reverse and forward genetic studies in plant species like rice, *Arabidopsis thaliana*, *Medicago truncatula* and potato (Duangpan *et al.*, 2013; Jeong *et al.*, 2002; O'Malley and Ecker, 2010; Tadege *et al.*, 2008; Ülker *et al.*, 2008). In tomato the analysis of an insertional

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collection of Micro-Tom mutants by applying the transcriptional activation strategy for gene tagging has led to the identification and cloning of an R2R3-MYB transcription factor, *ANT1*, involved in the biosynthesis and transport of anthocyanins (Mathews *et al.,* 2003). We have generated a collection of tomato T-DNA mutants from a commercial cultivar (cv. Moneymaker), which have been screened for salt tolerance (Pineda *et al.,* 2012). Within this collection, we identified the dominant *ars1* knock-out mutant, which contains a single T-DNA insertion tagging a MYB transcription factor belonging to the R1-MYB type. Here, we show that the *ars1* mutant has a normal growth pattern under control conditions, but it is salt-sensitive in the long-term on the basis of fruit yield. The high Na^+ accumulation in mutant leaves over time was related to lower reduction of stomatal conductance and transpiration rate under salt acclimation. Phenotype and physiological characterization of transgenic tomato lines either silencing or overexpressing *ARS1* proved that this gene is involved in the regulation of stomatal closure under salt stress.

RESULTS

Molecular and genetic characterization of the *ars1* tomato mutant

A screening for salt stress tolerance (200 mM NaCl for 20 days) has been performed in the tomato T-DNA mutant collection generated in the cv. Moneymaker (Pineda *et al.*, 2012). As result, a mutant showing higher salt-sensitivity than wild-type (WT) was identified in the first mutant generation (T_1), where tomato plants with altered phenotype showed higher degree of leaf rolling, loss of chlorophyll and even necrosis (Figure 1a). We named this mutant *altered response to salt stress 1* (*ars1*). Although similar plant fresh weights (FW) were found in WT and *ars1* T_1 plants without salt

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(around 440 g per plant), however FW experienced a higher reduction by salt stress in mutant than WT (235 ± 21 and 298 ± 14g per plant, respectively). The higher FW reduction in the mutant plant was associated to higher leaf Na^+ accumulation (1854 ± 180 and 1386 ± 131 mmol kg^{-1} DW in mutant and WT, respectively), as well higher leaf chlorosis, as observed in the chlorophyll measurements (32.1 ± 4.4 and 45.5 ± 5.2 SPAD units in mutant and WT, respectively).

Southern blot analysis showed that a single copy of the T-DNA was present in the genome of the T_1 mutant (Figure 1b). Flanking sequences of the T-DNA insertion were cloned by anchor-PCR and their sequences compared with the tomato genome sequence currently available in the SOL genomic database (http://www.solgenomics.net). Results revealed that the T-DNA is localized in chromosome one, and inserted into the second intron of a gene coding for a MYB-like transcription factor (Solyc01g095030.2), 2687 bp downstream from the predicted translation start site (Figure 1c). In addition, a 102 bp fragment including the 3'-end of intron 2 and the 5'-end of exon 3 were deleted during the T-DNA-mediated mutagenesis. As a consequence, T-DNA insertion changed the reading frame of the tagged gene, which in turn led to the translation of three new amino acid residues (VVC) and a premature stop codon before the SHAQKYF domain. The transcript sequence obtained by PCR amplification of cDNA from *ars1* mutant tissues confirmed the correct processing of intron 1 but not of intron 2, the latter carrying the mutation leading the premature stop codon. The truncated transcript would generate a non-functional protein in agreement with the dominant-negative nature of the *ars1* mutation. The tagged gene was 7781 bp-long and consisted of eight exons transcribed in a 1545 bp mRNA containing a 996 bp open reading frame. The *ARS1* gene encodes for a MYB-related protein of 331 amino acids, which contains a single

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MYB-like DNA binding domain (66 to 116 residues) with high similarity to SHAQKYF (pfam00249) (Baranowskij *et al.*, 1994; Rose *et al.*, 1999) or SANT (smart00717) class domains. The analysis of *ARS1* genomic sequence through the Tagger Prediction utility of the Tomato Functional Genomics Database (www.ted.bti.cornell.edu) revealed the presence of a putative ABA-responsive element located in the 5'UTR region of the gene (Figure 1c).

Comparative analysis of ARS1 encoded protein with protein databases showed the highest similarity to other plant SHAQKYF/MYB-like domain-containing proteins, particularly to those from members of the Solanaceae family such as *Solanum tuberosum* (97% similarity) (Shin *et al.*, 2011). Phylogenetic analysis of ARS1 indicated that the most similar protein to ARS1 was AT5G52660 (54% of sequence identity and 64% of sequence similarity) (Figure 2a). This protein has been classified within the CCA1-like subfamily of MYB-related proteins, which is the most abundant MYB-related subfamily in *Arabidopsis* (Yanhui *et al.*, 2006). Apart from *Arabidopsis* MYB proteins, ARS1 protein showed the highest homology with the rice OsI_08476 MYB protein, and both formed a specific clade together AT5G52660 (Figure 2a). The high homology in the conserved domains of the CCA1-like proteins, including tomato ARS1 and rice OsI_08476, has been observed after multiple alignment of the MYB-like and adjacent P-rich domains characteristic of proteins belonging to this phylogenetic clade (55-91% of identities and 67-98% similarities) (Figure 2b).

Genetic analysis performed in 14 plants of the T_2 segregating progeny confirmed the dominant inheritance of the *ars1* mutation, with a phenotypic segregation resulting in 11 individuals with *ars1* phenotype and 3 with WT phenotype yielding a $\chi^2 = 0.10$ ($P<0.001$). Phenotype segregation correlated with the genotype of T_2 plants since all

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wild-type phenotype plants lacked the *ars1* mutant allele (azygous), while 3 of the mutant plants were homozygous and 8 hemizygous for the *ars1* mutation (Figure S1a). The homozygous mutant plants showed higher rolling in their leaves after 12 days of 200 mM NaCl treatment and higher chlorosis after 20 days of treatment (Figure S1b). Moreover, Na^+ accumulation in leaves also co-segregated with the genotype of T_2 plants grown under salt stress (Figure S1c). All together, these results proved that the gene interrupted by the T-DNA was responsible for the mutant phenotype.

Subsequently, homozygous T_3 mutant plants were generated by selfing and used together with wild-type plants for characterization of the *ars1* mutant. Firstly, the spatial expression pattern of the *ARS1* gene was analyzed by RT-qPCR in vegetative and reproductive tissues of WT and *ars1* plants grown without and with salt-stress. The complete transcript of *ARS1* gene was not expressed in any tissue of mutant plants indicating that *ars1* is a knock-out mutation, as expected from the alterations in the ARS1 protein sequence caused by the T-DNA insertion (Figure 1c**)**. In WT plants *ARS1* is expressed in all analyzed organs of plants grown without salt, with the highest level of transcripts in flowers. Under salt stress, *ARS1* gene is highly induced in leaves but not in the remaining organs here analyzed (Figure S1d).

Phenotype and physiological responses of *ars1* mutant when salt stress is applied at long-term

Wild type and *ars1* mutant plants were grown in greenhouse without and with salt stress (100 mM NaCl) applied at the 10^{th}-leaf stage. Without stress, no morphological nor developmental alterations were observed in *ars1* mutant adult plants (Figure 3a), which was reflected in similar fruit yield between WT and mutant plants (Figure 3b).

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However, salt stress induced chlorosis, necrosis and senescence in leaves of mutant plants after 30 and, especially, 60 days of salt treatment (DST), which was not observed in WT plants (Figure 3a), as well as higher fruit yield reduction (Figure 3b), which was mainly due to the fruit number (18.1 ± 2.2 and 10.2 ± 1.5 in WT and mutant, respectively).

At physiological level, changes in leaf Na^+ concentration and leaf stomatal conductance (g_s) were measured to long-term. The Na^+ accumulation was much higher in *ars1* mutant than in WT leaves after 30 and, specially, 50 DST (Figure 3c**)**. After 30 DST, measurements of g_s were taken at dawn (between 6 and 7 h am) and after 2 h of light, since g_s varies over diurnal cycles and stomata tend to be closed at night and open during the day (Figure 3d). In both conditions, without and with salt, WT and mutant plants had similar leaf g_s at dawn, indicating that mutant closes the stomata to the same extent as WT in response to darkness. However, after 2 h of light, the g_s value was two-fold higher in the mutant than WT under salt stress, which was not observed in unstressed conditions. The differences in leaf g_s between WT and mutant plants were maintained after 50 DST, as shown in the evolution of g_s between 2 and 5 h of light (Figure 3e). Since the stomatal conductance is dependent on the number of stomatal pores, stomatal density was analyzed at this time in the adaxial surface of leaves (Table S2). Similar values were found in leaves of WT and *ars1* plants, which indicates that the increased g_s found in the mutant under salt stress is not due to differences in the number of stomata between mutant and WT.

The high leaf Na^+ accumulation in *ars1* mutant is related to reduced stomatal closure under salt stress

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It is still not clear whether the salt-sensitive phenotype of *ars1* mutant is attributable to ionic stress reflected by Na^+ toxicity or rather the mutant plants have higher root to shoot transport as a result of increased stomatal conductance under transpiring conditions. Indeed, when WT and mutant plants were grown hydroponically at high stress level (200 mM NaCl), both leaf g_s and transpiration rate (E) were significantly higher in the *ars1* mutant than WT after just 1 DST (Figure 4a). Given these results, the stomatal aperture and the number of open stomata were measured in detached leaves of *ars1* and WT plants grown without (control) and with NaCl for 3 and 7 DST (Figure 4b). Similar values in the stomatal aperture were found in WT (11.48 ± 0.10 µm) and *ars1* mutant plants grown without NaCl (11.76 ± 0.40 µm), while the *ars1* plants exhibited a lower reduction in stomatal aperture than WT after 3 DST, and this difference was maintained after 7 DST. Moreover, the percentage of open stomata was 3-4 times higher in the *ars1* mutant compared with WT after 3 and 7 DST, which corroborates that the increased salt-sensitivity of the mutant is associated to the low ability to close stomata in response to salt stress, as can be observed in the micrographs (Figure 4b). Since Na^+ is translocated from root to shoot through the transpiration stream, the lower degree of reduction in stomatal aperture of the mutant under salt stress should be inducing higher Na^+ accumulation in the shoot of the mutant, such as was observed when Na^+ partitioning was analyzed after 10 days of 200 mM NaCl treatment (Figure 4c). Interestingly, the higher Na^+ accumulation along the stem was associated to higher Na^+ accumulation in the mid and upper leaves, with the highest differences between WT and *ars1* being detected in the upper leaves (Figure 4c**),** a physiological trait related to salt sensitivity. Since leaf K^+ concentrations were quite similar in WT

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and *ars1* mutant (Figure S2a), the mutant plants showed a clear tendency to increase the leaf Na^+/K^+ ratio in the middle and upper leaves, with respect to WT (Figure S2b).

If the higher Na^+ transport to the shoot in the mutant is due to higher transpiration, the *ars1* mutant phenotype should be different when grown under non-transpiring conditions (*in vitro*). WT and mutant seedlings were grown *in vitro* with NaCl (100 mM) and LiCl (10 mM) in order to test the ionic toxicity component as well as with mannitol (200 mM) to test the osmotic component. It was evident that the mutant did not show phenotypic differences with WT in any of the conditions tested (Figure S3).

Stomatal closure of *ars1* mutant is also altered under dehydration and ABA treatments

Next, we studied whether the disruption of *ASR1* also altered the transpiration under drought conditions by subjecting WT and *ars1* mutant plants to dehydration by stopping irrigation. The response of mutant plants under non-stressful conditions was similar to WT regarding the values of g_s and E measured during four consecutive days, with mean values around 235 and 2.4 mmol m^{-2} s^{-1} for g_s and E, respectively. Under dehydration, *ars1* mutant plants showed higher values of g_s and E than WT from the first dehydration day, and the differences were maintained after 4 days of dehydration, in spite of the low values achieved in both parameters at this time (Figure 5a). Moreover, a water loss assay by using detached leaves showed that *ars1* mutant lost water significantly faster than the WT from the first 30 min, and continued losing water at a higher rate for 8 h (Figure 5b).

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Since ABA is a key regulator of stomatal closure, we investigated whether the *ars1* mutation affected the degree of stomatal closure in response to ABA. Results showed differences in the stomatal closure degree when treating detached leaves with different ABA concentrations under light conditions (Figure 5c). Thus, from 10 µM of ABA onwards, the reductions in the stomatal aperture were significantly lower in *ars1* than in WT leaves. These results indicate that *ARS1* gene regulates stomatal closure only under stress conditions and its role appears to be dependent on ABA signaling.

Characterization of tomato transgenic plants either silencing or overexpressing *ARS1* gene

Taking into account that *ARS1* gene is expressed in different plant organs but it is only induced by salt stress in leaves, it would be very interesting to corroborate the role of *ARS1* gene in the transpirational water loss by analyzing the salt stress response of lines with different levels of overexpression (OE lines) as well as lines that silence *ARS1* gene by an RNA interference strategy (RNAi lines), which should show a similar response to that found in the *ars1* mutant. At least 10 independent events of transformation were obtained for RNAi and OE lines. In a first assay carried out with T_1 plants, two RNAi lines, RNAi-L2 and RNAi-L3, were selected by their reduced level of *ARS1* expression (0.12 and 0.24 x-fold, respectively, relative to WT); moreover, two lines with different levels of overexpression were also selected, OE-L2 and OE-L17 (27.1 and 9.3 x-fold, respectively, relative to WT). Without salt, similar plant weights were found in all analyzed plants (WT, *ars1* mutant and OE and RNAi lines) (Figure S4a). After 10 days of salt stress (200 mM NaCl), plant weights were slightly lower in *ars1* mutant and RNAi lines, with respect to WT plants, contrary to the response

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observed in OE lines (Figure S4b). However, significant phenotype differences regarding ion toxicity symptoms (leaf chlorosis) were detected (Figure 6a), with RNAi lines and *ars1* mutant plants showing evident leaf chlorosis and rolling appearance. Contrarily, OE lines developed fully green leaves, which were more similar to those grown without stress, while WT leaves showed an intermediate phenotype between those of OE and RNAi lines. To confirm the role of *ARS1* gene in regulating stomatal closure under stress conditions, we monitored g_s and E immediately before applying the salt stress and after 3 DST (Table S3 and Figure 6b). No differences were found between WT and transgenic lines for both parameters in the absence of NaCl. However, under salt stress, RNAi and *ars1* mutant plants displayed higher relative values of g_s and E than WT plants, contrarily to the response observed in the OE lines.

Subsequently, homozygous transgenic lines (T_3) were obtained and those with only one insertion were selected. Two RNAi lines, RNAi-L2 and RNAi-L5, with reduction of *ARS1* expression higher than 80%, and two OE lines, OE-L4 and OE-L19, with high levels of *ARS1* transcripts (38.8 and 72.7 x-fold, respectively, relative to WT) were selected in this second salt stress assay. Firstly, g_s, E and the photosynthesis rate were measured after 7 DST in order to corroborate the role of *ARS1* gene in the stomatal closure (Figure S5), showing similar changes to those observed in the first assay with T_1 plants (Figure 6b). Moreover, the water use efficiency (WUE), calculated on the basis of stomatal conductance and photosynthesis (Shabala, 2013), slightly increased in the OE lines and decreased in the *ars1* mutant and RNAi lines (Figure S5).

In order to know whether the high Na^+ transport to the shoot of the *ars1* mutant was exclusively due to its excessive transpiration under stress and not to the altered expression of transporters genes, the expression levels of main genes involved in Na^+

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transport from root to shoot in tomato, *SlSOS1* and *SLHKT1;2* (Asins *et al.* 2013; Garcia-Abellan *et al.* 2014; Olias *et al.* 2009) were analysed prior to salt treatment and after 12 h at 100 mM NaCl and other 36 h at 200 mM NaCl (Figure 7). Interestingly, the expression pattern of *SlSOS1* and, especially, *SlHKT1;*2 showed opposite responses in roots of RNAi and OE lines, as their expression levels increased significantly with salinity in RNAi roots while it decreased in OE lines after 48 h of salt treatment. Furthermore, *ars1* mutant showed similar patterns to those of RNAi lines, while WT exhibited a similar response as OE-lines (*SlSOS1*) or intermediate (*SlHKT1;2*) between RNAi and OE lines.

DISCUSSION

ARS1, an R1-MYB gene involved in the tomato response to salt acclimation

Molecular and genetic characterization of *ars1*, a tomato dominant salt-sensitive mutant isolated in a T-DNA collection, allowed us to identify the *ARS1* gene, an R1-type member of the MYB protein family in tomato. The loss-of-function phenotype of *ars1* mutant plants agrees with the molecular characterization of the *ars1* mutation. Indeed, we determined that the T-DNA insertion changed the open reading frame of the *ARS1* gene just before the SHAQKYF motif, promoting a truncated protein, which in turn would be unable to carry out the DNA binding activity proposed for R1-MYB transcription factors (Feller *et al.,* 2011). Such molecular features would explain the dominant-negative nature of *ars1* mutation, in a similar way to other mutations described in plants (Veitia, 2007). Phylogenetic analysis demonstrates that the ARS1 protein belongs to the CCA1-like clade of R1-type proteins (Figure 2). A verified

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function has not been reported for all *Arabidopsis* R1-type proteins included in this clade, and the *Arabidopsis* and rice proteins most similar to ARS1 are among those with an unknown function. Nevertheless, two single *MYB* genes have been recently reported as involved in abiotic stress tolerance, i.e. *StMYB1R-1* from potato (Shin *et al.*, 2011) and *OsMYB48-1* from rice (Xiong *et al.*, 2014). Both genes, and particularly *OsMYB48-1,* are homologous to *ARS1*, suggesting that the functional role of the *ARS1* may be conserved in plants. In tomato, in spite of its importance at agronomic and scientific level, the functional role of MYB tomato genes in abiotic stress tolerance remains largely unknown. Zhao *et al.* (2014) recently identified a total of 121 R2R3-MYB genes in tomato, but relatively few were shown to respond to abiotic stress conditions. To date, the only tomato MYB gene reported as involved in abiotic stress tolerance is *SlAIM1*, which encodes an R2R3-MYB gene type (Abuqamar *et al.,* 2009).

Here, we show the role of *ARS1* gene, up to our knowledge the first R1-MYB type characterized in tomato, in salinity tolerance. The disruption of this gene did not affect plant growth and fruit yield under unstressed conditions, which make it a good candidate to improve abiotic stress tolerance from a point of view of biotechnological application (Chen *et al.*, 2015; Garcia-Abellan *et al.*, 2014). Generally the disruption or overexpression of most stress-related genes negatively affects plant growth and yield under optimal conditions due to the growth-defence response trade-off (Huot *et al.*, 2014). The salt-sensitivity of the *ars1* mutant is observed in adult plants grown under transpiring conditions, while no differential phenotype was observed in the mutant when grown *in vitro* (Figures 1, 3, S3). Other genes, either involved in stomatal closure under osmotic stress or even genes known to be involved in controlling root to shoot translocation of Na^+, such as *AtHKT1;1*, exhibit different responses when either mutant

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or overexpressing plants are grown in transpiring and non-transpiring conditions (Davenport *et al.*, 2007; Ding *et al.*, 2014). Since a stress situation during the flowering period results in high yield penalties in crop plants, which may not be reflected when experiments are conducted in vegetative growth phases, currently a priority in the research on abiotic stress tolerance is to evaluate the gene effects in natural conditions and to long-term, being crop yield the most important factor of agronomic interest (Cabello *et al.*, 2014; Roy *et al.*, 2014). Interestingly, the fruit yield was significantly reduced in the mutant with respect to WT plants only under saline conditions (Figure 3b).

ARS1 gene is involved in the transpirational water loss under salt stress

The salt sensitivity of the *ars1* mutant is mainly due to the toxic effect promoted by the so high degree of Na^+ transport to the shoot and its accumulation, especially in young leaves (Figures 3c, 4c), which could be associated to down-regulation of the expression of genes involved in the Na^+ transport from root to shoot (Hasegawa, 2013). However, the opposite response was observed, as the expression levels of the *SlSOS1* and *SlHKT1;2*, the main tomato genes involved in the Na^+ retrieval from xylem in roots (Asins *et al.,* 2013; Garcia-Abellan *et al.,* 2014; Olias *et al.,* 2009), are up-regulated in the *ars1* mutant and RNAi lines, while they are down-regulated in the OE lines (Figure 7). Therefore, the high Na^+ transport to the shoot in the *ars1* mutant is a consequence of the water loss via transpiration, as increased leaf g_s and E were observed in mutant and RNAi lines while the opposite occurs in OE lines (Figures 6 and S5). These results highlight the important role that the ability to avoid the water loss in salt stress may have in salt tolerance, as it has been recently observed in wild salt-tolerant species of

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Arabidopsis (Wu *et al.,* 2012) and tomato (Koenig *et al.*, 2013; Shabala, 2013). Although more studies are necessary to dissect the mode of action of *ARS1* tomato gene, these results presented here support the hypothesis that the *ARS1* tomato gene regulates stomatal closure under stress conditions, reducing transpiration and thus the massive Na^+ transport to the leaves, leading the whole response to NaCl acclimation over the long term. Since ABA is a key regulator of stomatal closure (Raghavendra *et al.*, 2010), we also demonstrate that the mutation affects the stomatal aperture in response to ABA, in a similar way to the response displayed under salt stress (Figure 5c). In summary, our results reveal that the R1-MYB transcription factor encoded by *ARS1* gene play an essential role in tomato response to salt acclimation.

EXPERIMENTAL PROCEDURES

Isolation of tomato *ars1* 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 (Atares *et al.,* 2011, Pineda *et al.,* 2012). Screening for salt tolerance was performed on plants coming from independent transformation events (T_1). The description of the screening protocol and salt stress treatment applied can be found in Supporting Experimental Procedures (Methods S1). T_2 segregating progenies were used for phenotype-genotype co-segregation analysis as well as for selection of homozygous T_3 progenies where phenotype and physiological characterization of *ars1* mutant was fulfilled.

The presence of a T-DNA in the *ars1* mutant genome was confirmed by standard PCR amplification of the *nptII* and *uidA* genes with specific primers (Table S1), while the number of T-DNA copies was analyzed by Southern blot hybridization experiments

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(Methods S1). For PCR amplification, DNA extractions were performed with Plant DNAzol Reagent (Invitrogen), following the manufacturer specifications.

Anchor-PCR and gene cloning

To determine the region of the genome affected by the insertion, the T-DNA flanking sequence was isolated by Anchor-PCR according to Schupp *et al.* (1999). Briefly, the genomic DNA was digested with different blunt ends restriction enzymes and the fragments obtained were ligated to a partially double-stranded DNA adapter. This anchor-ligated DNA was amplified by PCR using specific primers to the 5′ end of the adapter (Ad1) and the right border of the T-DNA (RB-1) (Table S1). Initial PCR products were re-amplified twice using innermost primers homologous to the adapters (Ad2 and Ad3) and RB regions (RB-2 and RB-3) (Table S1). The three PCR products sizes were analyzed by electrophoresis in a 1% agarose gel and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems - Foster City, CA), following manufacturer instructions.

Gene and protein sequence analysis

The sequences obtained by anchor-PCR were compared to the SGN Database (http://solgenomics.net). Protein domains were analyzed with the Conserved Domain Database (CDD) of the National Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi) and the InterProScan tool of the European Bioinformatics Institute (EMBL-EBI, http://www.ebi.ac.uk/Tools/pfa/iprscan). Homologous sequences of *ARS1* were obtained from the SGN (http://solgenomics.net/) and the NCBI using the predicted

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ARS1 protein sequence (SGN-P713408). Multiple sequence alignment was conducted with Clustal-X and the phylogenetic tree was constructed using the MEGA v5.1 software by means of the bootstrap method with 1000 replicates and the neighbor-joining option.

Generation of transgenic tomato lines

The complete *ARS1* open reading frame was amplified from the *S. lycopersicum* (cv. Moneymaker) cDNA sequence using the specific primers ARS1compF, which introduces a *Sac*I restriction site 83pb upstream of the start codon, and ARS1compR, which introduces a *Kpn*I restriction site 20pb downstream of the stop codon (Table S1). The PCR product was cloned and sequenced. The resulting plasmid was double digested with *Sac*I and *Kpn*I, and the *ARS1* cDNA was subcloned into the binary vector pROKII (Baulcombe *et al.*, 1986) to generate an overexpression (OE) (35S::*ARS1*) gene construct.

To generate *ARS1* silencing lines, a RNA interference (RNAi) approach was followed. With this aim, a 125bp fragment of the *ARS1* cDNA was amplified using the primers ARS1Fv and ARS1Rv (Table S1), and the PCR product was cloned in sense and antisense orientation separated by intronic sequences into the pKannibal vector (Wesley *et al.*, 2001) to generate a pKannibal-ARS1 plasmid. The resulting plasmid was digested with *Not*I and the entire construct was cloned into the binary vector pART27 (Gleave, 1992).

In all cases the binary plasmids generated were electroporated into *Agrobacterium tumefaciens* LBA 4404 strain for further use in genetic transformation experiments. Agrobacterium-mediated transformation was performed following the protocol

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described by Gisbert *et al.* (2000). For more details, please see Methods S1. At least 10 independent events of transformation were obtained for OE and RNAi lines, and the *ARS1* expression level was measured by qPCR as described below.

Stress assays

In the homozygous line (T_3) of *ars1* different salt stress assays were carried out for the mutant characterization, both in a greenhouse and in a controlled growth chamber, as described in Methods S1. Moreover, the drought characterization was carried out by withholding irrigation **(**Methods S1**)**.

Physiological measures, microscopy and gene expression analysis

Regarding physiological analyses, the methods for determination of chlorophyll, g_s, E, and concentrations of Na^+ and K^+ are given in Methods S1. Water loss rates were determined in detached leaflets from the 3rd leaf of *ars1* and WT adult plants, placed on open-lid Petri dishes, immediately weighted and incubated during 8 h. The decreases in fresh weight were monitored and results expressed as percentage of weight loss relative to initial weight. Microscopy analyses performed to determine no. of stomas and degree of stomatal aperture in control, salt stress and ABA treatment conditions, are described in detail in Methods S1. Finally, *ARS1*, *SlSOS1* and *SlHKT1;2* gene expressions were analysed according to the protocol also described in Methods S1.

Statistical analysis

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Data were statistically analyzed using the SPSS 13.0 software package by one-way ANOVA and Student's *t* tests ($P<0.05$). Significant differences between means were denoted by asterisks. All data are given as mean±SE (n=sample size).

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SUPPORTING INFORMATION

Additional Supporting information may be found in the online version of this article

Figure S1 Co-segregation phenotype-genotype analysis in T_2 plants grown under salt stress, and spatial expression of *ARS1* in WT and *ars1* mutant plants in control (without NaCl) and salt stress conditions.

Figure S2 Leaf K^+ content and leaf Na^+/K^+ ratio in WT and *ars1* mutant plants grown under salt stress.

Figure S3 Phenotype of *in vitro* WT and *ars1* mutant seedlings subjected to NaCl, LiCl and mannitol treatments.

Figure S4 Plant growth monitoring (plant weights) of *ars1* mutant, *ARS1*-silencing lines and *ARS1*-overexpressing lines in control (without NaCl) and salt stress conditions.

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Figure S5 Stomatal conductivity, transpiration rate, photosynthesis rate and water use efficiency in WT, *ars1*, *ARS1*-silencing lines, and *ARS1*-overexpressing lines in salt stress conditions.

Table S1 Primers used in this study.

Table S2 Stomatal densities of WT and *ars1* mutant leaves in control (without NaCl) and salt stress.

Table S3 Stomatal conductivity and transpiration rate in WT, *ars1*, *ARS1*-silencing lines, and *ARS1*-overexpressing lines in control (without NaCl) and salt stress conditions.

Methods S1 Supplementary experimental procedures.

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FIGURE LEGENDS

Figure 1 The dominant salt-sensitive *ars1* mutant identifies an R1-MYB gene of tomato. (a) Phenotype of WT and T_1 *ars1* mutant plants in control condition without NaCl and after 20 days of 200 mM NaCl treatment, in shoot and root. (b) DNA-blot analysis to determine no. of T-DNA insertions in the T_1 *ars1* mutant plant using the coding region of the *nptII* gene as probe. Single restriction fragments observed in genomic DNA digested with *BamHI* (12Kb) and *EcoRI* (1Kb) indicate the presence of a single T-DNA insertion in the *ars1* genome. (c) Identification of *ARS1*, a R1-MYB type gene tagged by the T-DNA and characterization of the insertional event. The presence in the 5'-untranslated region (5'UTR) of an ABA responsive element is indicated by vertical black straight line. Exons and UTRs are represented by black and grey boxes respectively, whereas introns are represented by horizontal lines. Start and stop codons for translation are indicated, as well as the SANT domain characteristic of this family of transcription factors. Positioning of primers designed for detecting presence of T-DNA insertion and for genotyping is showed in the *ARS1* genomic sequence as well as in the T-DNA insert.

Figure 2 Sequence analysis of ARS1. (a) Phylogenic tree constructed with MEGA5 software based on neighbor-joining method after sequences alignment with Clustal-X. Sequences of Arabidopsis CCA1-like single MYB-like domain proteins had previously been described (Yanhui *et al.*, 2006). The single MYB-like domain proteins from potato (StMYB1R1, Shin *et al.*, 2011) and rice (OsMYB48-1, Xiong *et al.*, 2014) implicated in salt-stress resistance were also included (bold letters). Proteins integrated in the CCA1-

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like clade are indicated with a bracket including ARS1 protein (bold letters). Scale indicates percentage of substitutions. (b) Multiple sequence alignment of the conserved MYB-like and adjacent P-rich domains of the CCA1-like proteins showed in (a). An arrow indicates the residue where the T-DNA insertion changes ARS1 reading frame including 3 amino acids (VVC) before a stop codon.

Figure 3 The null *ars1* mutant shows salt sensitivity to long-term. (a) Plants of WT and *ars1* mutant were grown in greenhouse. Salt stress (100 mM NaCl) was applied when the plants had ten true leaves. Pictures are representatives of the eight plants per treatment after 0, 30 and 60 days of salt treatment (DST). 0 DST means just before the start of the salt treatment. (b) Fruit yield of WT and *ars1* mutant without NaCl (control) and salt stress condition at the end of the assay. (c) Evolution of the Na^+ concentration in leaves of WT and *ars1* during 50 DST. (d) Stomatal conductance in leaves of WT and *ars1* plants without NaCl (control) and after 30 DST (salt). Measurements were taken at dawn and after 2 h of light. (e) Evolution of the stomatal conductance between 2 and 5 h of light in leaves of WT and *ars1* plants grown during 50 DST. Values are means ± SE of eight individual plants per line and condition. Asterisks indicate significant differences by Student *t*-test between WT and mutant plants ($P < 0.05$).

Figure 4 The *ars1* mutant shows increased stomatal aperture and Na^+ accumulation under salt stress. WT and *ars1* mutant plants were grown in hydroponic culture adding 200 mM NaCl to the Hoagland solution for 10 days when plants had developed ten true leaves. Measurements were taken in 3rd and 4th developed leaves. (a) Stomatal conductance and transpiration rate in leaves of WT and mutant without NaCl (control)

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and after 1 day of salt treatment (DST). (b) Stomatal aperture and percentage of open stomata in leaves of WT and *ars1* after 3 and 7 DST, and representative images of stomatal aperture in both genotypes and conditions without NaCl (control) and salt stress. (c) Shoot Na^+ partitioning in WT and mutant plants, in stem (left hand side graphic) and leaves (right hand side graphic) after 10 DST. Values are means ± SE of six individual plants per line. Asterisks indicate significant differences between WT and mutant plants by Student *t*-test ($P < 0.05$).

Figure 5 *ars1* mutant responses to dehydration and ABA. (a) Plants of WT and *ars1* were submitted to two successive cycles of withholding irrigation followed by 1 day of rewatering at the eight-leaf developmental stage, and stomatal conductance and transpiration rate were measured throughout the second dehydration cycle. (b) Water loss rate measured in detached leaf. The leaves were detached from light-grown plants with 8 fully developed leaves. Measures were taken during 8 hours of incubation at room temperature. (c) Stomatal aperture of WT and *ars1* mutant leaves treated with increasing ABA concentrations. Values are means ± SE of six individual plants per line. Asterisks indicate significant differences by Student *t*-test between WT and mutant plants ($P < 0.05$).

Figure 6 The overexpressing (OE) and silencing (RNAi) *ARS1* lines corroborate that *ARS1* gene is required in regulating stomatal conductance (g_s) and transpiration rate (E) under salt stress. (a) At the end of the salt stress assay (10 days at 200 mM NaCl), the leaves of OE lines did not show chlorosis, while *ars1* mutant and RNAi lines showed a high level of leaf chlorosis, with WT leaves showing an intermediate response. (b)

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Relative values with respect to WT of g_s and E in the 3rd developed leaf of *ars1*, OE and RNAi lines after 3 days of salt treatment. The measurements were taken as indicated in Table S3. Asterisks indicate significant differences between WT and each one of the other lines by Student *t*-test ($P < 0.05$).

Figure 7 The relative expression of *SlSOS1* and *SLHKT1;2* increases with salinity in roots of the *ars1* mutant and RNAi lines and decreases in roots of OE lines, compared with WT. Results of expression prior to salt treatment (no NaCl), after 12 h at 100 mM NaCl and other 36 h at 200 mM NaCl. The expression of WT prior to salt stress was set to 1. Values are means ± SE of six individual plants per line.

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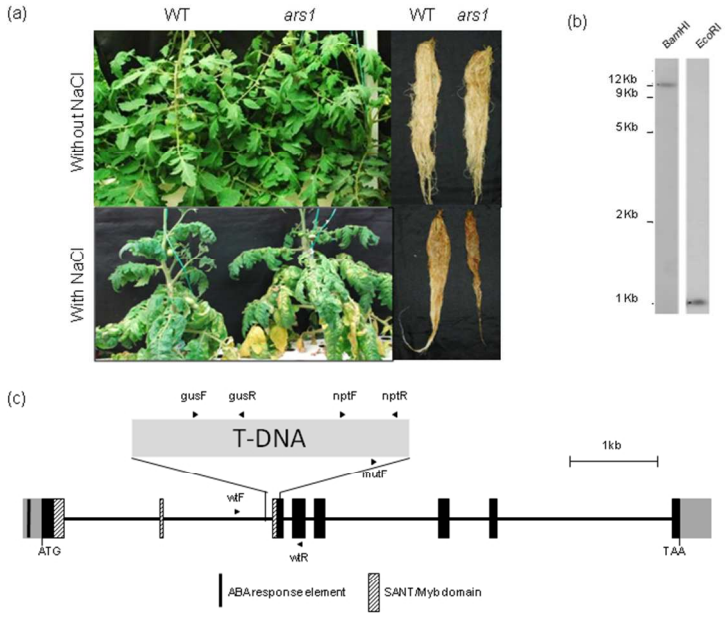

Figure 1 The dominant salt-sensitive ars1 mutant identifies an R1-MYB gene of tomato. (a) Phenotype of WT and T1 ars1 mutant plants in control condition without NaCl and after 20 days of 200 mM NaCl treatment, in shoot and root. (b) DNA-blot analysis to determine no. of T-DNA insertions in the T1 ars1 mutant plant using the coding region of the nptII gene as probe. Single restriction fragments observed in genomic DNA digested with BamHI (12Kb) and EcoRI (1Kb) indicate the presence of a single T-DNA insertion in the ars1 genome. (c) Identification of ARS1, a R1-MYB type gene tagged by the T-DNA and characterization of the insertional event. The presence in the 5'-untranslated region (5'UTR) of an ABA responsive element is indicated by vertical black straight line. Exons and UTRs are represented by black and grey boxes respectively, whereas introns are represented by horizontal lines. Start and stop codons for translation are indicated, as well as the SANT domain characteristic of this family of transcription factors. Positioning of primers designed for detecting presence of T-DNA insertion and for genotyping is showed in the ARS1 genomic sequence as well as in the T-DNA insert.
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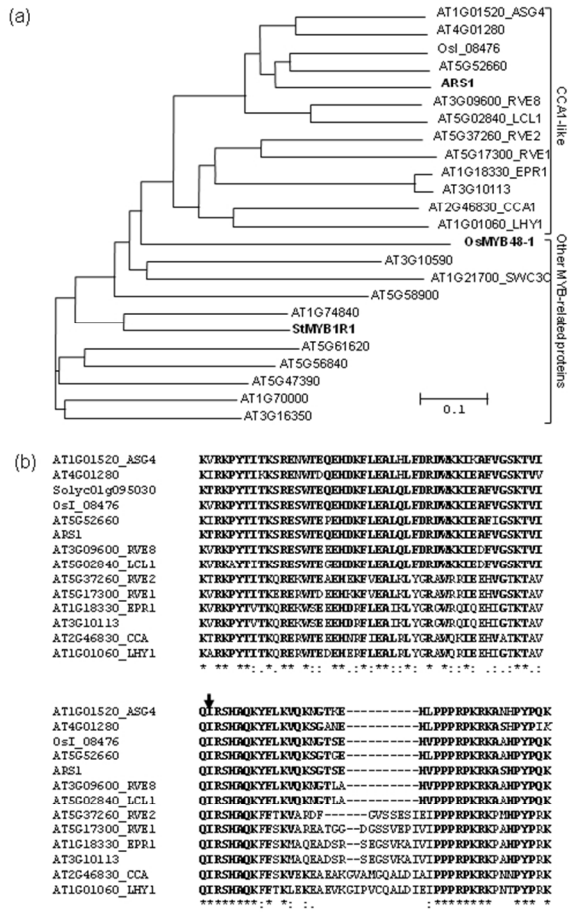

Figure 2 Sequence analysis of ARS1. (a) Phylogenic tree constructed with MEGA5 software based on neighbor-joining method after sequences alignment with Clustal-X. Sequences of Arabidopsis CCA1-like single MYB-like domain proteins had previously been described (Yanhui et al., 2006). The single MYB-like domain proteins from potato (StMYB1R1, Shin et al., 2011) and rice (OsMYB48-1, Xiong et al., 2014) implicated in salt-stress resistance were also included (bold letters). Proteins integrated in the CCA1-like clade are indicated with a bracket including ARS1 protein (bold letters). Scale indicates percentage of substitutions. (b) Multiple sequence alignment of the conserved MYB-like and adjacent P-rich domains of the CCA1-like proteins showed in (a). An arrow indicates the residue where the T-DNA insertion changes ARS1 reading frame including 3 amino acids (VVC) before a stop codon.
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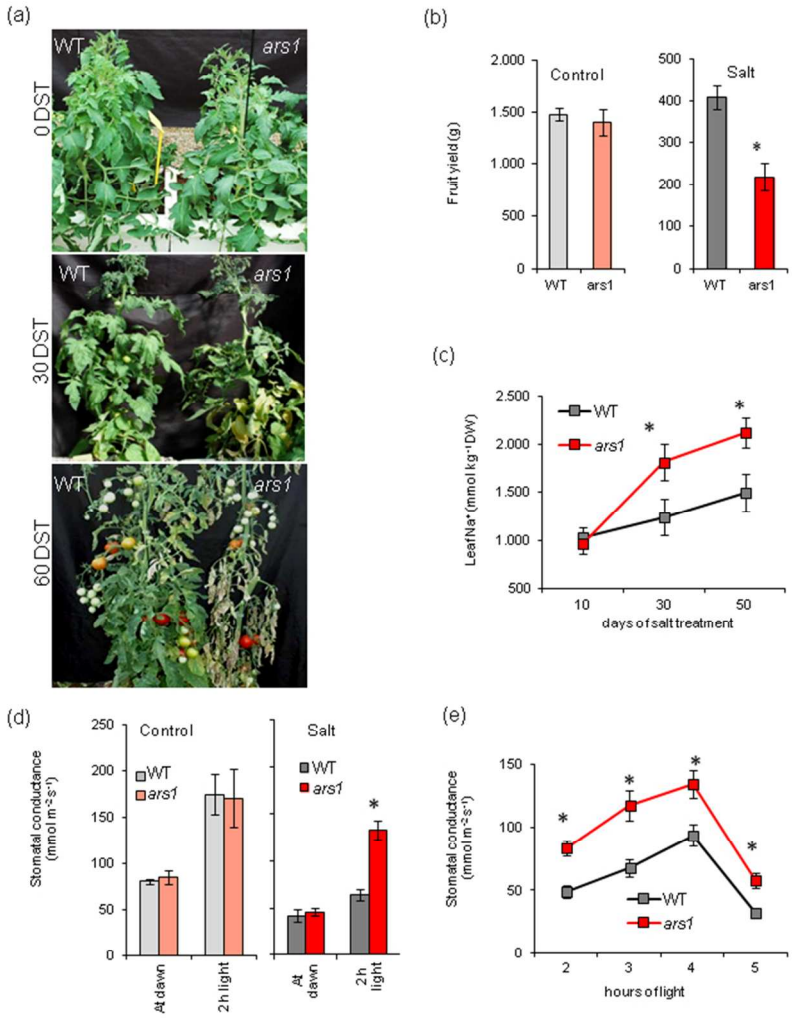

Figure 3 The null ars1 mutant shows salt sensitivity to long-term. (a) Plants of WT and ars1 mutant were grown in greenhouse. Salt stress (100 mM NaCl) was applied when the plants had ten true leaves. Pictures are representatives of the eight plants per treatment after 0, 30 and 60 days of salt treatment (DST). 0 DST means just before the start of the salt treatment. (b) Fruit yield of WT and ars1 mutant without NaCl (control) and salt stress condition at the end of the assay. (c) Evolution of the Na+ concentration in leaves of WT and ars1 during 50 DST. (d) Stomatal conductance in leaves of WT and ars1 plants without NaCl (control) and after 30 DST (salt). Measurements were taken at dawn and after 2 h of light. (e) Evolution of the stomatal conductance between 2 and 5 h of light in leaves of WT and ars1 plants grown during 50 DST. Values are means ± SE of eight individual plants per line and condition. Asterisks indicate significant differences by Student t-test between WT and mutant plants ($P < 0.05$).
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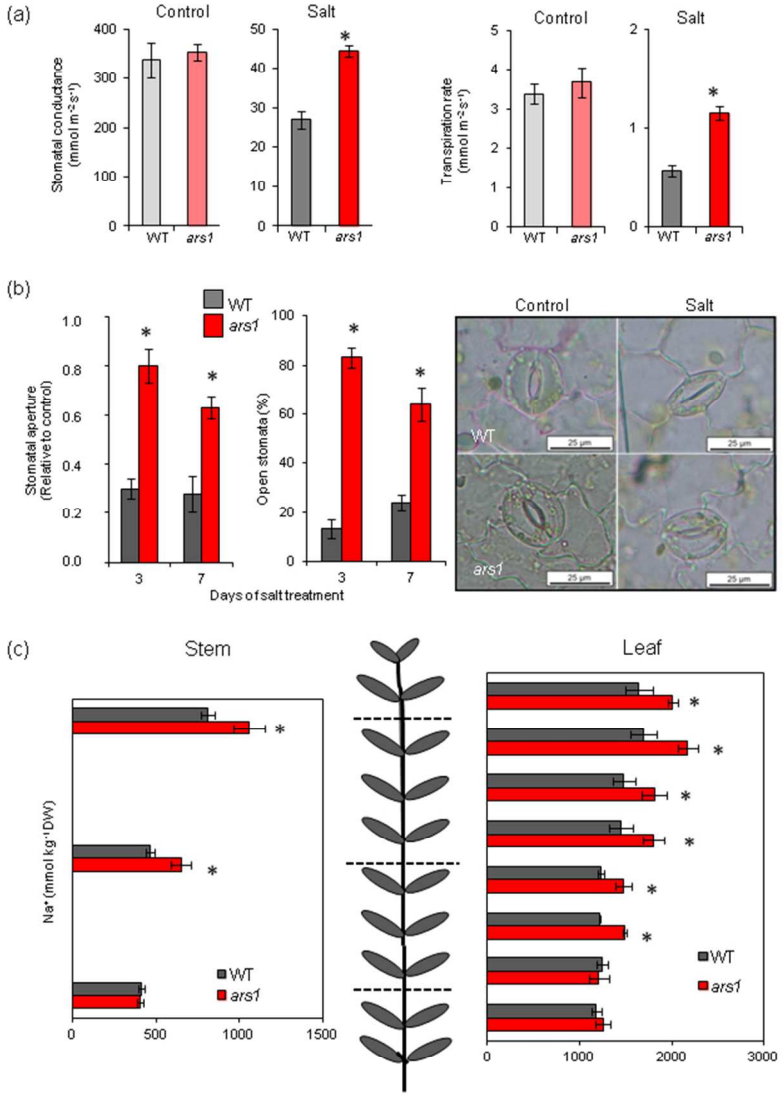

Figure 4 The ars1 mutant shows increased stomatal aperture and Na+ accumulation under salt stress. WT and ars1 mutant plants were grown in hydroponic culture adding 200 mM NaCl to the Hoagland solution for 10 days when plants had developed ten true leaves. Measurements were taken in 3rd and 4th developed leaves. (a) Stomatal conductance and transpiration rate in leaves of WT and mutant without NaCl (control) and after 1 day of salt treatment (DST). (b) Stomatal aperture and percentage of open stomata in leaves of WT and ars1 after 3 and 7 DST, and representative images of stomatal aperture in both genotypes and conditions without NaCl (control) and salt stress. (c) Shoot Na+ partitioning in WT and mutant plants, in stem (left hand side graphic) and leaves (right hand side graphic) after 10 DST. Values are means ± SE of six individual plants per line. Asterisks indicate significant differences between WT and mutant plants by Student t-test ($P < 0.05$).
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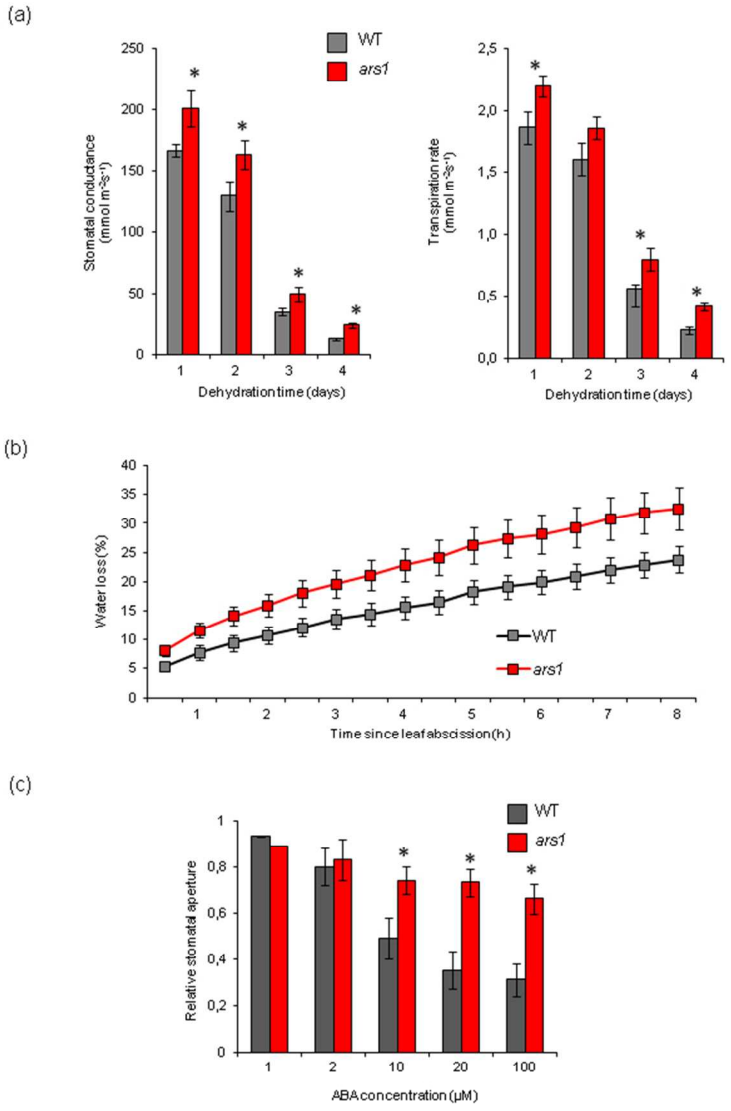

Figure 5 ars1 mutant responses to dehydration and ABA. (a) Plants of WT and ars1 were submitted to two successive cycles of withholding irrigation followed by 1 day of rewatering at the eight-leaf developmental stage, and stomatal conductance and transpiration rate were measured throughout the second dehydration cycle. (b) Water loss rate measured in detached leaf. The leaves were detached from light-grown plants with 8 fully developed leaves. Measures were taken during 8 hours of incubation at room temperature. (c) Stomatal aperture of WT and ars1 mutant leaves treated with increasing ABA concentrations. Values are means ± SE of six individual plants per line. Asterisks indicate significant differences by Student t-test between WT and mutant plants ($P < 0.05$).
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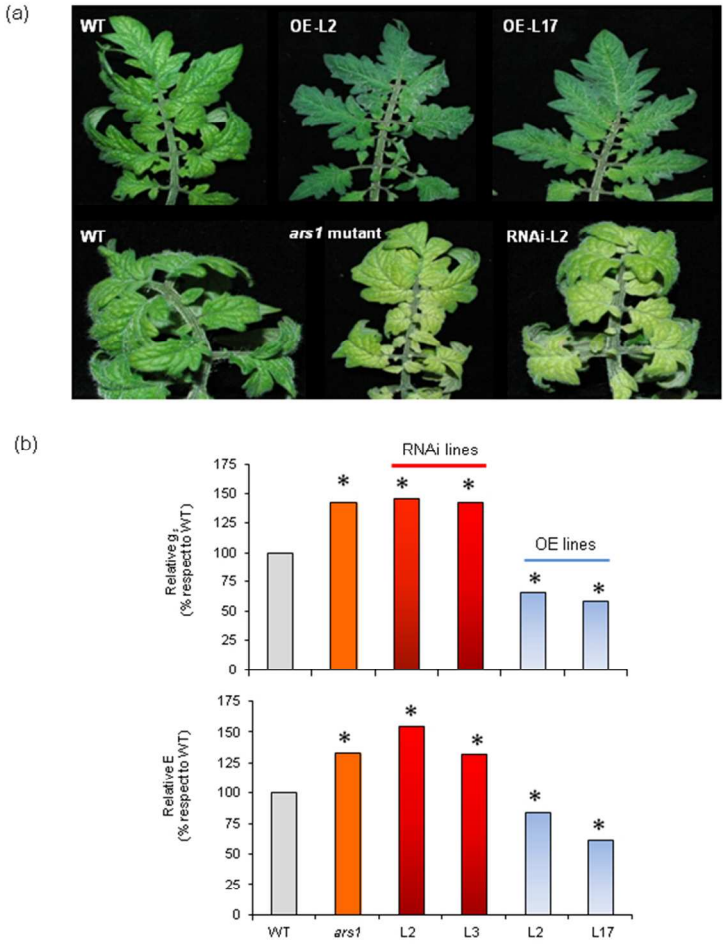

Figure 6 The overexpressing (OE) and silencing (RNAi) ARS1 lines corroborate that ARS1 gene is required in regulating stomatal conductance (gs) and transpiration rate (E) under salt stress. (a) At the end of the salt stress assay (10 days at 200 mM NaCl), the leaves of OE lines did not show chlorosis, while ars1 mutant and RNAi lines showed a high level of leaf chlorosis, with WT leaves showing an intermediate response. (b) Relative values with respect to WT of gs and E in the 3rd developed leaf of ars1, OE and RNAi lines after 3 days of salt treatment. The measurements were taken as indicated in Table S3. Asterisks indicate significant differences between WT and each one of the other lines by Student t-test ($P < 0.05$).
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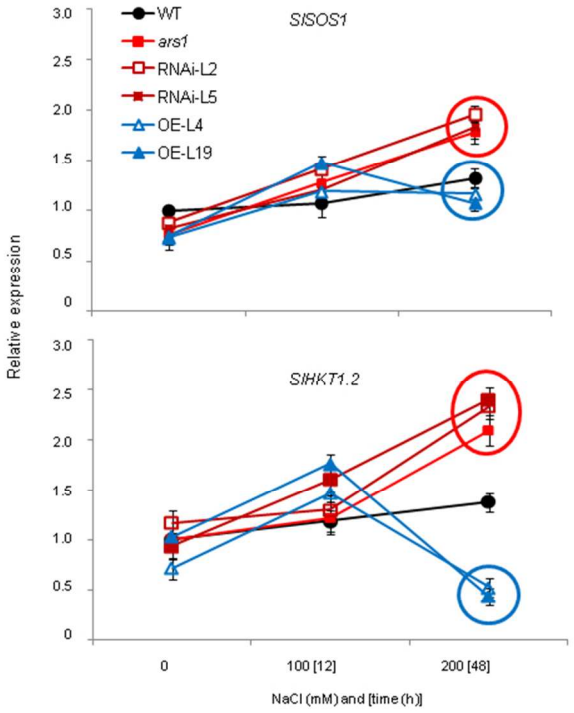

Figure 7 The relative expression of SlSOS1 and SLHKT1;2 increases with salinity in roots of the ars1 mutant and RNAi lines and decreases in roots of OE lines, compared with WT. Results of expression prior to salt treatment (no NaCl), after 12 h at 100 mM NaCl and other 36 h at 200 mM NaCl. The expression of WT prior to salt stress was set to 1. Values are means ± SE of six individual plants per line.
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