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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Received 20 July 2015; revised 18 September 2015; accepted 4 October 2015.
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Keywords: Solanum lycopersicum, insertional mutagenesis, salt stress, transpiration, stomatal aperture, MYB transcription factor.

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 abiotic stresses in this horticultural species are not well characterized, and only a very small number of genes playing key roles 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 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 biotic stresses (Lindemose et al., 2013; Nakashima et al., 2014). Among these transcription factors, members of the 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 tolerance 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

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 biotic stresses (Lindemose et al., 2013; Nakashima et al., 2014). Among these transcription factors, members of the 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 tolerance 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

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 abscisic acid (ABA). The characterization of tomato transgenic lines silencing and overexpressing ARS1 corroborates 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, because its manipulation could lead to improved stress tolerance without yield penalty under optimal culture conditions.

of MYB genes carrying four SANT domains (Dubos et al., 2010; Yanhui et al., 2006). 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 such as rice, Arabidopsis thaliana, Medicago truncatula and potato (Duangpan et al., 2013; Jeong et al., 2002; O’Malley and Ecker, 2010; Tadega et al., 2008; Ulker et al., 2008). In tomato, the analysis of an insertional 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 knockout 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 (T1), 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

![Figure 1](image-url)

**Figure 1** The dominant salt-sensitive ars1 mutant identifies an R1-MYB gene of tomato. (a) Phenotype of wild type 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 number 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 (12 kb) and EcoRI (1 kb) 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.
plant fresh weights (FW) were found in WT and ars1 T2 plants without salt (around 440 g per plant); however, FW experienced a higher reduction by salt stress in mutant than WT (235 ± 21 and 298 ± 14 g 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 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 T1 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 (https://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 (Soly01g095030.2), 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'sequence of exon 3 was 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 nonfunctional 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 MYB-like DNA-binding domain (66–116 residues) with high similarity to SHAQKYF (pfam00249) (Baranovskij 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 subfamily in Arabidopsis (Yanhai et al., 2006). Apart from Arabidopsis MYB proteins, ARS1 protein showed the highest homology with the rice OsL_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 OsL_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 T2 segregating progeny confirmed the dominant inheritance of the ars1 mutation, with a phenotypic segregation resulting in 11 individuals with ars1 phenotype and three with WT phenotype yielding a χ2 = 0.10 (P < 0.001). Phenotype segregation correlated with the genotype of T2 plants because all wild-type phenotype plants lacked the ars1 mutant allele (asygous), while three of the mutant plants were homozygous and eight hemizygous for the ars1 mutation (Figure 5a). 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 5b). Moreover, Na+ accumulation in leaves also co-segregated with the genotype of T2 plants grown under salt stress (Figure 5c). All together, these results proved that the gene interrupted by the T-DNA was responsible for the mutant phenotype.

Subsequently, homozygous T2 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 analysed 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 knockout 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 analysed 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 analysed (Figure 5d).

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 10th 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). 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;) 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; were taken at dawn (between 6 and 7 h am) and after 2 h of light, because g; 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; 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; value was twofold higher in the mutant than WT under salt stress, which was not observed in unstressed conditions. The differences in leaf g; between WT and mutant plants were maintained after 50 DST, as shown in the evolution of g; between 2 and 5 h of light (Figure 3e). As the stomatal conductance is dependent on the number of stomatal pores, stomatal density was analysed at this time in the abaxial surface of leaves (Table S2). Similar values were found in leaves of WT and ars1 plants, which indicates that the increased g; found in the mutant under salt stress is
to reduced stomatal closure under salt stress
mutant is attributable to ionic stress reflected by Na⁺ toxicity or
and WT.
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
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 g₀
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 three and seven 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 three DST, and this
difference was maintained after seven DST. Moreover, the
percentage of open stomata was 3–4 times higher in the ars1

Figure 2. Sequence analysis of ARS1. (a) Phylogenetic tree constructed with MEGAS
software based on neighbour-joining method after sequences alignment with ClustalX.
Sequences of Arabidopsis CCA1-like single MYB-like domain proteins had previously
been described (Yanhai 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 R-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
three amino acids (VVC) before a stop codon.
Salt stress (100 mM NaCl) was applied when the wild type (WT) and mutant plants 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’s t-test between WT and mutant plants (P < 0.05).

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 nontranspiring conditions (in vitro). WT and mutant seedlings were grown in vitro with NaCl (100 mM) and LiCl (10 mM) 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).

Figure 3 The null ars1 mutant shows salt sensitivity to long term. (a) Plants of wild type (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’s t-test between WT and mutant plants (P < 0.05).

Tomato ARS1 is involved in salt stress response

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 nonstressful conditions was similar to WT regarding the values of gs and E measured during four consecutive days, with mean values around 235 and 2.4 mmol/m²s⁻¹ for gs and E, respectively. Under dehydration, ars1 mutant plants showed higher values of gs 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 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).
As 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 signalling.

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 analysing 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 ten independent events of transformation were obtained for RNAi and OE lines. In a first assay carried out with T1 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 analysed 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 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 stems.

**Figure 4** The ars1 mutant shows increased stomatal aperture and Na+ accumulation under salt stress. Wild type (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 three and seven 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 ten DST. Values are means ± SE of six individual plants per line. Asterisks indicate significant differences between WT and mutant plants by Student’s t-test (P < 0.05).
Tomato ARS1 is involved in salt stress response

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 three 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 (T3) 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 seven DST to corroborate the role of ARS1 gene in the stomatal closure (Figure S5), showing similar changes to those observed in the first assay with T1 plants (Figure 6b). Moreover, the water use efficiency, 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).

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$^+$ transport from root to shoot in tomato, $SlSOS1$ and $SLHKT1;2$ (Asins et al., 2013; García-Abellán 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 $SLOS1$ and, especially, $SLHKT1;2$ showed opposite responses in

Figure 5  $ars1$ mutant responses to dehydration and ABA. (a) Plants of wild type (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 eight fully developed leaves. Measures were taken during 8 h of incubation at room temperature. (c) Stomatal aperture of WT and $ars1$ mutant leaves treated with increasing ABA concentrations. Values are means $\pm$ SE of six individual plants per line. Asterisks indicate significant differences by Student’s $t$-test between WT and mutant plants ($P < 0.05$).
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 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, that is *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

![Figure 6](image_url)
genes known to be involved in controlling root to shoot either involved in stomatal closure under osmotic stress or even in vitro mutant when grown conditions, while no differential phenotype was observed in the mutant is observed in adult plants grown under transpiring trade-off (Huot et al., 2014). Generally, the disruption or overexpression of most stress-related genes negatively affects plant growth and yield. The disruption of this gene did not affect plant growth the first R1-MYB type characterized in tomato, in salinity stress in roots of thears1 mutant, and RNAi lines, while they are down-regulated in the OE lines (Figure 7). Therefore, the high Na+ transport to the shoot in thears1 mutant is a consequence of the water loss via transpiration, as increased leaf g 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 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. As 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 plays 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 (Ataré et al., 2011; Pineda et al., 2012). Screening for salt tolerance was performed on plants coming from independent transformation events (T1). The description of the screening protocol and salt stress treatment applied can be found in Supporting Experimental Procedures (Methods S1). T2 segregating progenies were used for phenotype-genotype co-segregation analysis as well as for selection of homozygous T3 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 analysed by Southern blot hybridization experiments (Methods S1). For PCR amplification, DNA extractions were performed with Plant DNAzol Reagent (Invitrogen, Carlsbad, CA), following the manufacturer specifications.

Figure 7 The relative expression of SISOS1 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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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 adapter-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 analysed 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 (https://solgenomics.net). Protein domains were analysed with the Conserved Domain Database 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 (https://solgenomics.net) and the NCBI using the predicted 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 neighbour-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 primers ARS1Fv and ARS1Rv, which introduces a SacI restriction site 83-pb upstream of the start codon, and ARS1compR, which introduces a KpnI restriction site 20-pb downstream of the stop codon (Table S1). The PCR product was cloned and sequenced. The resulting plasmid was double digested with SacI and KpnI, 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 125-bp 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 pKanibal vector (Wesley et al., 2001) to generate a pKanibilal-ARS1 plasmid. The resulting plasmid was digested with Ncol, 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 described by Gisbert et al. (2000). For more details, please see Methods S1. At least ten 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 (T3) 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, gₛ, 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 number of stomas and degree of stomatal aperture in control, salt stress and ABA treatment plants were carried out in detail in Methods S1. Finally, ARS1, SISOS1 and SlHKTT1:2 gene expressions were analysed according to the protocol also described in Methods S1.

Statistical analysis

Data were statistically analysed 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).

Acknowledgements

This work was funded by a research project (AGL2012-40150-C01/ C02/03) from the Spanish Ministry of Economy and Competitiveness (MINECO). This work was also supported by grant RYC2010-06369 (Ramón y Cajal Programme) from the MINECO to NF-G and grant E-30-2011-0443170 (JAE-Doc Programme) from the Spanish Council of Scientific Research (CSIC) to IE and BP. The authors have no conflict of interests to declare.

References


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

Figure S1 Co-segregation phenotype-genotype analysis in T2 plants grown under salt stress, and spatial expression of ARST1 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, ARST1-silencing lines and ARST1-overexpressing lines in control (without NaCl) and salt stress conditions.

Figure S5 Stomatal conductivity, transpiration rate, photosynthesis rate and water use efficiency in WT, ars1, ARST1-silencing lines, and ARST1-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, ARST1-silencing lines, and ARST1-overexpressing lines in control (without NaCl) and salt stress conditions.

Methods S1 Supplementary experimental procedures.