# Loss of *AUXIN RESPONSE FACTOR 4* function alters plant growth, stomatal functions and improves tomato tolerance to salinity and water deficit

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**Summary**

Understanding physiological and molecular basis of plant response and tolerance to environmental stresses is important to improve plant survival, crop yield and quality. Auxin controls many aspects of plant growth and development. However, its role in stress responses remains so far poorly studied. Auxin acts on the transcriptional regulation of target genes, mainly through Auxin Response Factors (*ARF*). The current study aims to study the involvement of *SlARF4* in tomato tolerance to salinity and water deficit. Using a reverse genetic approach, we found that the down-regulation of *SlARF4* improves tomato tolerance to salt and drought stress by promoting root development and density, increasing soluble sugars content and maintaining chlorophyll content at high levels in stress conditions. Besides that, *ARF4*-as plants displayed a leaf curl phenotype, a low stomatal conductance coupled with an increase in leaf relative water content and ABA content under normal and stressful conditions. This increase in ABA content was correlated with the activation of ABA biosynthesis genes

and the repression of ABA catabolism genes. *cat1*, *Cu/ZnSOD* and *mdhar* genes were up-regulated in *ARF4*-as plants suggesting that *ARF4*-as mutant is more tolerant to salt and water stress.The data presented in this work strongly support the involvement of ARF4 as a key actor in tomato tolerance to salt and drought stresses.

**Keywords:** *ARF4*, Auxin, salt, drought, tomato, tolerance.

**Significance statement**

Down regulation of *Auxin Response Factor* 4 modify leaf structure, alters stomatal responses under normal conditions, and improves tomato tolerance to salt and drought stress.

## Introduction

Auxin is a key regulator of many plant growth and development processes throughout the plant life cycle (Vanneste and Friml, 2009). Auxins regulate diverse cellular and developmental responses in plants via three types of transcriptional regulators, auxin response factors (ARFs), Aux/IAA and TOPLESS (TPS) proteins (Reed, 2001) Szemenyei, Hannon & Long 2008). ARFs play a key role in regulating the expression of auxin response genes (Liscum and Reed, 2002). 23 *ARF* genes have been isolated from *Arabidopsis thaliana* (Liscum and Reed, 2002; Tom J Guilfoyle and Hagen, 2007). Most of them share a structure with conserved domains: an N-terminal DNA-binding domain (DBD), a variable central transcriptional regulatory region (MR), which can function as an activator or repressor domain, and a carboxy-terminal dimerization domain (CTD) that contributes to the formation of either ARF/ARF homo- and hetero-dimers or ARF/Aux/IAA hetero-dimers (Ulmasov *et al.*, 1999; Tom J. Guilfoyle and Hagen, 2007; Zouine *et al.*, 2014).

Since *ARF1*, the first *Arabidopsis* ARF gene, was cloned and its function investigated (Ulmasov *et al.*, 1997), the ARF gene family has been identified and well characterized in many crop species, including tomato (*Solanum lycopersicum*) (Wu et al., 2011; Zouine et al.,

2013), maize (*Zea mays*) (Xing et al., 2011), rice (*Oryza sativa*) (Wang et al., 2007; Jain and Jitendra P Khurana, 2009), poplar (*Populus trichocarpa*) (Kalluri et al., 2007), Chinese cabbage (*Brassica rapa*)(Mun et al., 2012), sorghum (*Sorghum bicolor*) (Ha et al., 2013), banana (Hu et al., 2015) and physic nut (*Jatropha curcas* L.) (Tang et al., 2018).

Genetic and molecular studies conducted in *Arabidopsis* and other plant species showed that ARF proteins are key factors in regulatory networks controlling plant growth and development (Chandler, 2016). Indeed, ARF mutations can affect plant morphology and physiology, and thus can affect plant development and interaction with the environment. In *Arabidopsis*, a loss-of-function allele of *AtARF2*, has pleiotropic effects on vegetative organ growth (Schruff *et al.*, 2005). An *Atarf8* mutation affects hypocotyl elongation and auxin homeostasis (Goetz *et al.*, 2006) while *AtARF7* loss of function mutation impairs the hypocotyl response to blue light and reduces the auxin response (Harper *et al.*, 2000). The *Atarf7*/*Atarf19* double mutant shows aberrant lateral root formation and abnormal gravitropism in both hypocotyls and roots (Narise *et al.*, 2010). Double mutant *Atarf3*/*Atarf4* plants display adaxialized tissues in the shoot, resembling mutations in the *KANADI* transcriptional repressor (Pekker *et al.*, 2005). In tomato, down-regulation of *ARF3* decreased the density of epidermal cells and trichomes (Zhang *et al.*, 2015). Transcriptional down-regulation of *ARF4* expression leads to severe leaf curling along the longitudinal axis (Jones *et al.*, 2002). The *ARF* gene family is also involved in the control of many physiological processes. In tomato, the *Slarf2A*/*B* mutation leads to a severe ripening inhibition with dramatically reduced ethylene production, while the over-expression of *ARF2A* resulted in a blotchy ripening pattern in fruit as a result of a significant accumulation of ripening-related genes and metabolites (Hao *et al.*, 2015; Breitel *et al.*, 2016). Down-regulation of *SlARF6* and *SlARF8* in transgenic tomato plants through the overexpression of *miR167a* may lead to floral development defects and female sterility (N., Liu *et al.*, 2014). The down-regulation of

*SlARF4* resulted in ripening-associated phenotypes such as enhanced firmness, sugar and chlorophyll content leading to dark green fruit and blotchy ripening (Jones *et al.*, 2002; Sagar *et al.*, 2013). This phenotype was also reported in *SlARF10* gain of function mutant (Mei *et al.*, 2018). *ARF* genes also seem to be implicated in plant responses to environmental stresses. It has been reported that both *OsARF11* and *OsARF15* show differential expression in salt stress conditions, suggesting that they are involved in the rice response to stress (Jain and Khurana, 2009). In banana, the expression of many *ARF* genes was altered in response to salinity and osmotic stresses (Hu *et al.*, 2015). In tomato, many *SlARF* genes are responsive to a wide range of abiotic stress conditions (Bouzroud *et al.*, 2018). Among them, *SlARF4* expression was significantly regulated by salinity and water deficit. *SlARF4* regulatory region was enriched in cis-*acting* elements specific to salinity and water deficit response (Bouzroud *et al.*, 2018). Moreover, *SlARF4* loss of function mutation induced modification in plant morphology and physiology. Indeed, *ARF4* down-regulated plants showed a severe upward curling along the longitudinal axis of leaves with an increase in chlorophyll content (Jones *et al.*, 2002). We carried out morphological, anatomical, physiological and molecular analyses to approach the possible involvement of *SlARF4* in tomato response to salinity and drought stress.

## Results and discussion

### An *SlARF4* downregulated line shows altered anatomical, morphological and physiological parameters

We analyzed previously published *AUXIN RESPONSE FACTOR 4* antisense RNA post-transcriptionally silenced lines (*ARF4*-as) (Sagar *et al.*, 2013). The *ARF4*-as plants exhibit a wide range of morphogenic phenotypes, namely delayed flowering, increased height and leaf curling as compared to their isogenic Micro-Tom (wild-type, WT) counterparts (Figure 1). Stem and root dry weight, on the other hand, showed a significant reduction in *ARF4*-as plants

compared to WT, along with an insignificant increase in leaf area and specific leaf area (Figure 2).

*ARF4*-as plants analyzed in this work displayed a severe upward curling along the longitudinal axis of leaves (Figure 1c), consistent with those observed in DR12-ASL lines generated in the Kemer cultivar by Jones *et al.* (2002) and those obtained in WT by Sagar *et al*. (2013). The curled leaf morphology observed in *ARF4*-as plants could result from modification in cell division or elongation of leaf tissues (Volkenburgh, 1999). The extent of the changes detected in leaf morphology prompted us to check leaf ultrastructure in WT and *ARF4*-as plants. Leaf thickness was clearly reduced in *ARF4*-as plants as compared to wild-type (Figure 3a, b). In fact, the average of leaf blade thickness was 209 ± 8 µm in WT compared to 181 ± 7 µm in *ARF4*-as (Figure 3c). The palisade parenchyma (PP) and spongy parenchyma (SP) tissue layers were thinner in *ARF4*-as plants than in WT plants (Figure 3d-e). The palisade:spongy mesophyll ratio was significantly higher in WT than in *ARF4*-as (Figure 3f). Meanwhile, the intracellular air spaces (IAS) were more conspicuous in *ARF4*-as compared to WT, while no visible difference was observed in adaxial or abaxial epidermis between genotypes (Figure 3g-h).

Consistent with the presence of thinner leaves in the *ARF4*-as mutant plants, photosynthetic performance estimated through photosynthetic $CO_2$ assimilation rate ($A$) was significantly lower than in the WT (Table 1). It is well known that $A$ can be limited by the slowest of two biochemical processes: (1) the maximum rate of ribulose 1,5-biphosphate (RuBP) carboxylase/oxygenase (Rubisco) catalyzed carboxylation ($V_{cmax}$) and (2) regeneration of RuBP controlled by electron transport rate ($J_{max}$) (Bernacchi *et al.*, 2009). Consistently, $V_{cmax}$ and $J_{max}$ exhibited a significant decrease in *ARF4*-as plants, indicating that photosynthesis is reduced due to the silencing of *SlARF4* (Figure 4a).

Stomatal conductance is one of the main parameters that affect photosynthesis under drought stress (Jiang *et al.*, 2006; Siddique *et al.*, 1999). Because stomatal behavior is closely linked to $CO_2$ uptake (Wong *et al.*, 1979), it is considered to be one of the main reasons for reduced photosynthesis (Ashraf and Harris, 2013; Medrano *et al.*, 2002). The response of stomatal conductance ($g_s$) to changing air vapor pressure deficit (*VPD*) was determined in *ARF4*-as and WT plants. At low *VPD*, wild-type plants exhibited high $g_s$ and tended to reduce $g_s$ as *VPD* increased from 1.25 kPa to 2 kPa and maintained a moderate $g_s$ values at higher *VPD*s. Meanwhile, *ARF4*-as plants presented a relatively low $g_s$ values at both low and high *VPD* compared to wild-type plants (Figure 4b). We verified a similarly impaired stomatal response in *ARF4*-as plants in response to step changes in irradiance and $CO_2$ levels (Figure S1), suggesting that *ARF4* could be an important player in the regulation of stomatal movements. Furthermore, intrinsic water-use efficiency was notably higher in *ARF4*-as plants (Table 1) suggesting that the down-regulation of *SlARF4* has the potential to improve the ration of carbon assimilation to transpirational water loss. Plants' tolerance to abiotic stress depends on their ability to adjust the relationship between water, transpiration, photosynthesis and water use efficiency through stomatal changes in order to maximize $CO_2$ assimilation (Zhang *et al.*, 2006). In fact, the decrease in stomatal conductance can enhance plant tolerance to drought stress of many plant species such as chickpea (*Cicer arietinum*), durum wheat (*Triticum turgidum*), Rice (*Oryza sativa*) and cotton (*Gossypium hirsutum*) (Flowers and Yeo, 1981) (Brugnoli and Lauteri, 1991) (Mafakheri *et al.*, 2010) (Rahnama *et al.*, 2011). *ARF4*-as plants showed a decrease in stomatal conductance coupled with an increase in water-use efficiency, however, this can generally lead to a yield penalty through stomatal limitation to carbon assimilation (Blum, 2009; Blum, 2005). Given this potential impact on carbon assimilation, we next sought to examine whether *SlARF4* under-expression might affect plant agronomic parameters. Yield, number of fruits and fruit fresh weight were not altered between WT and

*ARF4*-as plants, whereas fruit shape was altered in *ARF4*-as plants (Figure S3), as expected, given the well-known role of auxin in fruit development (De Jong *et al.*, 2009; Hendelman *et al.*, 2012; Chaabouni *et al.*, 2009; Su *et al.*, 2014; Hao *et al.*, 2015; Breitel *et al.*, 2016).

Successful growth of plants depends mostly on plasticity in leaf anatomical characteristics, which enables plants to cope with diverse stress environments (Pereira-Netto *et al.*, 1999). Leaf architecture plays a vital role in resisting a variety of stresses including drought stress (Balsamo *et al.*, 2006). Structural modifications in leaf anatomy contribute significantly to increasing drought tolerance, particularly under limited moisture conditions (Rhizopoulou and Psaras, 2003). Leaf rolling is a one of the common adaptation to water stress (Kadioglu *et al.*, 2012; Kadioglu and Terzi, 2007). It has been reported that greater leaf rolling may be an important indicator linked to drought tolerance and may have a positive impact on crop yield under water stress conditions (Fen *et al.*, 2015). Plants having leaf rolling mechanism exhibit a resistance to drought and high temperature and had higher water use efficiency (Kadioglu *et al.*, 2012). This was explained by the fact that leaf rolling reduced transpiration rate (Kadioglu and Terzi, 2007; Kadioglu *et al.*, 2012). The combination of a marked leaf curling phenotype associated with decreased $g_s$ in *ARF4*-as plants suggests that these plants might tolerate better water deficit. We thus endeavored to assess the influence of *ARF4* on plants responses to water and salt stress.

### ***SlARF4* expression is altered in response to salt and drought stresses**

The expression pattern of *SlARF4* was analyzed by real-time PCR in leaves and roots exposed to salt or drought stress. *ARF4* gene expression was significantly regulated by salt and drought stresses. *SlARF4* expression was significantly repressed in leaves and roots after 2hours of salt treatment while its expression was significantly induced in leaves and roots after 24 hours (Figure 5a-b). During drought treatment, *SlARF4* gene was slightly induced after 48 hours of stress application in leaves (Figure 5c). In drought stressed roots, *SlARF4* was although

significantly induced after 5 days of stress exposure (Figure 5d). Tomato lines harboring *pARF4*::*GUS* constructs were generated and used to assay *in planta* analysis of *ARF4* expression under stress conditions. We observed strong expression in leaves, primary root and root tip after 24 hours of salt stress exposure (Figure 5e). After five days of drought treatment, GUS activity was detected in the root system and in different parts of the leaf (Figure 5f).

Extensive research has shown that various environmental signals are associated with changes in auxin homeostasis, redistribution, and signaling (Navarro *et al.*, 2006; Park *et al.*, 2007). Accumulating evidence indicates that auxin plays a role in plant responses to abiotic stresses through complex metabolic and signaling networks. Genome-wide expression analyses have suggested that the expression of numerous *ARF* genes change when plants respond to abiotic stresses in various species including rice (Jain and Khurana, 2009), sorghum (Wang *et al.*, 2010), soybean (Van Ha *et al.*, 2013) and banana (Hu *et al.*, 2015). In tomato, expression profiling of the *ARF* family under abiotic stress conditions showed that most of the tomato *ARF*s were responsive and some of them were significantly regulated (Bouzroud *et al.*, 2018). Among them, *SlARF4* expression was significantly induced in tomato plants in response to salt (24 hours) or drought (five days). Additionally, *SlARF4* regulatory region was enriched in *cis*-acting elements specific to salinity and water deficit response (Bouzroud *et al.*, 2018). In this work, *ARF4* expression assessed both *in vitro* and *in planta* was affected by either salt or drought treatment which suggest that this gene might be involved in tomato response to those stresses. We thus evaluated the broad consequences of drought and salt stress in transgenic downregulated (*ARF4*-as) or constitutively expressing (*ARF*-ox) plants.

## ***ARF4* alters the plants response to salt and drought stress**

### ***Shoot and root fresh weight are differentially altered in ARF4 transgenic lines***

Shoot fresh weight was investigated in WT and *ARF4* transgenic plant lines under salt and drought stress conditions. In the absence of stress, fresh weight was significantly higher in the

*ARF4*-ox as compared to the other lines. The average of shoot fresh weight was 1,5 and 2 times higher than that of *ARF4*-as and WT plants respectively (Figure 6a). Two levels of NaCl (100 mM and 150 mM) were applied, and both led to reductions in shoot fresh weight in all genotypes. In response to 150 mM NaCl, shoot fresh weight decreased by 60% and 70% in WT and *ARF4*-ox plants respectively and only by 30% in *ARF4*-as plants (Figure 6a). In roots, the reduction in the fresh weight was around 55%, 28% and 65% for WT, *ARF4*-as and *ARF4*-ox plants lines respectively (Figure 6c). Similarly to salt stress, drought stress affected the growth of the three plant lines. Shoot fresh weight decreased by 66% and 82% respectively for WT and *ARF4*-ox plants and by 44% for *ARF4*-as plants when exposed to 15% of PEG 20000 (Figure 6b). Root fresh weight deceases significantly with the increase of PEG concentration. The decrease reached 57% respectively in WT and *ARF4*-ox plants while the reduction was around 40% in *ARF4*-as in response to 15% of PEG20000 (Figure 6d). The reduction in fresh weight was reported in many plant species which includes barley, sugar beet, cabbage and *sorghum* in response to salinity or water deficit (Jamil *et al.*, 2007; Farooq *et al.*, 2009; Fayez and Bazaid, 2014; Saddam *et al.*, 2014). The decrease in seedling growth (evaluated through the measurement of fresh weight) is due to restricted cell division and enlargement, as salt and drought stress directly reduces growth by decreasing cell division and elongation (Akıncı and Lösel, 2012). However, the reduction in fresh weight was less pronounced in the *ARF4*-as plants which suggest that *ARF4*-as plants might tolerate better salt and drought stress than the other plant lines.

***Root development and density are less affected in ARF4-as plants***

Root development was investigated in *ARF4* transgenic lines in response to different concentrations of NaCl or PEG. Our results showed a strong reduction in primary root length in *ARF4*-ox plants (by 65% in salt stress condition and by 42% in drought stress condition)

while the decrease was nearby 40% and 15% in WT and *ARF4*-as plants in response to salt and drought stress respectively (Figure 6e-f).

Root density was also investigated in the three lines under salt and drought stress conditions. Our results showed that root density significantly increased with the increase of NaCl or PEG concentrations in WT and in *ARF4*-as plants (Figure 6g-h). Root density increases by 57% in WT and 120% in *ARF4*-as, respectively while the *ARF4*-ox showed a significant reduction of in root density (10%) when exposed to 150 Mm of NaCl. In response to drought stress, the three plant lines showed a significant increase in root density. At 5% of PEG 20000, root density increased by 50%, 130% and 43% in WT, the *ARF4*-as and the *ARF4*-ox plants respectively.

Root system is the first organ to encounter salinity and drought stress. Root development can severely be affected by environmental stresses (Kurth *et al.*, 1986). In *Arabidopsis*, root length and development was significantly reduced in response to salinity (Burssens *et al.*, 2000). In this study, we had noticed a significant decrease in primary root length in WT and in *ARF4* transgenic lines in response to salt or drought stress conditions as compared with normal conditions. However, less reduction was reported in the *ARF4*-as. Deeper rooting has been shown to be beneficial for plant production and survival as it increases water uptake which confer the advantage to support plant growth during adverse conditions (Comas *et al.*, 2013). Numerous studies have linked plant stress tolerance with the increase in root length and density for several plant species such as barley, sunflower, wheat, rice and cotton (Munns *et al.*, 2006). *ARF4*-as plants showed less reduction in primary root length coupled with an increase in root density. *ARF4* was found to be implicated in defining root architecture in *Arabidopsis* under optimal growth conditions through the control of lateral root emergence (Marin *et al.*, 2010) which suggest that this gene might play an important role in root

architecture in stress conditions and thus contribute to the improvement tomato tolerance to salinity and water deficit.

***ARF4-as plants are less affected by salt and drought stress***

***Photosynthesis is less affected in ARF4-as plants***

Photosynthesis is among the primary processes to be affected by salinity and drought stress (Chaves, 1991; Munns *et al.*, 2006). Our results showed that total chlorophyll content decreased significantly in WT and *ARF4*-ox plants exposed to different concentrations of NaCl while no significant changes in chlorophyll content was detected in *ARF4*-as plants (Figure S4). At 150 mM of NaCl, the total chlorophyll content decreased only by 4% in the *ARF4*-as plants as compared to the control. Meanwhile, the diminution of total chlorophyll content was around 53,2% and 62,8% respectively in the *ARF4*-ox line and in WT at 150 mM of NaCl compared to the control (Figure S4). The drastic decline in the total chlorophyll content was also observed as a result of drought stress application on WT plants and *ARF4*-ox plants. The reduction in the total chlorophyll content averaged the 37% and the 15% in WT and *ARF4*-ox plants cultivated at 15% of PEG 20000 respectively (Figure S4). Meanwhile, total chlorophyll content increased significantly*ARF4*-as in response to drought stress. In fact, the average of chlorophyll content was 1,2 times higher in *ARF4*-as at 15% PEG 20000 than at 0% PEG 20000. Stress-induced decrease in chlorophyll content have been reported in several plant species including tomato and wheat (Dogan *et al.*, 2010; Marcińska *et al.*, 2012; Saeidi and Abdoli, 2015). Zarafshar *et al.*, (2014) explained this decrease as the result of pigment photo-oxidation and chlorophyll degradation. *ARF4*-as plants showed although a slight decrease in total chlorophyll content in response to salt stress and a slight increase in drought stress conditions. tomato and Pea tolerant genotypes showed less losses in photosynthetic pigments (Hernandez *et al.*, 2000; Furdi *et al.*, 2013). The lack of changes in

chlorophyll content in the *ARF4*-as plants shows the capacity to preserve the photosynthetic apparatus and thus indicates their better tolerance to salinity and drought stress.

***Sugars are highly accumulated in ARF4-as plants in stress conditions***

Soluble carbohydrates content was next investigated in tomato *ARF4* transgenic and WT lines in response to different concentrations of NaCl or PEG. In response to salt stress, leaf soluble sugar content was decreased by 57, 5% and 69, 3% in WT and *ARF4*-ox lines respectively and was increased by 146% in *ARF4*-as line exposed to 150 mM of NaCl for 2 weeks (Figure S4). In roots, the soluble sugar content was also decreased in WT and *ARF4*-ox lines by 49, 4% and 51, 46% respectively, while an increase around the 191% in soluble sugar content was detected in *ARF4*-as line. PEG induced drought stress imposed to plants significantly decreased soluble sugar contents at all the stress levels in WT, *ARF4*-as and *ARF4*-ox lines. In fact, leaf soluble sugar content decreased with the increase of PEG concentration. Leaf soluble sugar content significantly decreased by 42,8%, 43% and 18% respectively in WT, *ARF4*-ox and *ARF4*-as plants exposed to 15% of PEG 20 000. In roots, PEG application induced a significant decrease in soluble sugar content by 41, 1% and 62, 27% (at 15% of PEG 20 000) in WT plants and in *ARF4*-ox plants respectively. Meanwhile, a significant increase in soluble carbohydrates content was although reported in *ARF4*-as plants and reached the 67% at the high level of the PEG as compared to the control (Figure S4). Accumulation of soluble carbohydrates has been associated with drought and salt tolerant mechanisms in many plant species. Sugar accumulation help plants to tolerate abiotic stresses by improving their ability to preserve osmotic balance within the cells (Atkinson & Urwin 2012). Tolerant species and varieties accumulate more soluble sugars in leaves and growing tissues than sensitive ones (Pattanagul and Thitisaksakul, 2008). Soluble sugars lead to buffer cellular redox potential, help to stabilize membranes and protein structures and also provide possible energy source under severe constraints (Keunen *et al.*, 2013; Rosa *et al.*, 2009; Sami

*et al.*, 2016). Sugar accumulation in the *ARF4*-as plants might improve tolerance to salinity and water stress by increasing cellular osmolarity which promotes the influx of water into cells, or at least a reduced water efflux providing the turgor necessary for cell expansion.

Carbohydrates produced in leaves during photosynthesis are transported throughout the plant by SUTs in order to support plant growth. The *SUT1* proteins are involved in the movement of sucrose into and out of the source and sink tissues and through the phloem via apoplastic pathways. Thus, it is accepted that SUTs are important for plant growth and stress tolerance (Jia *et al.*, 2015). Investigating the expression of the sucrose transporter *SlSUT1* under salt and drought stresses had revealed that its expression was induced in WT and the *ARF4*-as leaves and roots exposed to salt stress while *SlSUT1* was significantly repressed in the *ARF4*-ox plant line (Figure S5). These findings were also detected in response to drought stress. In drought stressed leaves, *SlSUT1* was significantly induced in WT and *ARF4*-as plants whereas its expression was significantly down-regulated in *ARF4*-ox plants. In roots, the expression of *SlSUT1* was also up-regulated significantly. The sucrose transporters have also been shown to be regulated by abiotic stresses such as salt stress in potato celery and rice (Kühn *et al.*, 1997; Noiraud *et al.*, 2000). *SUT1* transcript increased by 40% in sugarcane leaves after 24 hours PEG treatment (Patade *et al.*, 2012). An increase in *SUT1* expression was also reported in sweet sorghum salt tolerant genotype (Sui *et al.*, 2015). Thus, the up-regulation of *LeSUT1* in *ARF4*-as might improve its tolerance to drought and salinity through the stimulation of sucrose transport required for the osmoregulation and for the cellular energy demands during drought and salinity stresses.

***ARF4-as plants showed lower stomatal conductance***

Stomata play an imminent role in plant response to environmental changes as they control both water losses and $CO_2$ uptake (Damour *et al.*, 2010). Stomatal conductance was estimated in WT and *ARF4*-as plants in salt or drought stress (Figure 7a-b). Under normal conditions,

*ARF4*-as plants exhibited low stomatal conductance as compared to WT plants. Salt and drought stress application induces a significant decrease in stomatal conductance in WT. In fact, the reduction in stomatal conductance observed in the WT was around 32% and 46% in response to salt and drought stress respectively. Meanwhile, no significant changes in stomatal conductance were detected in *ARF4*-as plants that still maintained the same values of stomatal conductance observed in normal conditions Salt and drought tolerance was correlated with a decline in stomatal conductance in many plant species such as chickpea, maize, common bean and cotton (Brugnoli and Lauteri, 1991; Heschel and Riginos, 2005; Miyashita *et al.*, 2005; Mafakheri *et al.*, 2010; Anjum *et al.*, 2011) .

Stomatal closure can be trigged by various internal and external factors. Schultz, (2003) have shown that the decline in leaf water potential can trigger stomatal closure under stressful conditions. Relative water content (RWC) was investigated in WT and *ARF4*-as plants. A remarkable decrease in RWC was observed in the three plant lines (Figure 7c). Up to 25% and 15% of water losses was recorded during the first 30 minutes of dehydration in WT and *ARF4*-as plants respectively. RWC reached 72% in WT and after 4 hours of dehydration while a 50% decrease of water losses was found in *ARF4*-as plants. The maintenance of a relatively high RWC during mild drought is indicative of drought tolerance in wheat, barley, sugarcane, rice and maize (Roy and Basu, 2009). The maintenance of higher RWC in *ARF4*-as under stress conditions can possibly be the result of the stomatal closure and suggest that these plants might better tolerate dehydration stress than WT.

**_ARF4_-as plants exhibited a high ABA content**

Abscisic acid is identified as the key hormone that mediates plant responses to adverse environmental stimuli. Stomatal conductance can also be trigged by the subsequent accumulation of ABA (Tombesi *et al.*, 2015). Endogenous ABA content of 5 weeks *ARF4*-as and WT plants in normal or under salt and drought conditions were then measured in leaf

tissues. Our results showed that the ABA content in *ARF4*-as increased by 120% and 101% under salt and drought treatment respectively whereas in WT plants increased by only 20% in salt conditions and decreased by 26% in drought treatment (Table 2). The lowest stomatal conductance observed in *ARF4*-as as compared with WT plants may be associated with the accumulation of relatively more ABA in *ARF4*-as stressed leaves and suggests that *ARF4*-as plants might tolerate better salt and drought stresses than WT plants.

Endogenous ABA content is related to the balance between its biosynthesis and its degradation. In tomato, *SlNCED1* and *SlNCED2* encoding 9-cis-epoxycarotenoid dioxygenase are the primary genes responsible for ABA biosynthesis, whereas *SlCYP707A1*, *SlCYP707A2*, *SlCYP707A3* and *SlCYP707A4* encoding ABA 8′-hydroxylase are main genes for ABA catabolism (Yang *et al.*, 2014). Investigating the expression of these genes in WT and the *ARF4*-as plants had revealed that their expressions were significantly regulated by salt or drought stress application (Figure 8). Indeed, *SlNCED1* expression was significantly induced in *ARF4*-as leaves after 2 hours and 24 hours of salt stress exposure while no significant changes was observed in roots. In WT, the expression of *SlNCED1* was significantly up-regulated in leaves and roots after 24 hours of salt treatment. In response to drought stress, the expression of *SlNCED1* was significantly up-regulated in *ARF4*-as roots while no significant change in *SlNCED1* expression was detected in WT plants. *SlNCED2* expression was significantly induced in the *ARF4*-as leaves after 2 hours and 24 hours of salt application and in WT after 24 hours. A significant increase in the expression of *SlNCED2* was detected in *ARF4*-as plants in response to drought stress. Water stress is known to induce the expression of *NCED* genes in several plant species which include tomato, maize, common bean, maize, Arabidopsis, cowpea and avocado (Tan *et al.*, 1997; Burbidge *et al.* 1999; Qin & Zeevaart 1999; Chernys and Zeevaart, 2000; Iuchi, Kobayashi, Yamaguchi-Shinozaki & Shinozaki 2000; Iuchi *et al.* 2001). Moreover, transgenic plants overexpressing the *NCED* gene

accumulated large amounts of ABA and were more resistant to drought stress (Thompson *et al*. 2000; Wan & Li 2006).

The expression pattern of *SlCYP707A1*, *SlCYP707A2* and *SlCYP707A3*, key ABA degradation genes was also assessed in *ARF4*-as and WT plants under salt and drought stress conditions (Figure 8). Quantitative RT-PCR results showed that the expression of the ABA metabolism genes was up-regulated in WT leaves after 2 hours and 24 hours of salt stress exposure. In roots, the expression of *SlCYP707A1* and *SlCYP707A3* was up-regulated in WT plants in response to salt stress while a significant repression was reported in the expression of *SlCYP707A2*. Although, the expression of the ABA metabolism genes expression was repressed in the *ARF4*-as salt stressed roots. In response to drought stress, the expression of *CYP707A1*, *CYP707A2* and *CYP707A3* was repressed in *ARF4*-as plants while their expression was up-regulated in WT roots and down-regulated in leaves. An increase in the transcript of *CYP707A* genes was reported in response to salt or drought stress in many plant species including common bean, Arabidopsis and Peanut (Saito *et al*. 2004; Yang & Zeevaart 2006; Liu *et al*. 2014). *Arabidopsis* knock-out mutant *cyp707a3-1* accumulated higher endogenous ABA levels coupled with a reduced transpiration rate, thereby resulting in a phenotype exhibiting enhanced tolerance to drought stress (Umezawa *et al.* 2006). Down-regulation of *CYP707A* genes in *ARF4*-as plants in response to salt and drought stress might prevent ABA degradation. The significant increase in *SlNCED1* expression in *ARF4*-as leaves and roots along with the repression of the three ABA catabolism genes in normal and salt stress conditions might explain the increase in the concentration of ABA in leaves.

**Antioxidant genes expression is altered in response to salinity and drought stress**

In plants, abiotic stresses induce the overproduction of reactive oxygen species (ROS), a highly reactive and toxic molecules that cause damage to proteins, lipids, carbohydrates and DNA and results ultimately in oxidative stress (Das and Roychoudhury, 2014). The ability of

plants to mitigate the negative effects of abiotic stresses relays on the efficiency of the antioxidant defense systems to protect plant cells from oxidative damage by scavenging ROS accumulation The key enzymatic antioxidants are catalase, superoxide dismutase (SOD), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) (Choudhury *et al.* 2013).

***Cat1 (Catalase) expression in response to salt and drought stress***

Catalases are tetrameric heme-containing enzymes with the potential to directly dismutate $H_2O_2$ into $H_2O$ and $O_2$ and essential for ROS detoxification (Das and Roychoudhury, 2014). In angiosperms, catalase is encoded by three genes known as *Cat1*, *Cat2* and *Cat3* (Choudhury *et al.*, 2013). We investigated *Cat1* expression in WT and *ARF4* transgenic lines exposed to salt and drought stresses. Our results showed that *Cat1* expression was induced in *ARF4*-as plants exposed to salt stress while its expression was slightly repressed in the *ARF4*-ox plants. A high up-regulation in *Cat1* expression was although detected in the wild type plant roots in response to salt stress (Figure S6). In drought stressed leaves, *Cat1* expression was significantly induced in WT while a remarkable but not significant repression was observed in *ARF4* transgenic lines. Meanwhile, *Cat1* gene expression was highly up-regulated in WT and *ARF4*-as plant roots (Figure S6). Numerous studies have linked abiotic stress tolerance to an overproduction of catalase in many plant species such maize, potato and rice (Moriwaki *et al.*, 2008; M'Hamdi *et al.*, 2009; Gondim *et al.*, 2012). An increase in *Cat* transcript and activity was reported in wheat in response to drought stress and sweet potato exposed to salt and drought stresses (Luna *et al.* 2005; Yong *et al.* 2017). The overproduction of a bacterial *cat1* gene improved tolerance to salinity in tomato and tobacco (Al-Taweel *et al.* 2007; Mohamed *et al.* 2003). Introduction of the same catalase gene into Rice was reported to increase resistance to salt (Moriwaki *et al.*, 2008). *Cat1* gene was significantly up-regulated in

*ARF4*-as plants might contribute to the improvement of their tolerance to salinity and water stress.

***SOD (Superoxide dismutase) expression in response salt and drought stress***

Superoxide dismutase (SOD) is an enzyme that belongs to the family of metalloenzymes ubiquitous in all aerobic organisms. It is one of the first defense against ROS induced damages by catalyzing the removal of $O_2^-$ by dismutating it into $O_2$ and $H_2O_2$ (Choudhury *et al.*, 2013; Das and Roychoudhury, 2014). Based on the metal ion it binds, SOD are classified into three isozymes; Mn-SOD (in mitochondria), Fe-SOD (in chloroplasts) and Cu/ZnSOD (in cytosol, peroxisomes and chloroplasts) (Das and Roychoudhury, 2014). Investigating the expression of *Cu/ZnSOD* had revealed that its expression was remarkably repressed in WT plants and *ARF4*-ox plants subjected to salt and drought stresses. Meanwhile, *Cu/ZnSOD* gene expression was induced in *ARF4*-as leaves in response to salt and drought stresses while its expression was repressed in the roots (Figure S6).

Abiotic stresses such as salinity and water deficit induce the generation of superoxide in plant cells because of impaired electron transport in the chloroplasts due in part to the accumulation of high concentrations of iron (Wang, Wang, Kwon, Kwak & Su 2005). Several studies had stated a significant increase in SOD activity in response to osmotic stress in many plant species such as tomato (Gapińska *et al.*, 2007), Chickpea (*Cicer arietinum*) (Kukreja *et al.*, 2005), potato (Aghaei *et al.*, 2009) and mulberry (Ahmad *et al.*, 2014). Pea (*Pisum sativum*) tolerant genotypes responded to NaCl stress by increasing mitochondrial Mn-SOD and chloroplastic CuZn-SOD and ascorbate peroxidase activities (Hernández *et al.*, 1993). Szőllősi, (2014) reported an increase in the activity of total SOD in *Olea europaea* leaves or in shoots of *Oryza sativa* cultivars in response to drought stress. An increase in SOD activity was also detected in both tolerant and non-tolerant tomato species subjected to water deficit (Rahman *et al.* 2004). This finding was also demonstrated in *P. vulgaris* (Zlatev *et al.*, 2006),

*Trifolium repens* (Chang-Quan and Rui-Chang, 2008) and *Picea asperata* (Yang *et al.*, 2008). Genetic manipulation of genes encoding SOD isoforms increased plant tolerance to abiotic stress conditions. In *Arabidopsis*, the constitutive expression of *MnSOD* gene improved salt tolerance in transgenic plants (Wang *et al.*, 2004). The introduction of tomato Cu/ZnSOD in potato plants showed increased tolerance to paraquat (Perl *et al.*, 1993). The overexpression of Rice cytosolic Cu/Zn-SOD in chloroplasts of tobacco plant improved their photosynthetic performance during photooxidative stresses such as high salt or drought stresses (Badawi *et al*. 2004). Knowing that SOD iso-enzymes act as an early scavenger, keeping the superoxide radical at low level, preventing oxidative damage and higher photosynthetic rate which improves plant growth in limited conditions. The up-regulation of Cu/ZnSOD in the *ARF4*-as might reduce oxidative stress caused by abiotic stress and might increase tomato survival under salt and drought stress conditions.

***mdhar (Monodehydroxyascorbate reductase) expression in response to salt and drought stress***

Monodehydroxyascorbate reductase (MDHAR) is another enzymatic antioxidant indirectly involved in the ROS scavenging through regenerating Ascorbic acid, indispensable for the dismutation of $H_2O_2$ by APX enzyme (Das and Roychoudhury, 2014). Investigating the expression pattern of *mdhar* gene in salt stress conditions had revealed a significant up-regulation in *ARF4*-as leaves and roots while its expression was repressed in *ARF4*-ox and WT plants (Figure 18). In response to drought stress, the expression of *mdhar* was significantly up-regulated and repressed respectively in WT and the *ARF4*-ox plant leaves. In roots, *mdhar* is significantly up-regulated and down-regulated in the *ARF4*-as and *ARF4*-ox plant line respectively. Several studies had underlined the protective role of *mdhar* in plant tolerance to various abiotic stresses. (Sharma and Shanker Dubey, 2005) had reported an increase in MDHAR activity in rice seedlings exposed to drought stress. This finding was also

stated in wheat (*Triticum aestivum*) and in mulberry (*Morus alba*) when subjected to drought stress (Reddy *et al.*, 2004). The overexpression of *mdhar* in transgenic tobacco increased the tolerance against salt and osmotic stresses (Eltayeb *et al.*, 2006). MDHAR plays an important role in the Ascorbate- Glutathione ROS detoxification pathway through restoring ascorbate (AsA) used as an electron donor to scavenger $H_2O_2$ molecules (Anjum *et al.*, 2010). Thus, the up-regulation of *mdhar* gene in the ARF4-as plants might increase their tolerance to salinity and water deficit by providing the necessary substrate for the degradation of $H_2O_2$.

## Conclusion

In the face of a global scarcity of water resources and the increased water and soil salinization, abiotic stresses present major challenges in sustaining crop yield. Plant tolerance to these stresses relays on the implementation of molecular mechanisms for cellular adjustments, including signal perception and transduction cascades, transcriptional networks and adaptive metabolic pathways. Plant hormones such as ABA, ethylene and SA play an important role in mediating plant responses to stresses. Auxin, the key regulator of many aspects of plant growth and development, was identified as an actor in plant response to biotic and abiotic stress. ARFs play crucial role in auxin signaling. *ARF4* is involved in the regulation of several processes such as lateral root development, fruit quality and development (Marin *et al.*, 2010; Jones *et al.*, 2002; Sagar *et al.*, 2013). In this work, we investigated the involvement of this gene in the acquisition of salt and drought tolerance in tomato. Using a reverse genetic approach, we found that the down-regulation of *SlARF4* increases tomato tolerance to salinity and drought stress. Indeed, the down-regulation of *SlARF4* increases root length and density resulting in a better development of root system which might increase water acquisition and minimize water losses and thus stimulate plant growth under adverse conditions. Furthermore, *ARF4*-as plants displayed a severe leaf curling, a lower stomatal conductance, a higher water use efficiency under normal conditions

and maintained high level of chlorophyll content even in stress conditions suggesting that their photosynthetic activity was less affected by the stress application. Total carbohydrates were accumulated in high proportion in photosynthetic tissues of *ARF4*-as plants. At the molecular level, the expression of the sucrose transporter *LeSUT1* was significantly up-regulated in *ARF4*-as which might explain carbohydrates accumulation in root tissues. *cat1*, *Cu/ZnSOD* and *mdhar* genes were found to be up-regulated in *ARF4*-as plants suggesting that *ARF4*-as mutant is more tolerant to salt and water stress. Furthermore, *SlARF4*-as plants showed a significant increase in ABA content associated with a low stomatal conductance under stressful conditions. This increase in ABA content is due to the activation of ABA biosynthesis genes and the repression of ABA catabolism genes. Taking together, the data presented in this work brings new elements on auxin involvement in stress tolerance in tomato and underline the role of *ARF4* in this process and provides new insights into tomato selection and breeding for environmental stress-tolerant.

## Experimental procedure

### Plant material

Wild-type tomato (*Solanum lycopersicum*, L.) cv Micro-Tom (WT), and the following *AUXIN RESPONSE FACTOR 4* lines were used in this study: an *ARF4* RNAi antisense silenced line (*ARF4*-as), a line with *ARF4* expression driven by the constitutive cauliflower mosaic virus (CaMV) promoter 35S (*ARF4*-ox), and a reporter line with the β-glucoronidase (GUS) gene driven by the native *ARF4* promoter region (pARF4::GUS). *ARF4*-as plant lines and *ARF4*-ox plant lines were previously generated and well-characterized by Sagar *et al.*, (2013). For each mutant, the most representative plant line was chosen.

### Characterization of *SlARF4*-as plants

#### *Plant growth conditions*

Seeds of WT and *ARF4*-as transgenic plants were sown in germination trays with commercial substrate (Tropstrato HT Hortaliças, Vida Verde) and supplemented with 1g $L^{-}1$ 10:10:10 NPK and 4 g $L^{-1}$ dolomite limestone ($MgCO_3$ + $CaCO_3$). After the emergence of the first pair of true leaves, the seedlings were transferred to plastic pots (350mL) with the same substrate described above, supplemented with 8 g $L^{-1}$ 10:10:10 nitrogen: phosphorus: potassium (N:P:K) and 4 g $L^{-1}$ lime. The experiment was conducted in greenhouse localized at Universidade Federal de Viçosa (642 m asl, 20°45' S; 42°51' W), Minas Gerais, Brazil, under semi-controlled conditions (mean temperature of 28°C and 450-500 μmol $m^{-2}$ $s^{-1}$ PAR irradiance). Irrigation was performed twice a day, where each plastic pot received the same volume of water.

***Growth analyses***

Plant height and internode length were measured 35 days after germination (DAG). At 45 DAG the plants were harvest and divided into leaves, stem and root. Leaf area was measured using leaf area meter (Li-Cor Model 3100 Area Meter, Lincoln, NE, USA). Leaf, stem and root were then packed separately in paper bags and oven dried at 70°C for 72h until they reached constant weight. Shoot and root biomass were measured from the dry weight of leaves, stem and root. Specific Leaf Area (SLA) was determined as described by Hunt, (1982).

***Microscopy***

For anatomical analysis, leaf discs were collected from the medium point of the fifth lateral leaflet and fixed in FAA50 (Formaldehyde, acetic acid and ethanol 50%) for 48 h, and then stored in ethanol 70%. Samples were infiltrated with historesin (Leica Microsystems, Wetzlar, Germany) and cut in cross sections with ~5 μm (RM2155, Leica Microsystems, Wetzlar, Germany). Leaf cross sections were mounted in water on glass slides and stained with toluidine blue. Histological sections were observed using an optic microscope (AX-70 TRF,

Olympus Optical, Tokyo, Japan) and then photographed using digital photo camera (Zeiss AxioCam HRc, Göttinger, Germany). Anatomical features, as leaf thickness and thickness cell layers, were analyzed using Image-Pro Plus® software (version 4.5, Media Cybernetics, Silver Spring, USA).

***Measurements of Photosynthetic Parameters***

Photosynthetic parameters were performed using an open-flow infrared gas exchange analyzer system (Li 6400XT, Li-Cor, Lincoln, USA) equipped with an integrated fluorescence chamber (Li-6400-40; Li-Cor Inc.). All measurements were made on terminal leaflets of intact and completely expanded leaves. The analysis was conducted under common conditions for photon flux density (1000 μmol m-2 s -1), leaf temperature (25 ± 0.5ºC), leaf-to-air vapor pressure difference (16.0 ± 3.0 mbar), air flow rate into the chamber (500 μmol s-1), reference $CO_2$ concentration of 400 ppm using an area of 2 $cm^2$ in the leaf chamber.

*A*/Ci curves were determined initiated at an ambient $CO_2$ concentration of 400 μmol $mol^{-1}$ under a saturating photosynthetic Photon Flux Density (PPFD) of 1000 μmol $m^{-2}$ $s^{-1}$ at 25°C under ambient $O_2$ supply. $CO_2$ concentration was decreased to 50 μmol $mol^{-1}$ of air in step changes. Upon the completion of the measurements at low $C_a$, $C_a$ was returned to 400 μmol $mol^{-1}$ of air to restore the original *A*. Next, $CO_2$ was increased stepwise to 1600 μmol $mol^{-1}$ of air. The maximum rate of carboxylation ($V_{cmax}$), maximum rate of carboxylation limited by electron transport ($J_{max}$) and triose-phosphate utilization (TPU) were estimated by fitting the mechanistic model of $CO_2$ assimilation proposed by Farquhar *et al.*, (1980). Corrections for the leakage of $CO_2$ into and out of the leaf chamber of the LI-6400 were applied to all gas-exchange data as described by Rodeghiero *et al.*, (2007).

***Kinetics of stomatal conductance***

The evaluation of stomatal behavior in response to light and $CO_2$ variation was performed in WT and *ARF4*-as plants 40 DAG, under laboratory conditions (temperature 22-25°C; relative

humidity 50-60% and radiation of 150-200 µmol $m^{-2}$ $s^{-1}$), using an open-flow infrared gas exchange analyzer system (Li 6400XT, Li-Cor, Lincoln, USA). The variation of $g_s$ was performed on the fifth or sixth fully expanded leaf. For step-change evaluation of *g*s in response to light intensity (Lawson *et al.*, 2010), leaves were stabilized for 30 min in the dark and then radiation was increased to 1000 µmol $m^{-2}$ $s^{-1}$, and allowed to stabilized for 240 min. Subsequently, light was turned off and observations recorded for another 100 min. The alternation in the amount of light was based on the results presented by. The response of $g_s$ to $CO_2$ was performed in a similar manner to those described above for light response. The analysis was performed in a range of 240 minutes, with changes in $CO_2$ concentration (400-800-400 µmol $CO_2$ $m^{-2}$ $s^{-1}$).

***Productivity traits***

The productivity parameters were measured in eight plants per genotype (WT and *ARF4*-as). The average fruit weight was determined after individual weighing of each fruit, using a semi analytical balance with a sensitivity of 0.01 g (Shimadzu® AUY220 model). Equatorial and polar diameter was measured using a digital pachymeter (Jomarca *STAINLESS HARDENED*). The determination of the soluble solids content (°Brix) was performed in 10 fruits per plant using a digital temperature-compensated refractometer, model RTD 45 (Instrutherm®, São Paulo, SP).

**Salt and drought stress analyses**

***Plant growth and stress conditions***

WT tomato seeds were sterilized for 10 min in 50% sodium hypochlorite, rinsed four times with sterile distilled water and sown in pots containing peat. Then they were incubated in a culture room with 16h light/ 8h dark photoperiod and 25± 2°C temperature. After 3 weeks, plants were subjected to salt and drought stresses. Salt stress was performed by watering daily the plants with 250mM of NaCl solution. Control plants were daily watered with distilled

water. Leaves and roots samples were harvested after 2 and 24 hours of salt stress application. Drought stress was performed on three week-old plants by water holding for 48 hours and for 5 days. Watering continued normally throughout for control plants. Leave and root samples were collected after drought stress application. Three biological replicates were done for each condition.

***RNA extraction***

Total RNA was extracted from leaves and roots samples by using The Plant RNeasy extraction kit (RNeasy Plant Mini Kit, Qiagen, Valencia, CA, USA). To remove any residual genomic DNA, the RNA was treated with an RNase-Free DNase according to the manufacturer's instruction (Ambion® DNA-*free*™DNase). The concentration of RNA was accurately quantified by spectrophotometric measurement and 1µg of total RNA was separated on 2% agarose gel to monitor its integrity. DNase-treated RNA (2µg) was then reverse-transcribed in a total volume of 20µl using the Omniscript Reverse Transcription Kit (Qiagen,).

***Real time PCR***

The real-time quantification of cDNA corresponding to 2µg of total RNA was performed in the ABI PRISM 7900HT sequence detection system using the QuantiTech SYBR Green RT-PCR kit (Qiagen). The Gene-specific primers used are listed in Supplementary Table 1. The reaction mixture (10µl) contained 2µg of total RNA, 1,2 µM of each primer and appropriate amounts of enzymes and fluorescent dyes as recommended by the manufacturer. Actin gene was used as reference. Real-Time PCR conditions were as follow: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min, and finally one cycle at 95°C for 15 s and 60°C for 15 s. Three independent biological replicates were used for real-time PCR analysis. For each data point, the $C_T$ value was the average of $C_T$ values obtained from the three biological replicates.

***Histochemical analysis of GUS expression***

Transgenic plants expressing pARF4::GUS were generated by *A. tumefaciens*-mediated transformation according to H., Wang *et al.*, (2005). For that, PCR was performed on the genomic DNA of tomato 'Micro-Tom' (10 $ng.ml^{-1}$) using specific primers. The corresponding amplified fragment was cloned into the pMDC162 vector (Curtis and Grossniklaus, 2003)containing the GUS reporter gene using Gateway technology (Invitrogen). The cloned *SlARF* promoter was sequenced from both sides using vector primers in order to see whether the end of the promoter is matching with the beginning of the reporter gene. Sequencing results analyses were carried out using the Vector NTI (Invitrogen) and ContigExpress software by referring to ARF promoter sequences.

After being surface sterilized, pARF4::GUS seeds were cultivated in Petri dishes containing half strength Murashige & Skoog medium for 7 days in a growth chamber at 25°C with 16h light/ 8h dark cycle. One week plants were then grown hydroponically for two weeks in Broughton & Dillworth (BD) liquid medium (Broughton and Dilworth, 1971). Three week-old plants were subjected to salt and drought treatment. Salt stress was performed by adding 250 mM of NaCl to the culture medium. After 24 hours of the salt stress application, plants were incubated overnight at 37°C in GUS buffer (3 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide [Duchefa Biochemie, Haarlem, The Netherlands], 0.1% v/v Triton X-100 [Sigma, Steinhaim, Germany] 8 mM β-mercaptoethanol, 50 mM $Na_2HPO_4/NaH_2PO_4$ [pH 7.2]) then followed by a destaining in 70% EtOH.. Drought stress was conducted by adding 15% PEG 20000 to the liquid culture solution. Plants were collected after five days of stress application and were GUS-stained as described above. For each stress condition, control plants were cultivated in BD liquid medium for the same period.

***Stress tolerance assays in the transgenic tomato plants***

WT, *ARF4*-as and *ARF4*-ox seeds were first surface-sterilized for 10 min in 50% sodium hypochlorite, rinsed four times with sterile distilled water. They were then cultured in petri dishes containing half strength Murashige & Skoog (MS) medium for 7 days in a growth chamber at 25° with 16h light/ 8h dark cycle. One week plants were then grown hydroponically in pots containing 1L of BD medium for two weeks in the same growth conditions (25° with 16h light/ 8h dark cycle) (Broughton and Dilworth, 1971). Three weeks plants were then subjected to salt and drought stresses. Salt stress was performed by adding 100 mM or 150 mM NaCl to the liquid BD medium. Leaves and roots samples were harvested after 2 weeks of treatment. Drought treatment was conducted as follows: three weeks plants were subjected to drought stress by adding 5% or 15% PEG 20 000 corresponding to final osmotic potentials of -0,09 MPa and -0,28 MPa to the hydroponic solution for 2 weeks. Meanwhile, the control plants were grown normally in BD medium. Leaves and roots samples were collected after 2 weeks of culture for both stressed and unstressed plants. For each treatment, the hydroponic solution was renewed each 3 days.

***Determination of shoot and root fresh weights***

Leaf and root fresh weights were determined as followed: stressed and unstressed plants from each line were harvested and rinsed thoroughly with distilled water (DW). The plants were blot dried on blotting sheet and cut into shoots and roots. The fresh weight of each part of the plant was measured and the mean of shoots and roots weights was determined based on at least twelve plants per line in each condition.

***Determination of primary root length and lateral root density***

Primary root length was determined was determined as followed. Pictures for each plant were analyzed using ImageJ software in order to determinate the root length. Twelve independent plants from each line under control and stress conditions were used to calculate the mean.

The lateral root number was determined by counting the number of emerging roots and the mean was calculated based on at least twelve plants per line in each condition. The lateral root density (LR density) was determined using the following equation: number of LRs / the length of the root.

***Determination of chlorophyll content***

Determination of chlorophyll content of each line under stressful and normal conditions was performed as described in Bassa *et al.*, (2012). A 100 mg aliquot of leaves collected from stressed and unstressed plants was weighted and ground with 1 ml of 80% acetone. The liquid obtained was centrifuged for 1 min at 10000 rpm to remove any remaining solid tissue. Samples were analyzed by spectrophotometry at two wavelengths, 645 and 663 nm, using 80% acetone as the blank. The total chlorophyll content was determined using the following equations: Total Chlorophyll Content = 20.2 × Chl a + 8.02 × Chl b ( Chl a = 0.999A663 – 0.0989A645 and Chl b = –0.328A663+1.77A645)

***Determination of soluble sugar content***

Total soluble sugar content was determined as in Dubois *et al.*, (1956). 100 mg of fresh leaves and roots of each sample were homogenized in 5 ml of 80 % ethanol in test tubes. Test tubes were kept in water bath of 80 °C for 1 hour, and sample extracts were transferred to another test tube, and 0.5 ml distilled water and 1 ml of 5 % phenol then added and allowed to incubate for 1 hour. Finally after 1 hour, 2.5 ml sulphuric acid was added to the test tubes, and shaken well on an orbital shaker. Absorbance was read at 485 nm on Spectrophotometer. Sucrose was used as standard.

***Determination of leaf stomatal conductance***

Leaf abaxial stomatal conductance of the youngest fully expanded leaf (usually the fifth leaf, counting from the base) of stressed and well-drained plants of each studied line were determined after two weeks of stress treatment using a hand-held leaf diffusion poromoter

(SC-1 LEAF POROMETER, Decagon, USA). For each line, three biological replicates were conducted at each condition.

***Determination of ABA content***

ABA measurement assays were performed as described (Forcat *et al.*, 2008). Briefly, 110 mg of frozen tissue were extracted at 4°C for 30 min with 400 µl of $H_2O$ with 10% methanol + 1% acetic acid. The internal standard was 2H6 ABA. The extract was centrifuged at 13,000 g for 10 min at 4°C. The supernatant was carefully removed and the pellet re-incubated for 30 min with 400 µl of methanol-acetic acid mix. Following the centrifugation, the supernatants were pooled. Extracts were then analyzed by LC-MS using an Acquity UPLC coupled to a XevoQtof (Waters, Massachusetts, USA). Analysis parameters were described in (Jaulneau *et al.*, 2010).

***Determination of Relative water content (RWC)***

Tomato fully expanded leaves were excised and fresh weight (FW) was recorded every 30 minutes. The excised leaves were then allowed to float on deionised water for about 24 h and turgid weight (TW) was recorded. Leaves were dried at 80 °C for 24 h and dry weight (DW) recorded. Finally, RWC was calculated according to Smart and Bingham, (1974).

***Quantitative expression assays***

Three weeks WT, *ARF4*-as and *ARF4*-ox plants grown hydroponically in BD medium were subjected to either 150 mM of NaCl or 15% of PEG. Leaves and root samples were harvested after 2 hours and 24 hours for salt stress and after 48 hours for drought. RNA extraction, cDNA synthesis and real time PCR were performed as previously described in paragraph "ARF4 gene expression under salt and drought stress conditions". The Gene-specific primers used are listed in Supplementary Table1.

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**Conflict of interest**

All Authors have read, edited and approved the final version of the manuscript. They have declared that no competing interests exist.

**Supporting information**

**Figure S1. stomatal responses to irradiance and $CO_2$ levels in Micro-Tom (WT) and *ARF4*-as transgenic line.** Stomatal conductance ($g_s$) response in tomato plants cv. Micro-Tom (WT) and isogenic *ARF4* antisense transgenic line (*ARF4*-as) from dark-adapted (0 µmol $m^{-2}$ $s^{-1}$) leaves exposed to light (1000 µmol $m^{-2}$ $s^{-1}$) and on subsequent transfer back to darkness (0 µmol $m^{-2}$ $s^{-1}$) in 350 minutes interval (n=5). Stomatal conductance in response to $CO_2$ elevation and subsequent decrease to ambient $CO_2$ (400-800-400 µmol $CO_2$ $m^{-2}$ $s^{-1}$) in 240 minute interval (n=4). Measurements were performed using a LI-6400; LI-COR gas exchange chamber in plants aged 40 days after germination. Data presented are mean ± SE obtained using the 5$^{th}$ leaf totally expanded.

**Figure S2. Net $CO_2$ assimilation rate (*A*) and stomatal conductance ($g_s$) in Micro-Tom (WT) and isogenic *ARF4* antisense transgenic line (*ARF4*-as).** (a) $CO_2$ assimilation rate (*A*) as a function of stomatal conductance (*g*s), each point represents one measurement on an individual plant. Line fitted by linear regression. (b) net $CO_2$ assimilation rate (*A*). (c) stomatal conductance ($g_s$). (d) intrinsic water efficiency ($A/g_s$). Values are means ± s.e.m (n=8). Asterisks indicate values that were determined by Student's *t* test to be significantly different ($P < 0.05$) between genotypes.

**Figure S3. Productive parameters in tomato Micro-Tom (WT) and *ARF4*-as transgenic line.** (a) and (b) representative tomato plants in reproductive stage. (c) representative fruits of a single plant. (d) Number of fruit. (e) Fruit fresh weight. (f) and (g) equatorial and polar diameter of the fruit, respectively Values are means ± s.e.m (n=8 plants).Asterisks indicate values that were determined by Student's *t* test to be significantly different ($P < 0.05$) from Micro-tom (WT). Scale bar = 1cm.

**Figure S4. Photosynthesis and sugar accumulation in WT, *ARF4*-as and *ARF4*-ox plant lines exposed to different concentrations of NaCl or PEG.** (a) and (b) total chlorophyll content in salt and drought stress conditions respectively, (c) and (d) leaf soluble sugars in salt and drought stress conditions respectively, (e) and (f) root soluble sugars content in salt and drought stress conditions respectively. Salt and drought stresses were performed on three weeks tomato plants for two weeks by adding 100mM of NaCl or 150 mM of NaCl for salt stress or 5% or 15% of PEG 20 000 for drought. Values are mean ± SD of three biological replicates. Bars with different letters indicate the statistical significance ($p<0,05$) according to Student Newman-Keuls test.

**Figure S5. Expression of sucrose transporter *LeSUT1* in WT, *ARF4*-as and *ARF4*-ox plants exposed to salt or drought stresses.** (a) gene expression in leaves and roots exposed to 150mM of NaCl, (b) gene expression in leaves and roots leaves exposed to 15% PEG. ΔΔCt refers to fold differences in gene expression relative to untreated plants. Values are mean ± SD of three biological replicates. Stars (*) indicate the statistical significance ($p<0,05$) according to Student's t-test.

**Figure S6. Expression of *Cat1*, *mdhar* and *SOD* in WT, *ARF4*-as and *ARF4*-ox plants exposed to salt or drought stresses.** (a) gene expression in leaves and roots exposed to 150mM of NaCl, (b) gene expression in leaves and roots leaves exposed to 15% PEG. ΔΔCt

refers to fold differences in gene expression relative to untreated plants. Values are mean ± SD of three biological replicates. Stars (*) indicate the statistical significance (p<0,05) using Student's t-test.

**Supplementary Table S1:** Gene ID and quantitative RT-PCR primers

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## Tables

**Table 1**. **Characterization of photosynthetic parameters in tomato cv. Micro-Tom (WT) and isogenic *ARF4* antisense transgenic line (*ARF4*-as).** A: photosynthetic $CO_2$ assimilation rate; gs: stomatal conductance; $V_{cmax}$: *maximum Rubisco carboxylation rate* ; $J_{max}$: maximum electron transport rate; TPU: triose phosphate utilization.. Mean values (*n=5*) ± s.e.m. Significant differences by *t*-test at *0.01 and **0.001

| | **WT** | ***ARF4*-as** |
|---|---|---|
| ***A* (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | 18.30 ± 0.81 | 11.59** ± 0.59 |
| **$g_s$ (µmol $m^{-2}$ $s^{-1}$)** | 0.18 ± 0.02 | 0.09** ± 0.01 |
| **$A/g_s$** | 101.52 ± 4.62 | 122.76* ± 3.16 |
| **$V_{cmax}$ (µmol $m^{-2}$ $s^{-1}$)** | 80.37 ± 2.34 | 70.43* ± 3.01 |
| **$J_{max}$ (µmol $m^{-2}$ $s^{-1}$)** | 156.40 ± 7.74 | 140.70* ± 6.30 |
| **TPU (µmol $m^{-2}$ $s^{-1}$)** | 11.84 ± 0.57 | 10.82 ± 0.53 |

**Table 2: ABA content in WT and ARF4-as plants in response to salt or drought stress conditions.** Values presented are mean ± SD of three biological replicates (except for data related to *ARF4*-as plants in drought stress conditions, where only two biological replicates were done).

| | **Salt stress** | | **Drought stress** | |
|---|---|---|---|---|
| | 0 mM NaCl | 150 mM NaCl | 0% PEG | 15% PEG |
| **WT** | 0,1272±0 | 0,1454±0,007 | 0,0681±0,007 | 0,05±0,007 |
| ***ARF4*-as** | 0,1151±0,015 | 0,2636±0,02 | 0,0636±0,014 | 0,1272±0,012 |

## Article figures

**Figure 1. Phenotype of cv. Micro-Tom tomato plants (wild-type, WT) and isogenic *ARF4* antisense transgenic line (*ARF4*-as), 35 days after germination**. (a) WT and *ARF4*-as plants (b) at the same stage of development. (c) Upward-curled leaf phenotype of *ARF4*-as compared to WT (d) number of leaves to first inflorescence, (e) plant height and (f) plant internode length. Bars are mean values (n=7) ± s.e.m. Asterisks indicate values that were determined by Student's *t* test to be significantly different ($P < 0.05$) from WT.

**Figure 2. Dry weight and parameters related with leaf area for Micro-Tom (WT) and isogenic *ARF4* antisense transgenic line (*ARF4*-as), 45 days after germination.** (a) leaf dry weight. (b) stem dry weight. (C) root dry weight. (d) total dry weight. (e) total leaf area. (f) specific leaf area (SLA). Values are means ± s.e.m (n=8). Asterisks indicate values that were determined by Student's *t* test to be significantly different ($P < 0.05$) from the Micro-tom (WT).

**Figure 3. Leaf anatomy and morphology for Micro-Tom (WT) and *ARF4*-as transgenic line**. Representative leaf cross-sections of Micro-Tom (WT, a) and *ARF4*-antisense line (*ARF4*-as, b). Scale bars= 20 µm. Total thickness of the leaf blade (c)Thickness of palisade (d), spongy (e) and ratio of palisade to spongy mesophyll (f), proportion of intercellular air spaces (g) and adaxial (h) and abaxial (i) and mesophyll (j) thickness in WT and *ARF4*-as plants. Values are means ± s.e.m (n=6).

**Figure 4**. **Photosynthetic assimilation rate and stomatal conductance in Micro-Tom (WT) and *ARF4*-antisense silencing line.** (a) Net photosynthesis (*A*) curves in response to sub-stomatal ($C_i$) $CO_2$ concentration in WT and *ARF4*-as plants. Values are presented as means ± s.e.m. (n=5) obtained using the fully expanded fifth leaf. Two-branch curves: the biochemically based leaf photosynthesis model (Farquhar et al., 1980) was fitted to the data

based on $C_i$, values of $A/C_i$ for five plants of WT (gray) and *ARF4-as* (black). (b) Response of stomatal conductance ($g_s$) to changes in leaf-to-air vapor pressure deficit (VPD). Vapor pressure difference was varied by changing the humidity of air, keeping leaf temperature constant. Each point represents one measurement on an individual plant.

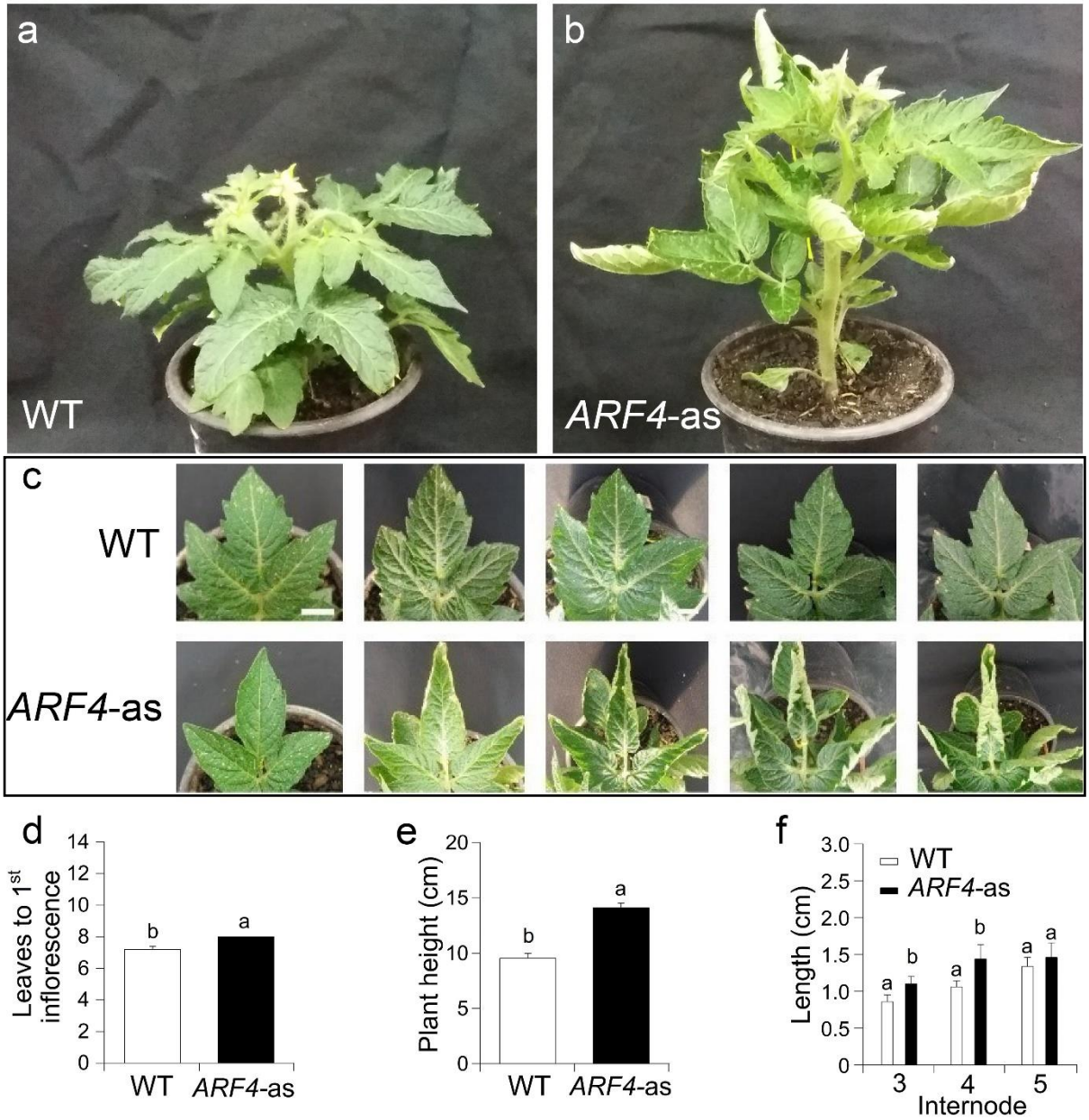
**Figure 5**. ***SlARF4* expression in response to salt and drought stresses.** Gene expression in leaves (a) and roots (b) of WT plants exposed to salt stress, in leaves (c) and roots (d) of WT plants exposed to drought stress. GUS activity in pARF4::GUS tomato lines in salt (e) or drought (f) stress conditions. Stars (*) indicate the statistical significance (p<0,05) according to Student's t-test.

**Figure 6. Growth parameters of tomato wildtype (WT), *ARF4*-as and *ARF4*-ox in response to salt and drought stresses.** (a) and (b) shoot fresh weight in salt and drought stress conditions respectively, (c) and (d) root fresh weight in salt and drought stress conditions respectively, (e) and (f) primary root length root in salt and drought stress conditions respectively, (g) and (h) root density in salt and drought stress conditions respectively. Salt and drought stresses were performed on three weeks tomato plants for two weeks by adding 100mM of NaCl or 150 mM of NaCl for salt stress or 5% or 15% of PEG 20 000 for drought. Values are mean ± SD of three biological replicates. Bars with different letters indicate the statistical significance (p<0,05) according to Student Newman-Keuls test.

**Figure 7. Stomatal conductance and relative water content in WT and *ARF4*-as plants.** (a) and (b) stomatal conductance in salt and drought stress conditions, (c) relative water content in response to dehydration. Bars with different letters indicate the statistical significance (p<0,05) according to Student Newman-Keuls test.

**Figure 8. Expression of *SlNCED1, SlNCED2, SlCYP707A1, SlCYP707A2* and *SlCYP707A3* genes in WT and *ARF4*-as leaves and roots after 2H and 24H of salt stress**

**and 48H of drought stress application.** ΔΔCt refers to fold differences in gene expression relative to untreated plants. Values presented are mean ± SD of three biological replicates. Asterisks (*) indicate the statistical significance ($p<0,05$) according to Student's t-test.

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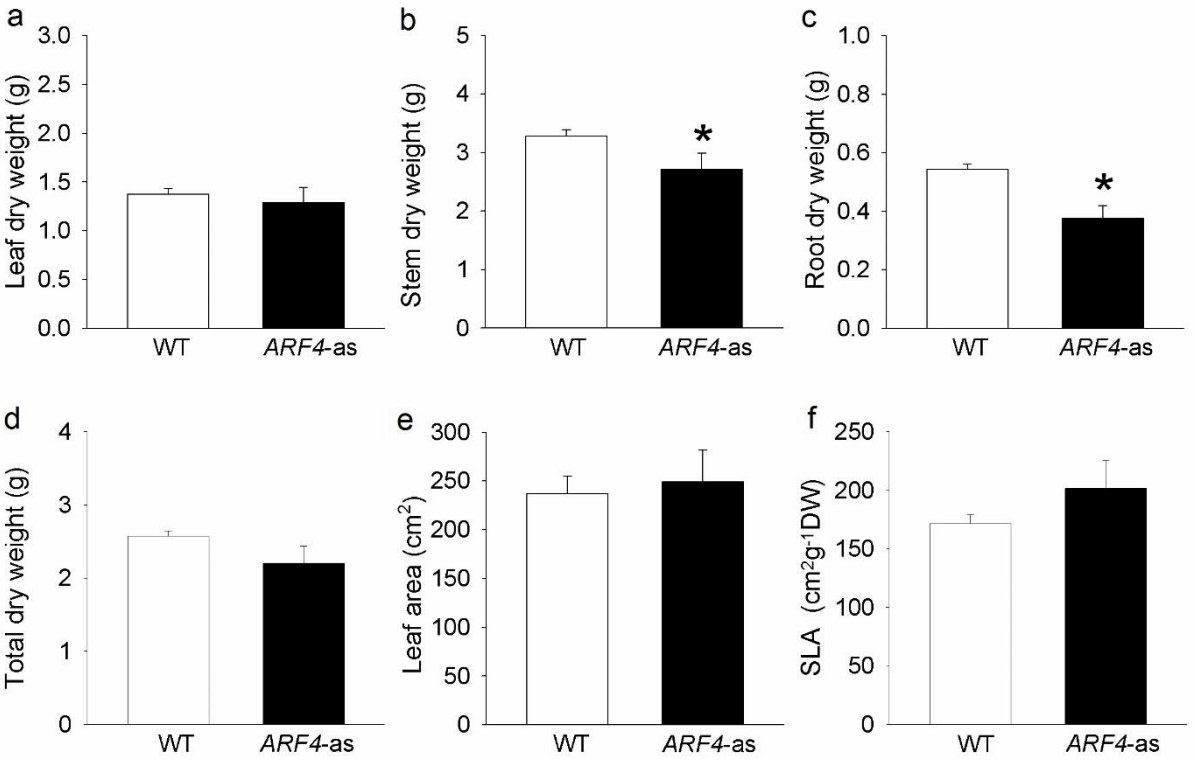


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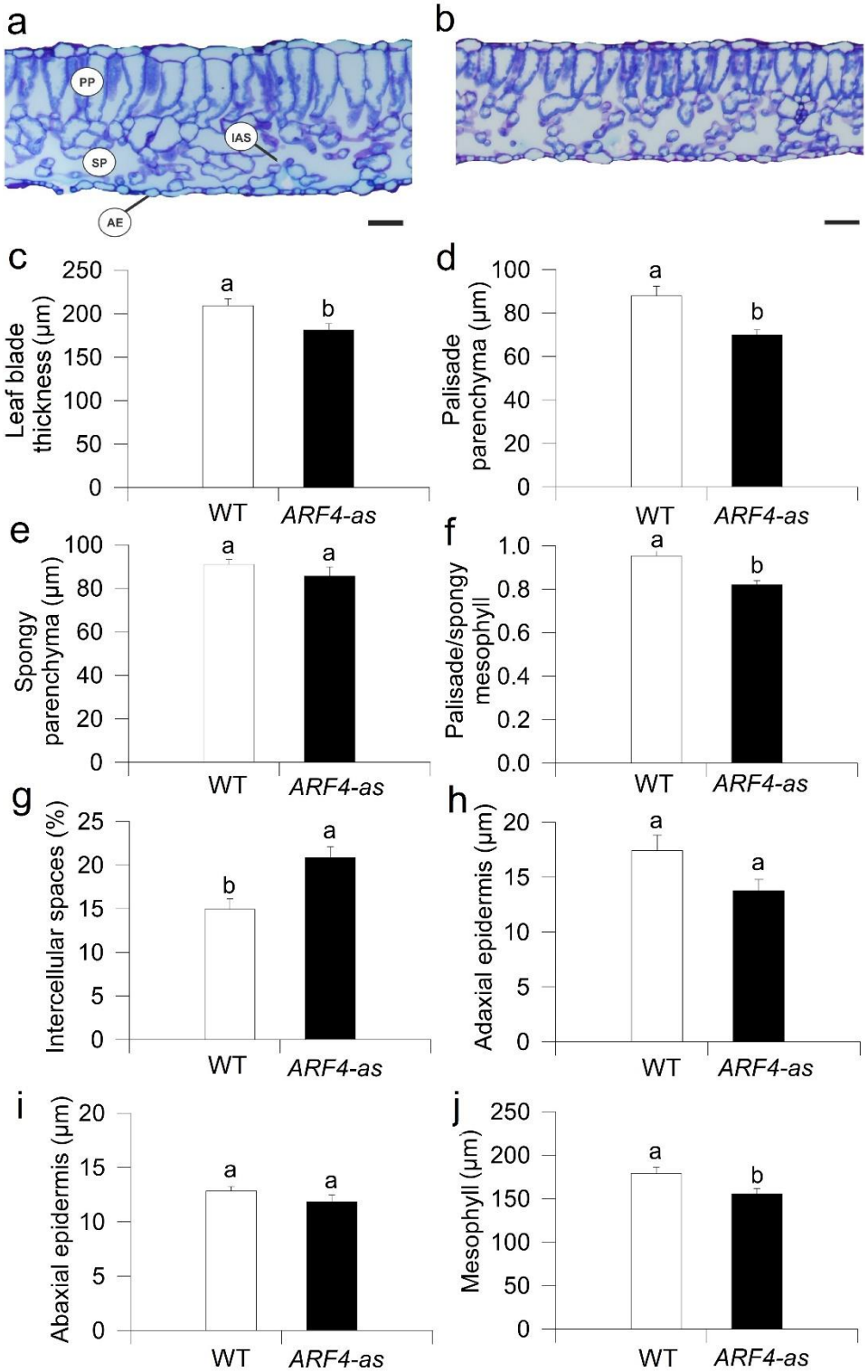


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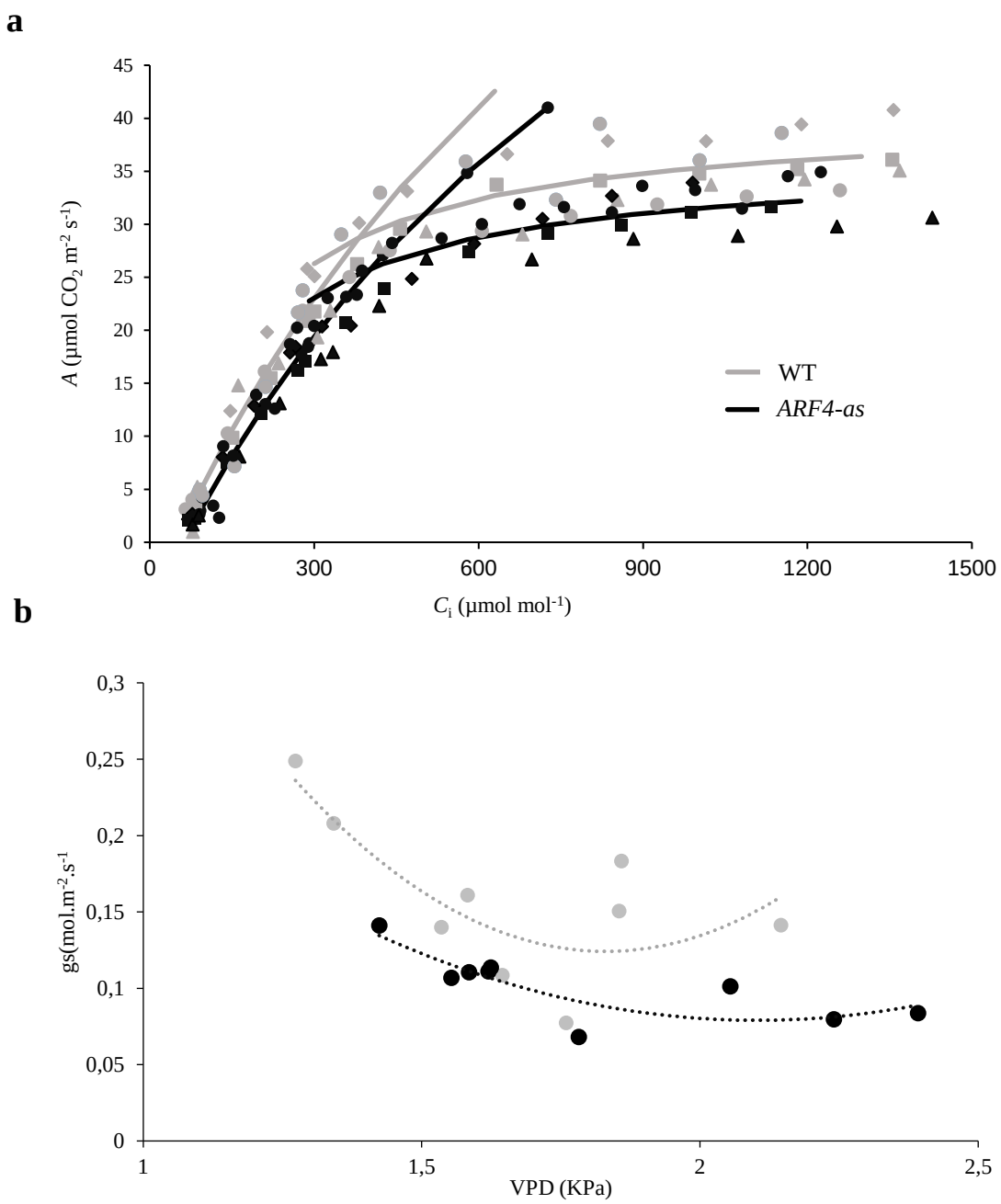


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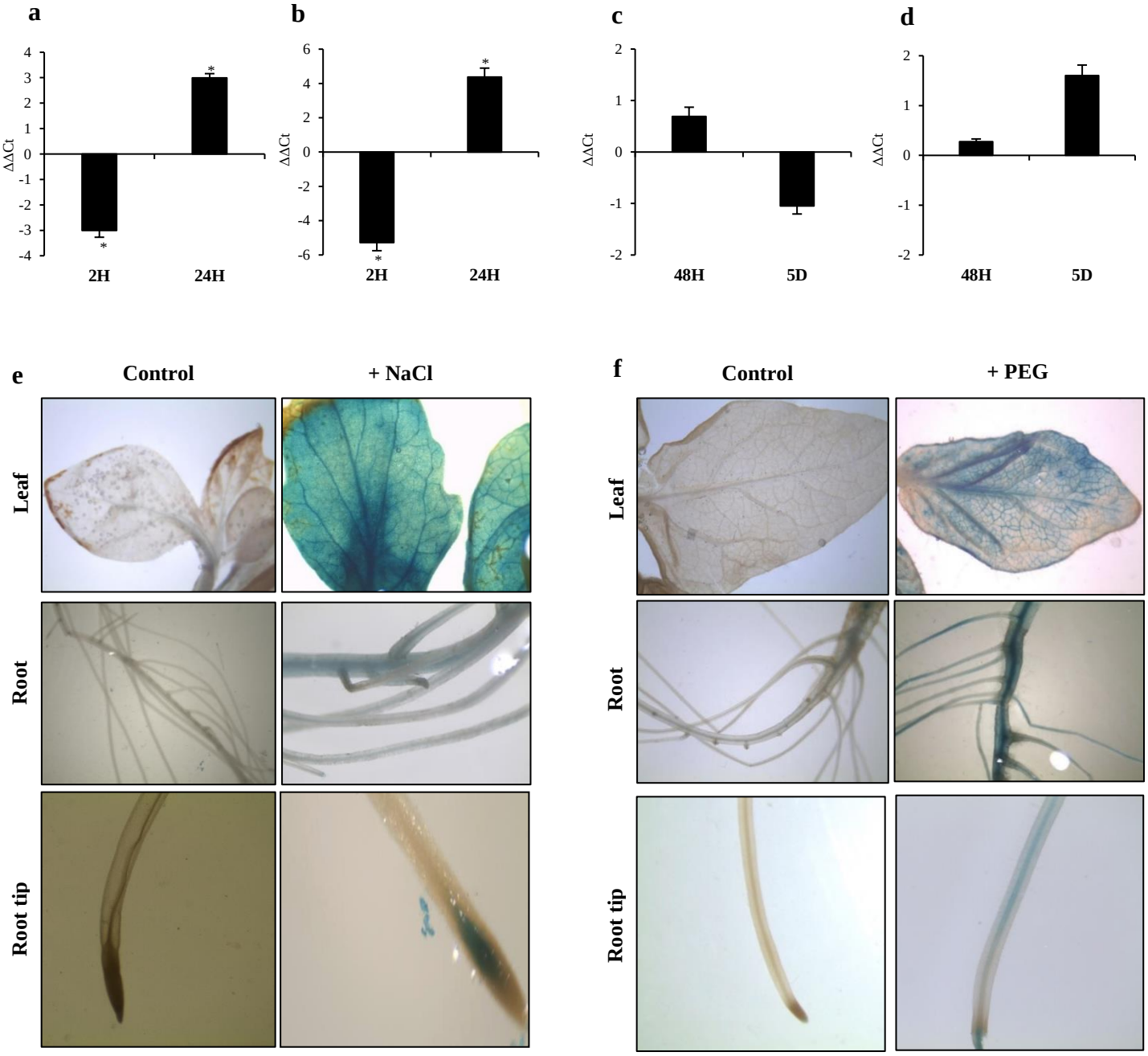


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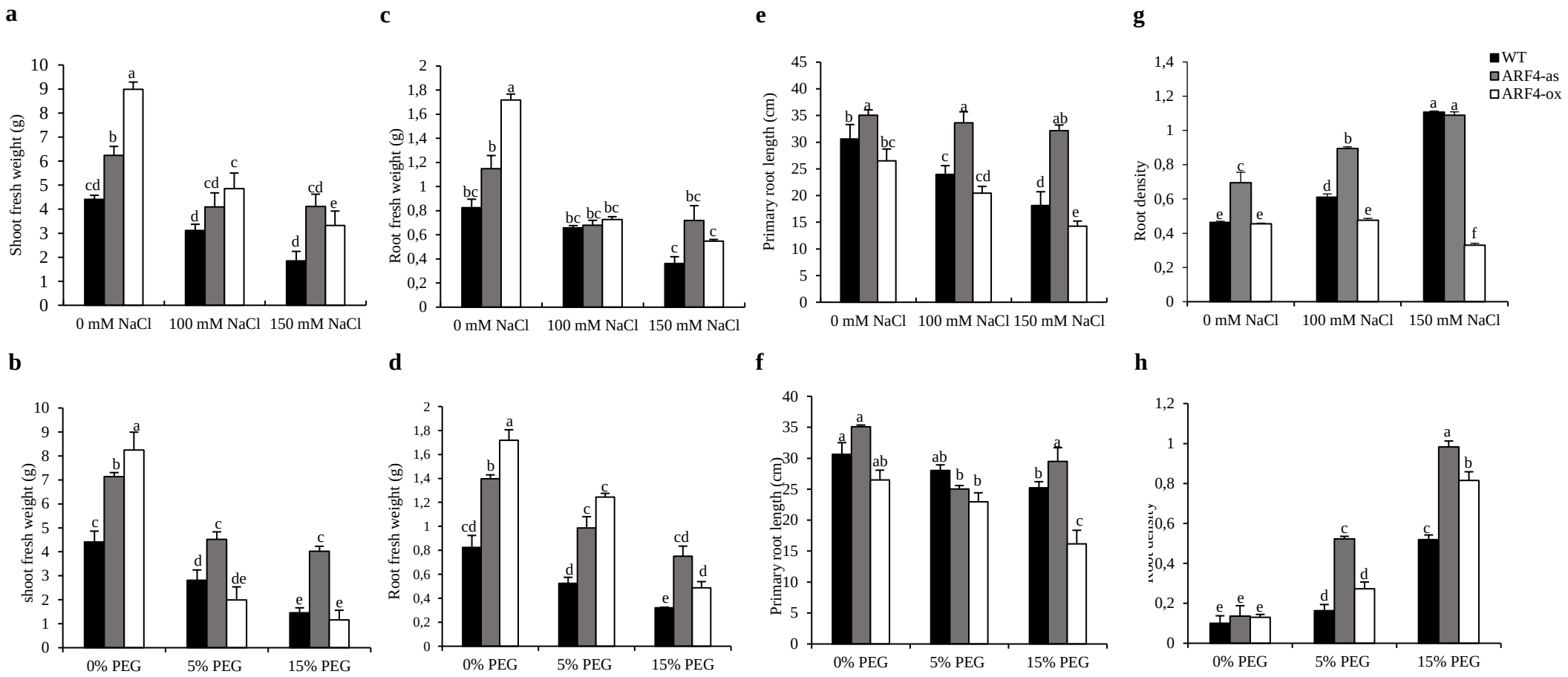


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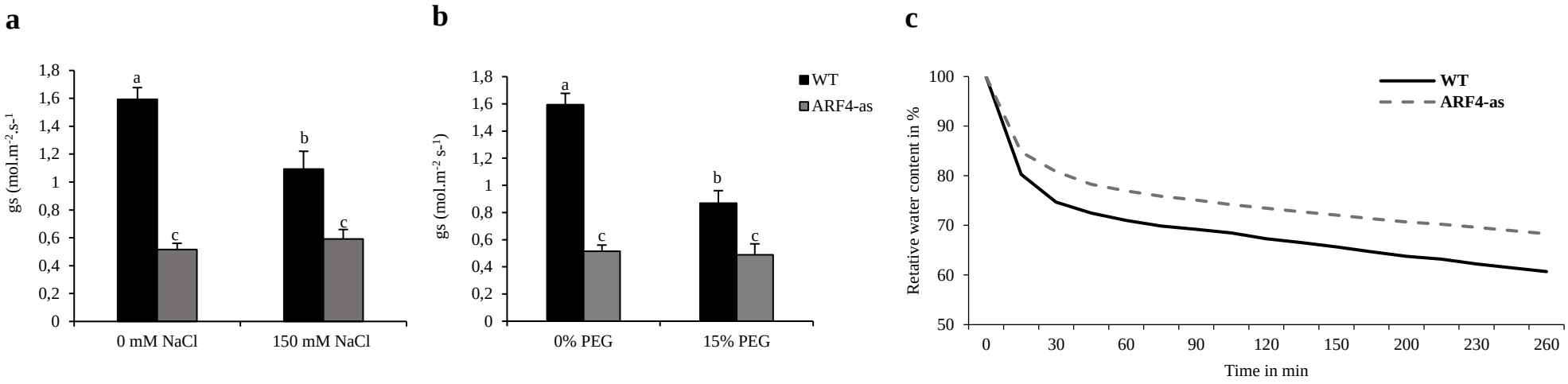


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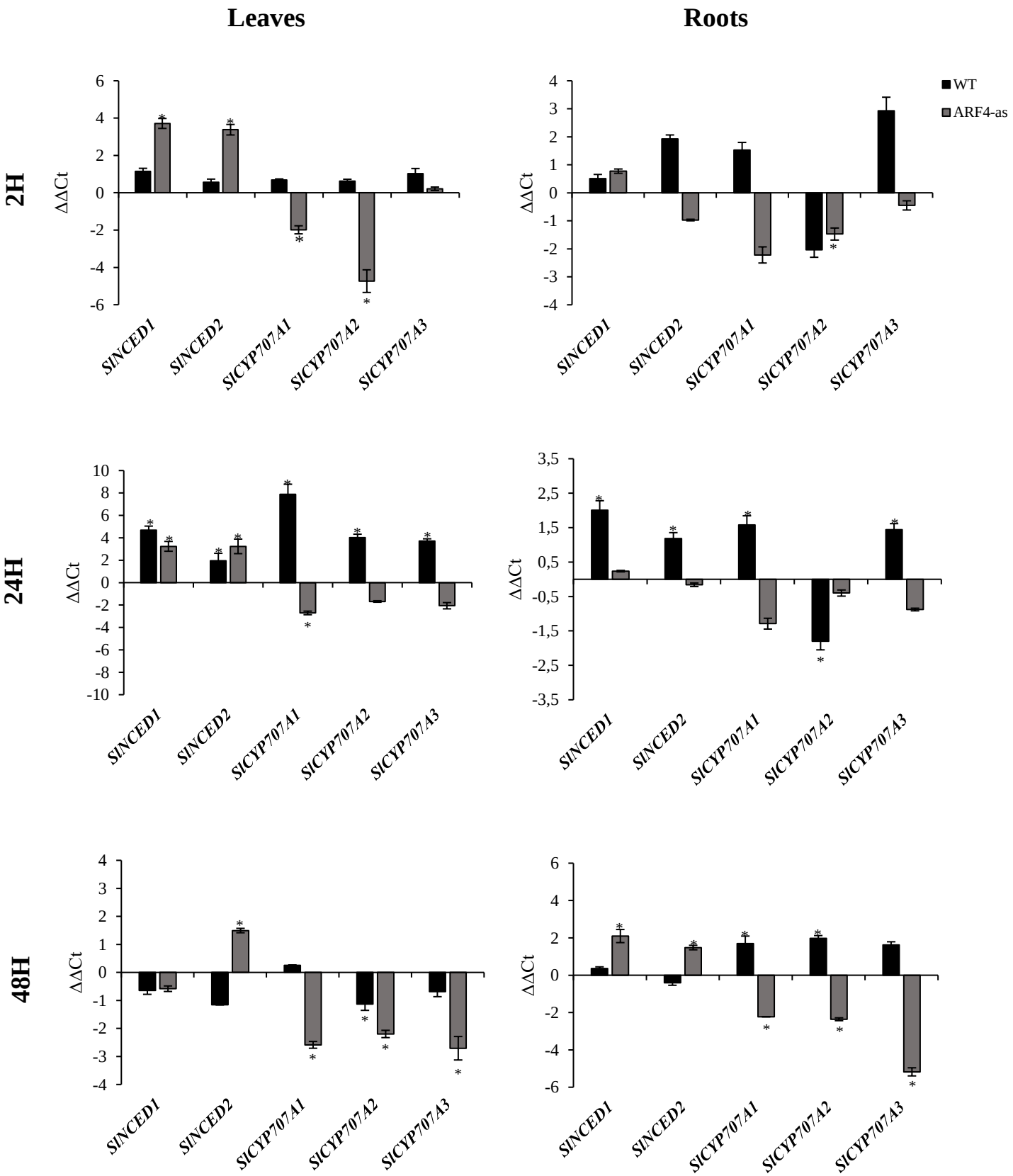


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