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Research

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Summary

- Salinity stress limits plant growth and has a major impact on agricultural productivity. Here, we identify NAC transcription factor SlTAF1 as a regulator of salt tolerance in cultivated tomato (*Solanum lycopersicum*).
- While overexpression of *SlTAF1* improves salinity tolerance compared with wild-type, lowering *SlTAF1* expression causes stronger salinity-induced damage. Under salt stress, shoots of *SlTAF1* knockdown plants accumulate more toxic Na^+ ions, while *SlTAF1* overexpressors accumulate less ions, in accordance with an altered expression of the Na^+ transporter genes *SlHKT1;1* and *SlHKT1;2*. Furthermore, stomatal conductance and pore area are increased in *SlTAF1* knockdown plants during salinity stress, but decreased in *SlTAF1* overexpressors.
- We identified stress-related transcription factor, abscisic acid metabolism and defence-related genes as potential direct targets of SlTAF1, correlating it with reactive oxygen species scavenging capacity and changes in hormonal response. Salinity-induced changes in tricarboxylic acid cycle intermediates and amino acids are more pronounced in *SlTAF1* knockdown than wild-type plants, but less so in *SlTAF1* overexpressors. The osmoprotectant proline accumulates more in *SlTAF1* overexpressors than knockdown plants.
- In summary, SlTAF1 controls the tomato's response to salinity stress by combating both osmotic stress and ion toxicity, highlighting this gene as a promising candidate for the future breeding of stress-tolerant crops.

Introduction

Salt stress adversely affects plant growth, development and crop productivity and is a major challenge to agriculture production (Munns & Tester, 2008; Shabala, 2013). Stress engenders both osmotic and ionic stress in plants. Excess soil salinity hinders water uptake by the plant roots and decreases turgor pressure due to water efflux from the vacuole, thereby resulting in an insufficient osmotic adjustment. Furthermore, high salinity stress enforces the accumulation of Na^+ ions, leading to tissue toxicity. Na^+ ion concentration increases gradually in aerial parts of the plants via transportation from root to shoot through the transpiration stream. Salinity-induced stress results in an immediate reduction in growth mainly via reduction of cell expansion in root tips and younger leaves, and stomatal closure in leaves, whereas salinity-induced ion toxicity promotes premature senescence or programmed cell death (Munns and Tester, 2008; Shabala, 2009).

To endure salinity stress, diverse adaptive mechanisms have evolved in plants including, for example, an efficient exclusion of Na^+ ions from cells, their compartmentalisation in the vacuole by specific transporters, adjustment of the osmotic balance of the cells by accumulating osmoprotectants, a change in photosynthetic activity, enhanced antioxidant and reactive oxygen species (ROS) scavenging capacity, and changes in hormonal responses (Munns and Tester, 2008; Shabala, 2013; Deinlein *et al.*, 2014; Maathuis, 2014).

Salt stress induces massive changes in gene expression in different species, underscoring the importance of transcriptional regulators in salt stress responses (Golldack *et al.*, 2011; Deinlein *et al.*, 2014). Transcription factors (TFs) are fundamental elements of transcriptional regulatory units. In cooperation with the basal transcriptional machinery and chromatin modifying proteins, they modulate gene expression and fine tune biological responses.

Among the TF families known in plants, the NAC (NAM (no apical meristem), ATAF (Arabidopsis transcription activation

factor), CUC2 (cup-shaped cotyledon)) family has attracted particular attention due to its roles in responses to diverse environmental stresses (Olsen et al; 2005; Jensen *et al.*, 2010; Pérez-Rodríguez *et al.*, 2010; Puranik *et al.*, 2012). The NAC family typically encompasses more than a 100 members in higher plants (Jin *et al.*, 2017). NAC TFs have a highly conserved N-terminal NAM domain that includes a dimerisation motif and confers DNA-binding activity, while their C-terminal region has a transcription activation function and shows high sequence variability (Ooka *et al.*, 2003; Ernst *et al.*, 2004; Jensen *et al.*, 2010). In different plant species, NAC TFs control responses to biotic and abiotic stresses, including salinity. For example, in *Arabidopsis thaliana*, JUNGBRUNNEN1 (JUB1), Arabidopsis NAC transcription factor 19 (ANAC019), ANAC055 and ANAC072 (also called RD26, RESPONSIVE TO DESICCATION 26) positively regulate the tolerance to salt stress (A. Wu *et al.*, 2012; Li *et al.*, 2014), while ANAC092 (also called ORESARA1, ORE1) and ANAC016 are negative regulators of the response to salinity (Balazadeh *et al.*, 2010; Kim *et al.*, 2013). JUB1 directly controls the expression of *DREB2A* (*DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2A*), an Apetala 2/ethylene-responsive element-binding protein (AP2/EREBP) TF with an important function in the regulation of drought, salinity and osmotic stress tolerance (Dubouzet *et al.*, 2003; Sakuma *et al.*, 2006; Chen *et al.*, 2008; Lata and Prasad, 2011; Zhang *et al.*, 2016). ANAC019, ANAC055 and RD26 bind to the promoter of *ERD1* (*EARLY RESPONSE TO DEHYDRATION1*, a drought responsive gene) and enhance drought stress tolerance when overexpressed in Arabidopsis (Tran *et al.*, 2004). Loss-of-function mutants of *ANAC019*, *ANAC055* and *RD26* exhibit increased sensitivity to salinity stress (Li *et al.*, 2014).

In rice, *STRESS REPONSIVE NAC1* (*SNAC1*), *SNAC2* (*OsNAC6*), *OsNAC045, OsNAC5, OsNAC106* and *ONAC022* function as positive regulators of salt tolerance (Hu *et al.*, 2006; Nakashima *et al.*, 2007; Zheng *et al.*, 2009; Takasaki *et al.*, 2010; Sakuraba *et al.*, 2015; Hong *et al.*, 2016). Enhanced salt tolerance of *ONAC022* overexpression plants was accompanied by reduced levels of Na^+ ions in roots and shoots, and enhanced expression of abscisic acid (ABA) biosynthetic and signalling genes and several stress-responsive TFs, including *OsDREB2A* (Hong *et al.*, 2016). By contrast, OsNAC2 functions as a negative regulator of the response to severe salinity. OsNAC2 directly activates transcription of *OsAP37* (*Oryza sativa ASPARTIC PROTEASE 37*, encoding a caspase-like protease), but triggers repression of *OsCOX11* (*Oryza sativa CYTOCHROME OXIDASE 11*, involved in ROS scavenging), leading to enhanced caspase activity and accumulation of ROS and subsequently programmed cell death during severe salinity stress (Mao *et al.*, 2018).

Tomato (*Solanum lycopersicum*) is an important vegetable crop that is rich in antioxidant molecules such as carotenoids, vitamin E, vitamin C, ascorbic acid and phenolic compounds, mainly flavonoids (Frusciante *et al.*, 2007). Seed germination, growth, biomass allocation and fruit yield of tomato plants are negatively affected by salinity stress (Sholi, 2012; Zhang *et al.*, 2016; Massaretto *et al.*, 2018). Attempts have been made to enhance salinity tolerance in tomatoes by genetic engineering of genes that encode the plasma membrane Na^+/H^+ antiporter SlSOS1 (*S. lycopersicum* SALT OVERLY SENSITIVE 1; Olias *et al.*, 2009), the endosomal Na^+/H^+ antiporter LeNHX2 (Huertas *et al.*, 2013), and also regulatory proteins including serine/threonine protein kinase SlSOS2 (*S. lycopersicum* SALT OVERLY SENSITIVE 2; Belver *et al.*, 2012; Huertas *et al.*, 2012) and TFs of diverse families (e.g. SlAREB1, *S. lycopersicum* ABA-responsive element-binding protein 1; SlARS1, *S. lycopersicum* altered response to salt stress 1; SlDREB2, *S. lycopersicum* dehydration-responsive element-binding protein 2; and SlbZIP1, *S. lycopersicum* basic leucine zipper 1; Orellana *et al.*, 2010; Campos *et al.*, 2016; Hichri *et al.*, 2016; Zhu *et al.*, 2018). Additionally, some NAC TFs, including SlNAC4, SlNAC35 and SlNAC11, have been shown to affect salt tolerance in tomato (Zhu *et al.*, 2014; Wang *et al.*, 2016; Wang *et al.*, 2017). Silencing of *SlNAC4* has led to an increased sensitivity of plants to drought and salt stress, and a decreased expression of stress-responsive genes including genes encoding antioxidants (*CATALASE 1*, *CAT1* and *CAT2*) and proline biosynthesis enzymes (*PYRROLINE-5-CARBOXYLATE SYNTHASE*, *P5CS*; Zhu *et al.*, 2014). Similarly, silencing of *SlNAC11* reduces salt stress tolerance in tomato (Wang *et al.*, 2017), while ectopic expression of *SlNAC35* elevates salt tolerance in tobacco (Wang *et al.*, 2016). However, molecular knowledge of the signalling pathways and downstream targets of those TFs is scarce.

Here, we demonstrate an important role of tomato NAC transcription factor SlTAF1 (*Solanum lycopersicum* Transcription Activation Factor 1, Solyc06g060230) for establishing tolerance to salinity stress. We show that enhanced salt tolerance conferred by SlTAF1 is associated with increased levels of the osmolyte proline, reduced stomatal conductance and stomatal pore area, reduced accumulation of Na^+ ions in shoots, and upregulation of salt stress-responsive and ABA biosynthesis genes, including various TFs. Collectively, our results demonstrated that SlTAF1 is a key regulatory hub that controls diverse circuitries of defence-related events in the salinity stress response in tomato, highlighting this gene as a promising candidate for breeding stress-tolerant crops.

Materials and Methods

Plant material and growth conditions

Solanum lycopersicum cv Moneymaker wild-type was used as the control in this study. Seeds of the wild tomato species *S. pimpinellifolium* and *S. cheesmaniae* were obtained from the Tomato Genetics Resource Center (https://tgrc.ucdavis.edu).

For seed production, phenotyping and detached leaf experiments, Murashige and Skoog (MS) medium-grown wild-type and *SlTAF1* transgenic tomato seedlings were transferred to soil, as previously reported (Schwarz *et al.*, 2014; Thirumalaikumar *et al.*, 2018) and grown in a glasshouse under a 16 h : 8 h, day : night regime, 450 µmol photons m^{-2} s^{-1} light, 24°C, and 65% relative humidity.

Hydroponic culture system and liquid nutrient medium

For aerated hydroponics, standard nutrient medium was used for *S. lycopersicum* cv Moneymaker. Briefly, macronutrients: 1.25 mM $Ca(NO_3)_2.4H_2O$; 0.83 mM K_2HPO_4; 1.5 mM KNO_3; 0.75 mM $MgSO_4$, and micronutrients: 50 μM $Na_2FeEDTA$; 11.6 μM H_3BO_3; 2.4 μM $MnSO_4.H_2O$; 200 nM $ZnSO_4$; 100 nM $CuSO_4.5H_2O$; 100 nM $Na_2MoO_4.2H_2O$. After preparation of the nutrient medium, the pH was adjusted to 5.8 using H_2SO_4. Generally, roots of tomato plants are sensitive to hypoxia; they need adequate air around the root zone for proper growth (Klaring & Zude 2009). To this end, an air pump was utilised to achieve an aeration for healthy root growth in the hydroponic culture system via the formation of air bubbles and waves. Aerated hydroponic trays comprising nutrient medium with tomato seedlings were grown in a controlled growth chamber (photoperiod 16 h : 8 h, day : night; light 350 μmol photons m^{-2} s^{-1}; temperature 22°C : 18°C, day : night; and 70% relative humidity). After 7 d of seedling transplantation, medium was replenished every third day to avoid depletion of nutrients.

Salt treatment in a hydroponic culture system and by salt-water irrigation

For salinity treatment in hydroponics, 1-wk-old MS medium-grown tomato seedlings (wild-type and *SlTAF1* transgenic plants) were transplanted to an aerated hydroponic culture system containing nutrient medium, and grown in a growth chamber. Salt treatment was induced by supplementing nutrient medium with NaCl; plants grown in nutrient medium without NaCl (0 mM) were used as controls. For salt-water irrigation, wild-type and *SlTAF1* transgenic tomato seedlings were grown in a growth chamber and supplemented with NaCl (200 mM) or without NaCl.

Treatments

For gene expression analysis in different tomato species (*S. lycopersicum, S. pimpinellifolium* and *S. cheesmaniae*) after salt treatment, 2-wk-old seedlings were transferred to MS liquid medium containing 120 mM NaCl (NaCl was omitted in control treatments) and incubated for 4 h. Dehydration treatment was performed as previously described (Thirumalaikumar *et al.*, 2018). For gene expression analysis upon different treatments, 2-wk-old seedlings of wild-type *S. lycopersicum* cv Moneymaker were initially grown on MS medium and thereafter transferred to liquid MS medium flasks and treated with salt (NaCl 120 mM; for 2, 4, 6 or 10 h), H_2O_2 (10 mM; for 4 h), and ABA (100 μM; for 0, 2, 4, 6, 12, 24 or 36 h). For expression analysis of SlTAF1 early responsive genes, 3-wk-old seedlings of *SlTAF1-IOE* were treated with 15 μM estradiol (EST) for 6 h in liquid MS medium (mock treatment: 0.15% (v/v) ethanol, used to dissolve EST). To test salt-dependent expression of potential target genes of SlTAF1, 3-wk-old *SlTAF1-IOE* seedlings were transferred to liquid MS medium containing 200 mM NaCl and 15 μM EST and incubated for 6 h on a shaker (without EST in mock treatment). After the treatments, samples were harvested and immediately plunged into liquid nitrogen.

Determination of sodium (Na^+) and potassium (K^+) ions

Na^+ and K^+ ion concentrations were measured in tomato shoot and root using ion chromatography (Dionex ICS-3000). Briefly, oven dried (shoot and root) plant material was ground into fine powder using a Retsch mill (Retsch, Haan, Germany). Next, 20 mg of ground material was weighed using an analytical weighing balance and homogenised in 1 ml of ULC/MS grade deionised water by vortexing for 2 min. Subsequently, ultrasonication was performed for 10 min. Afterwards, samples were centrifuged for 30 min and supernatant was filtered through Nanosep Centrifugal Devices (Pall Corp.; VMR International, Darmstadt, Germany). Filtered samples were diluted 1 : 100 in ULC/MS water. Ion chromatography was calibrated by injecting different concentration solutions of NaCl and KNO_3 (3.125, 6.25, 12.5, 25, 50 and 100 μM). Data were collected and processed using CHROMELEON v.6.8 software (Dionex). Standard curves for Na^+ and K^+ ions were calculated from standard solutions. An equation derived from the standard curve was used to calculate the ion concentration in the samples.

Metabolite profile analysis by GC-MS

To perform metabolite profiling, plants were grown in a controlled growth chamber. Leaf samples were harvested and immediately frozen in liquid nitrogen. After grinding samples, the extraction and relative levels indicated in metabolite profile results were obtained by gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) (Osorio *et al.*, 2012). Both, chromatograms and mass spectra were evaluated using CHROMATOF v.4.51.6 (LECO Corp., St Joseph, MI, USA) and TAGFINDER v.4.0. (Luedemann *et al.*, 2008). Each compound was annotated based on its unique mass spectrum (Kopka *et al.*, 2005).

Data availability statement

Gene IDs are listed in Supporting Information Table S1.

Detailed descriptions of DNA constructs, plant transformation, identification of the SlTAF1 binding motif, RNA extraction, gene expression analysis by qRT-PCR, ABA determination and others are available in supporting Methods S1.

Results

SlTAF1 expression is induced by abiotic stresses

SlTAF1 is a tomato NAC transcription factor. It is a close homologue of Arabidopsis *ATAF1* (*Arabidopsis thaliana ACTIVATING FACTOR 1*, also called *Arabidopsis NAC002*, *ANAC002*; 68% identity and 72% similarity at the amino acid level) whose expression is induced during leaf senescence and by various abiotic stresses, such as H_2O_2 treatment, drought, salinity, prolonged darkness and that mediates multiple functions in

the adaptation to abiotic and biotic stresses (Jensen *et al.*, 2007; Lu *et al.*, 2007; Jensen *et al.*, 2008; Wang *et al.*, 2009; Garapati *et al.*, 2015; Li *et al.*, 2016) (PLAZA 3.0; http://bioinformatics.psb.ugent.be/plaza).

SlTAF1 is expressed in all organs throughout tomato development, however its expression is considerably higher in roots, open flowers and during fruit ripening than in other organs (Tomato eFP Browser; Rohrmann *et al.*, 2011; Shinozaki *et al.*, 2018). In leaves, expression of *SlTAF1* is induced during leaf senescence (Fig. S1a). To assess the response of *SlTAF1* to abiotic stresses, we examined the effect of H_2O_2, drought and salinity on its expression in tomato (cv Moneymaker) by qRT-PCR. As shown in Fig. 1a, *SlTAF1* expression was significantly enhanced after exposure of 2-wk-old tomato seedlings to a 4 h H_2O_2 treatment. Also, dehydration (2 h) resulted in a significant increase in *SlTAF1* transcript level (Fig. 1b). With respect to salinity stress, we analysed 2-wk-old tomato seedlings subjected to 120 mM NaCl for different times (2, 4, 6 or 10 h). *SlTAF1* transcript abundance highly (*c.* 13-fold) increased already after 2 h of salt stress, and it steadily increased peaking at the end of the treatment (*c.* 57-fold increase at 10 h). (Fig. 1c) We also examined *SlTAF1* expression in 2-wk-old seedlings of the wild tomato species *S. pimpinellifolium* and *S. cheesmaniae*. After 4 h of NaCl treatment (200 mM), *SlTAF1* expression was strongly enhanced in both species, as well as in *S. lycopersicum*, compared with the control (Fig. 1d).

Salinity and drought led to an accumulation of ABA (Takahashi *et al.*, 2018). Accordingly, treatment with ABA (100 μM) stimulated *SlTAF1* expression (Fig. 1e), suggesting that SlTAF1 acts downstream of ABA. Taken together, *SlTAF1* is an early dehydration-responsive and salinity stress-responsive gene suggesting that it plays a role in the response to these stresses in tomato.

SlTAF1 promotes tolerance to salt stress

The rapid and strong transcriptional response of *SlTAF1* to salinity stress in both, cultivated and wild tomato prompted us to investigate its potential function in combating this environmental stress. To characterise the function of SlTAF1 for the response to salt stress, transgenic lines with altered expression of *SlTAF1* were generated. First, we obtained several transgenic tomato lines that overexpressed *SlTAF1*, compared with wild-type, under the control of the largely constitutive cauliflower mosaic virus (CaMV) *35S* promoter (Fig. S1b). All *35S* overexpression lines exhibited a severe growth retardation and dwarf phenotype (Fig. S1c,d). To uncouple the pleiotropic effects of SlTAF1 on plant growth and its role in stress tolerance, we next generated plants expressing an *SlTAF1* in-frame fusion to green fluorescent protein (*GFP*), under the control of the native *SlTAF1* promoter (from this point forward, *pTAF1:TAF1-GFP).* Two lines (*L1* and *L2*), exhibiting increased expression of *SlTAF1* and *GFP* after 4 h of NaCl treatment, were selected for further investigation (Fig. S2a, b). Confocal microscope visualisation illustrated a SlTAF1-GFP signal in the nucleus of leaf epidermal cell after 4 h of NaCl treatment in agreement with the function of SlTAF1 as a transcription factor (Fig. S2b).

To generate *SlTAF1* transgenic lines with reduced *SlTAF1* expression level, the artificial micro-RNA (amiRNA) silencing technology was used. A 21-bp amiRNA sequence was chosen to target the third exon of *SlTAF1* (Fig. S2c). Two independent transgenic lines (hereafter named *kd-L1* and *kd-L2*) with reduced expression of *SlTAF1* were selected for analysis of salt tolerance. Expression of the closely homologous genes *SlNAC1* and *SlNAC4* (*c.* 62% and 72% identity with *SlTAF1* at the nucleic acid level; PLAZA 3.0) were not altered in the selected *SlTAF1* knockdown lines (Fig. S2c), indicating that the *amiRNA* which we designed specifically targets *SlTAF1*.

To evaluate the function of *SlTAF1* in salinity tolerance, *SlTAF1* transgenic lines (*pTAF1:TAF1-GFP-L1* and *L2, kd-L1* and *kd-L2)* and wild-type plants were subjected to salinity stress. For this purpose, 10-d-old seedlings grown on agar were transferred to an aerated hydroponic nutrient solution and, after 10 d, a subset of plants was subjected to salinity stress (120 mM NaCl) for 5 d. As shown in Fig. 2, all genotypes showed symptoms of salt stress, such as yellowing, reduction of chlorophyll content and biomass. However, the effects were remarkably stronger in the *kd* plants: wild-type and *pTAF1:TAF1-GFP-L1* plants showed a *c.* 45% decrease in total wet biomass, while a 70% reduction of biomass was observed for the *kd* lines (Fig. 2c). The chlorophyll content was significantly higher in the *pTAF1:TAF1-GFP-L1* line, but in direct contrast lower in the *kd-L1* and *kd-L2* lines compared with wild-type upon 5 d of salt stress (Fig. 2d). Accordingly, the expression of the senescence-associated genes *SAG13* and *SAG15* was reduced in *pTAF1:TAF1-GFP-L1* but enhanced in *SlTAF1-kd* lines compared with the wild-type plants following 5 d of salt stress (Fig. 2e). Importantly, hypersensitivity of *SlTAF1-kd* lines to salinity became even more evident when the exposure to salt stress was extended to 8 d (Fig. S3), strongly supporting the role of SlTAF1 for protecting against the otherwise deleterious effects of salinity stress.

Abiotic stresses including salinity cause an accumulation of ROS which eventually leads to programmed cell death (Petrov *et al.*, 2015). Detection of H_2O_2 by diaminobenzidine (DAB) staining revealed reduced ROS (H_2O_2) levels in *pTAF1:TAF1-GFP-L1* plants but increased levels in *kd-L1* lines compared with wild-type after 5 d of salt stress (Fig. 2f,g).

We also tested the effect of salt stress on the transgenic lines grown in soil. To this end, 25-d-old soil-grown tomato plants were subjected to salt stress (200 mM NaCl) at three intervals (each time 72 h) for a period of 10 d. As illustrated in Fig. S4a,b, *kd-L1* and *kd-L2* were severely affected by salt stress while *pTAF1:TAF1-GFP-L1* and *-L2* plants showed a less sensitive phenotype compared with wild-type. The chlorophyll content remained significantly higher in *pTAF1:TAF1-GFP-L1* and *-L2* than wild-type after salt treatment, while it dropped in *kd-L1* and *kd-L2* plants (Fig. S4c). Ion leakage (as an indicator of membrane integrity) was significantly higher in *kd-L1* and *kd-L2* plants than in wild-type, while it was lower in *pTAF1:TAF1-GFP-L1* and *-L2* plants (Fig. S4d). These data provide further support for the

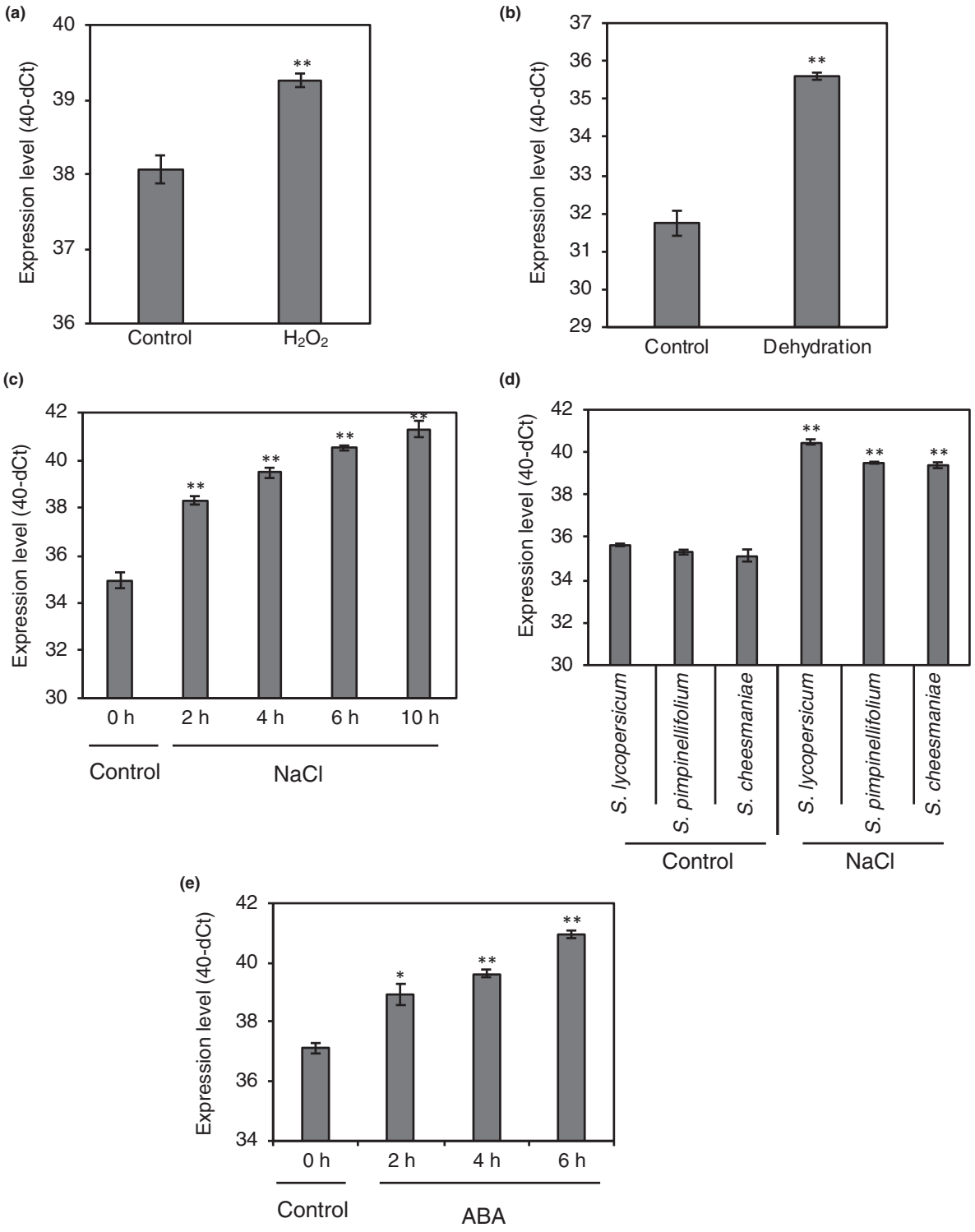

Fig. 1 *SlTAF1* expression under different stress treatments in tomato. (a) Transcript level of *SlTAF1* (*Solanum lycopersicum TRANSCRIPTION ACTIVATION FACTOR 1*) in 2-wk-old wild-type seedlings after H_2O_2 treatment for 4 h. (b) Transcript level of *SlTAF1* in detached leaflets (terminal leaflet of leaf no. 5) of 42-d-old wild-type plants after 2 h dehydration. (c) Expression level of *SlTAF1* in 2-wk-old wild-type seedlings (*S. lycopersicum* cv Moneymaker) at different time points after NaCl (120 mM) treatment. (d) Expression of *SlTAF1* in 2-wk-old wild-type seedlings of *S. lycopersicum*, *S. pimpinellifolium* and *S. cheesmaniae* after 4 h of 200 mM NaCl treatment. (e) *SlTAF1* expression upon ABA treatment. Two-week-old wild-type seedlings were treated with 100 μM ABA for 2, 4 or 6 h; 0 h indicates the time point before treatment (control). (a–e) Data represent the means of three biological replicates ± SE. Asterisks indicate significant difference (Student's *t*-test; **, $P < 0.01$) from controls. Expression analysis was carried out using qRT-PCR. Expression of *SlTAF1* was determined relative to the *SlGAPDH* (*S. lycopersicum GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE*; *Solyc04g009030*) reference gene. The Y-axis indicates expression level (40-dCt). Values are expressed as the difference between an arbitrary value of 40 and dCt, so that high 40-dCt values indicate high gene expression levels.

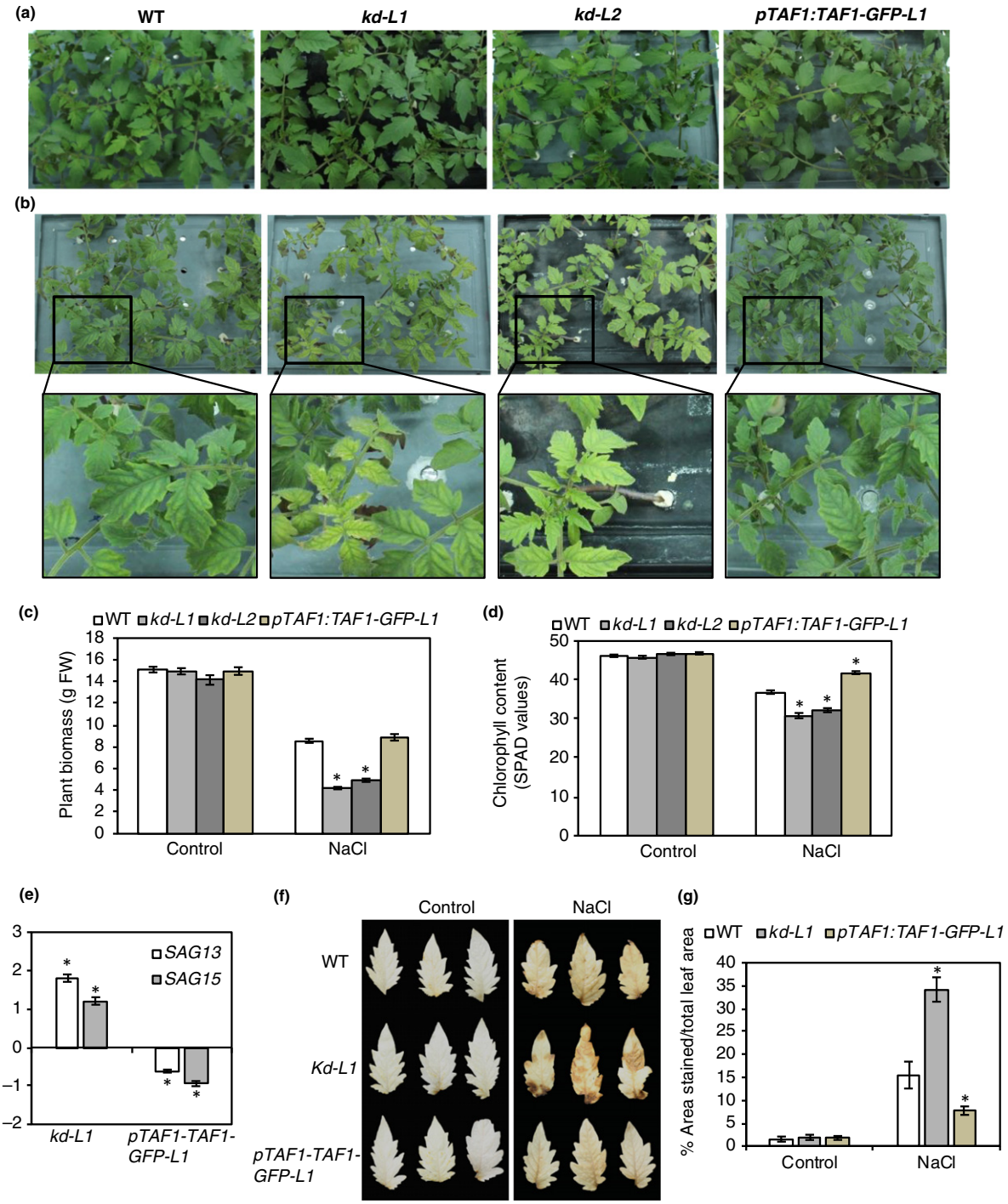

Fig. 2 *SlTAF1* promotes salt stress tolerance in tomato. (a) Representative images of 22-d-old wild-type and *SlTAF1* transgenic plants grown in aerated hydroponics nutrient solutions for 15 d. Tomato wild-type (*Solanum lycopersicum* cv Moneymaker) and transgenic seeds (T3, homozygous) were germinated on MS medium and grown for 7 d before transfer of the seedlings to the hydroponics system. (b) Wild-type and *SlTAF1* transgenic plants were grown as in (a) for 10 d in aerated hydroponics and later supplemented with 120 mM NaCl for 5 d. The black-boxed areas are enlarged in the lower panels. Note the generally less healthy phenotype of *SlTAF1-kd* lines (*kd-L1* and *kd-L2*). (c) Total plant biomass (FW) of control plants as shown in (a) and salt-treated plants as shown in (b). Values are means of eight biological replicates ± SE. (d) Chlorophyll content of the third leaf (counted from the bottom of the stem) of plants grown in (a) and (b). Chlorophyll content was measured by SPAD meter. Values are means of eight biological replicates ± SE. (e) Expression of *SENESCENCE-ASSOCIATED GENES* (*SAGs*) in *SlTAF1-kd-L1* and *pTAF1:TAF1-GFP-L1* plants compared with wild-type after 5 d of salt treatment (120 mM NaCl). Expression analysis was carried out using qRT-PCR. Values were normalised to those determined in the control plants. Y-axis denotes expression values on a $\log_2$ fold change (FC) scale. Data represent means of three biological replicates ± SE. (f) DAB (3,3′-diaminobenzidine) staining after 5 d of salt stress in wild-type and *SlTAF1* transgenic plants. (g) Percentage of dark-brown spot coloration relative to the total leaf area after DAB staining. Note, stronger DAB staining indicates higher level of H_2O_2. Values are means of three biological replicates ± SE in control and of five biological replicates ± SE in NaCl treatment experiments. Asterisks (c–e,g) denote a significant difference between transgenic lines and wild-type (Student's *t*-test; *, $P < 0.05$).

model that SlTAF1 functions as a positive regulator of salt tolerance in tomato plants also when grown in soil.

To further evaluate the role of *SlTAF1* for the response to salinity stress, we generated transgenic lines with a deletion at the *SlTAF1* locus, using CRISPR/Cas9 editing (Belhaj *et al.*, 2013; Brooks *et al.*, 2014), and evaluated their response to salt stress (Fig. S5a–d). Like *SlTAF1 kd* plants, *CR-taf1-L18* plants were significantly more sensitive to salt than wild-type when grown in a hydroponics system (120 mM NaCl for 5 d) or in soil (200 mM NaCl for 6 d) (Fig. S5e–j).

Taken together, the results presented provide compelling evidence that SlTAF1 is a key regulatory component of salinity stress tolerance in tomato.

SlTAF1 regulates ion homeostasis under salinity stress

During salinity stress, the excessive accumulation of sodium (Na^+) ions in leaves leads to ion toxicity which negatively affects plant growth (Maathuis, 2014). Here, we quantified Na^+ and K^+ levels in shoots (youngest leaves number 4, 5 and 6, counted from the bottom of the stem) and roots of 22-d-old wild-type and *SlTAF1* transgenic plants exposed to salinity stress, 120 mM NaCl, for 5 d (and no NaCl as control). As expected, salt stress led to higher accumulation of Na^+ and decreased K^+ levels in shoots and roots of the wild-type plants (Fig. 3 and Fig. S6). In shoots, Na^+ accumulation was drastically higher in *SlTAF1-kd* plants than in wild-type. By contrast, significantly lower Na^+ level was detected in *pTAF1:TAF1-GFP-L1* compared with wild-type (Fig. 3a). No considerable difference in the level of K^+ was observed between the transgenic lines (Fig. 3b). As a result, the deduced Na^+/ K^+ ratio was higher in *SlTAF1-kd* and lower in *pTAF1:TAF1-GFP-L1* than in wild-type (Fig. 3c). The higher accumulation of Na^+ in the leaves of *SlTAF1 kd* plants could explain their salinity-hypersensitive phenotype.

In roots, no significant differences were observed for Na^+ and K^+ contents between wild-type and the *SlTAF1* transgenic plants. Similarly, the Na^+/ K^+ ratios were not altered (Fig. S6).

To investigate the mechanisms underlying the altered accumulation of Na^+ in leaves of *SlTAF1* transgenic lines, we determined the expression of the xylem parenchyma localised Na^+ transporters *SlHKT1;1* (*S. lycopersicum HIGH-AFFINITY K(+) TRANSPORTER 1;1*) and *SlHKT1;2* (Asins *et al.*, 2013), plasma membrane-localised Na^+/H^+ antiporter *SlSOS1* (Olias *et al.*, 2009), and vacuolar antiporter *LeNHX4* (Gálvez *et al.*, 2012) in shoots (sixth leaf) and roots of *SlTAF1* transgenic lines subjected to 120 mM NaCl for 2 d in hydroponic culture. Expression of *SlHKT1;1* and *SlHKT1;2* was significantly lower in *SlTAF1-kd* than wild-type, but higher in *pTAF1:TAF1-GFP-L1* in both, shoots and roots. Expression of *SlSOS1* was higher in *SlTAF1-kd* than wild-type shoots, while no difference was observed in roots. *LeNHX4* expression was slightly upregulated in *SlTAF1-kd* compared with wild-type shoots, while no change was detected in roots (Fig. 3d,e).

In tomato, it has been reported that Na^+/K^+ homeostasis in the aerial part is mainly regulated by the Na^+ transporter SlHKT1;2. Silencing of *SlHKT1;2* increased the leaf Na^+/K^+ ratio and resulted in hypersensitivity to salinity (Asins *et al.*, 2013). Differential accumulation of Na^+ in the leaves of *SlTAF1 kd* and *pTAF1:TAF1-GFP-L1* could be a consequence of altered *HKTs* expression in those lines.

SlTAF1 controls stomatal aperture in response to salinity stress

Na^+ moves from roots to shoots via the transpiration stream. Enhanced leaf transpiration and, therefore, water loss leads to massive transport of Na^+ to leaves (Campos *et al.*, 2016). Therefore, the ability to prevent water loss is one of the mechanisms to enhance salinity tolerance (H. J. Wu *et al.*, 2012; Koenig *et al.*, 2013; Shabala, 2013).

To test whether the elevated Na^+ level in shoots of *SlTAF1-kd* plants may be due to alteration in water loss via transpiration, we determined the stomatal pore area in *SlTAF1* transgenic and wild-type plants. To this end, the abaxial leaf epidermis of wild-type, *SlTAF1-kd* and *pTAF1:TAF1-GFP-L1* was imprinted with dental resins after 48 h of 120 mM NaCl treatment (and without NaCl as control) and analysed by microscopy. As shown in Fig. 4a,b, *SlTAF1-kd* displayed significantly larger stomatal pore area than wild-type, whereas pore area in *pTAF1:TAF1-GFP-L1* was significantly lower. We did not observe a difference in stomatal pore area between wild-type and *SlTAF1* transgenic lines at the control condition (Fig. 4a,b). We also assessed stomatal conductance of *SlTAF1* transgenic lines during salinity stress. A significantly higher stomatal conductance was observed in *SlTAF1-kd* after salt stress (48 h and 15 d) than wild-type, while *pTAF1:TAF1-GFP-L1* exhibited lower stomatal conductance (Fig. 4c).

As ABA is an important phytohormone involved in stomatal closure, we checked whether treatment of ABA affects stomatal response in *SlTAF1* transgenic plants. Peeled abaxial epidermal leaf strips of wild-type, *SlTAF1-kd* and *pTAF1:TAF1-GFP-L1* plants were treated with ABA (100 µM) and examined for stomatal closure. Application of ABA led to reduction of stomatal pore area in all genotypes, however the reduction was significantly lower in *SlTAF1-kd-L1* than wild-type, but substantially higher in *pTAF1:TAF1-GFP-L1* (Fig. S7). These results indicated that SlTAF1 is involved in ABA-mediated stomatal closure during salinity stress.

SlTAF1 alters primary metabolism upon salt treatment in tomato

To elucidate potential additional mechanisms involved in the regulation of salinity tolerance by SlTAF1, we investigated the primary metabolite profile of *SlTAF1* transgenic and wild-type plants following salt stress treatment by gas chromatography coupled to mass spectrometry (GC-MS). To this end, 54 primary metabolites were characterised in the leaves (sixth leaf counted from the bottom of the stem) of 22-d-old wild-type and transgenic lines after 5 d of salt (120 mM NaCl) stress. In wild-type plants, 39 metabolites were significantly altered upon salt stress, of those the levels of 11 metabolites increased while 28 metabolites decreased (Table S2). Those metabolites that were present at

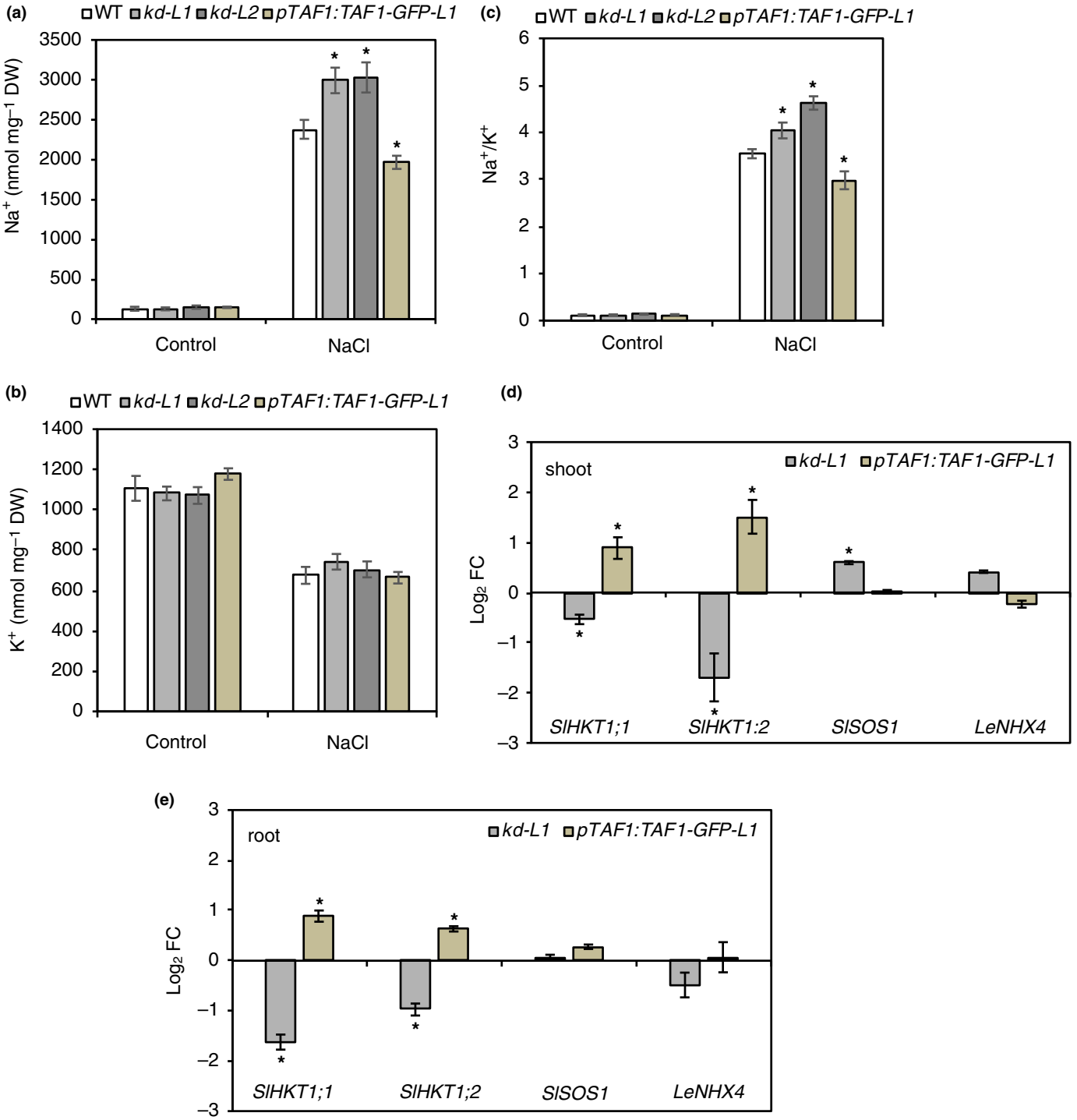

Fig. 3 Effect of salt stress on Na^+ and K^+ ions in leaves of *SlTAF1* transgenic tomato plants. (a) Na^+ and (b) K^+ concentrations (nmol/mg DW) in leaves no. 4, 5 and 6 (counted from the bottom of the stem; top three leaves) of wild-type and *SlTAF1* transgenic plants (as grown in Fig. 2a,b) after 5 d of salt (120 mM NaCl) treatment and under control condition (no salt added). (c) Ratio of Na^+ (a) and K^+ (b) concentrations in leaves of wild-type and *SlTAF1* transgenic lines. Data represent the mean of four biological replicates ± SE. (d,e) Expression of ion transporter genes *SlHKT1;1* (*Solanum lycopersicum HIGH-AFFINITY K(+) TRANSPORTER 1;1*), *SlHKT1;2*, *SlSOS1* (*S. lycopersicum SALT OVERLY SENSITIVE 1*) and *LeNHX4* (*Lycopersicon esculentum Na^+/H^+ ANTIPORTER 4*) in (d) sixth leaf (counted from the bottom of the stem) and (e) roots of *SlTAF1-kd-L1* and *pTAF1:TAF1-GFP-L1* plants compared with wild-type after treatment for 2 d with NaCl (120 mM) in a hydroponic culture system. Expression analysis was carried out using qRT-PCR. Expression values were normalised to those in the corresponding control plants. *Y*-axis denotes $\log_2$ fold change. Expression values represent means of three biological replicates ± SE. Asterisks indicate a significant difference between wild-type and *kd-L1*, and *pTAF1:TAF1-GFP-L1* plants (Student's *t*-test; *, $P < 0.05$).

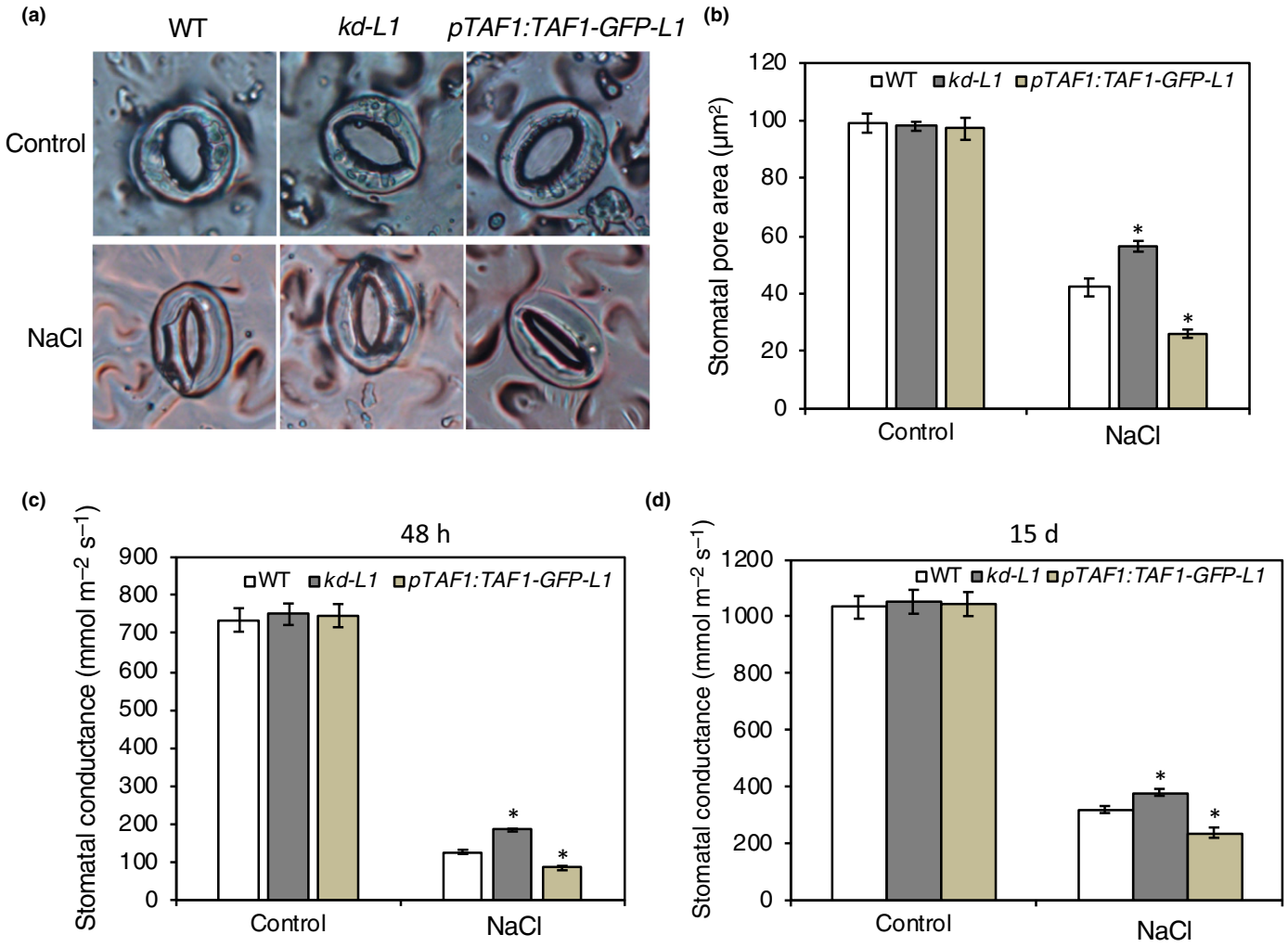

Fig. 4 Stomatal aperture and conductance in wild-type and *SlTAF1* transgenic tomato plants during salinity stress. Wild-type, *SlTAF1-kd-L1* and *pTAF1:TAF1-GFP-L1* plants were grown hydroponically as shown in Fig. 2(a, b). (a) Representative images of stomatal aperture of terminal leaflets of the third leaf after 48 h of 120 mM NaCl treatment (without NaCl as control). (b) Stomatal pore area of wild-type and *SlTAF1* transgenic plants. Measurements were taken at 3 h after the beginning of the photoperiod. Data are means of three biological replicates ± SE in control; while four biological replicates ± SE in salt treatment. Each biological replicate included the measurement of *c.* 120 stomata. (c, d) Stomatal conductance, determined with a porometer, of the third leaf of wild-type, *SlTAF1-kd-L1* and *pTAF1:TAF1-GFP-L1* plants after 48 h and 15 d of 120 mM NaCl treatment, respectively (without NaCl treatment as control). (c, d) Data represent means of six biological replicates ± SE. Asterisks indicate a significant difference between wild-type and *kd-L1*, and *pTAF1:TAF1-GFP-L1* plants (Student's *t*-test; *, $P < 0.05$).

significantly different abundances between wild-type and *SlTAF1* transgenic plants *(kd-L1, kd-L2* and *pTAF1:TAF1-GFP-L1)* upon salt stress were plotted in histograms (Fig. 5 and Table S2). Among all examined metabolites, the compatible osmolytes proline and 4-hydroxyproline showed dramatic induction upon salt stress in wild-type plants. Accumulation of proline by salt stress has been reported in several plant species including tomato (Verbruggen and Hermans, 2008; Gharsallah *et al.*, 2016). However, upregulation of proline and 4-hydroxyproline by salt treatment was significantly diminished in *SlTAF1-kd*. By contrast, induction of proline by salt stress was considerably higher in *pTAF1:TAF1-GFP-L1* plants compared with wild-type.

The level of the majority of other amino acids decreased in wild-type plants after salt stress, with the exception of tyrosine and serine. This reduction was less significant in *pTAF1:TAF1-GFP-L1* but more prominent in *SlTAF1-kd* plants (in comparison with wild-type).

Among the sugars, xylose and rhamnose declined in *SlTAF1-kd*, but remained unchanged in *pTAF1:TAF1-GFP-L1* compared with wild-type, while maltose increased in *SlTAF1-kd*, but decreased in *pTAF1:TAF1-GFP-L1*. Tricarboxylic acid (TCA) cycle intermediates malate and fumarate were significantly lower in the *SlTAF1-kd* plants and higher in *pTAF1:TAF1-GFP-L1* compared with wild-type upon salt treatment.

When taken together these data suggested that SlTAF1 is an important component of the control of cellular metabolism under salt stress since modification of its expression levels either dampens (in the case of deficiency of *SlTAF1* expression) or exacerbates (in the case of *SlTAF1* overexpression) the wild-type metabolic response to salt stress.

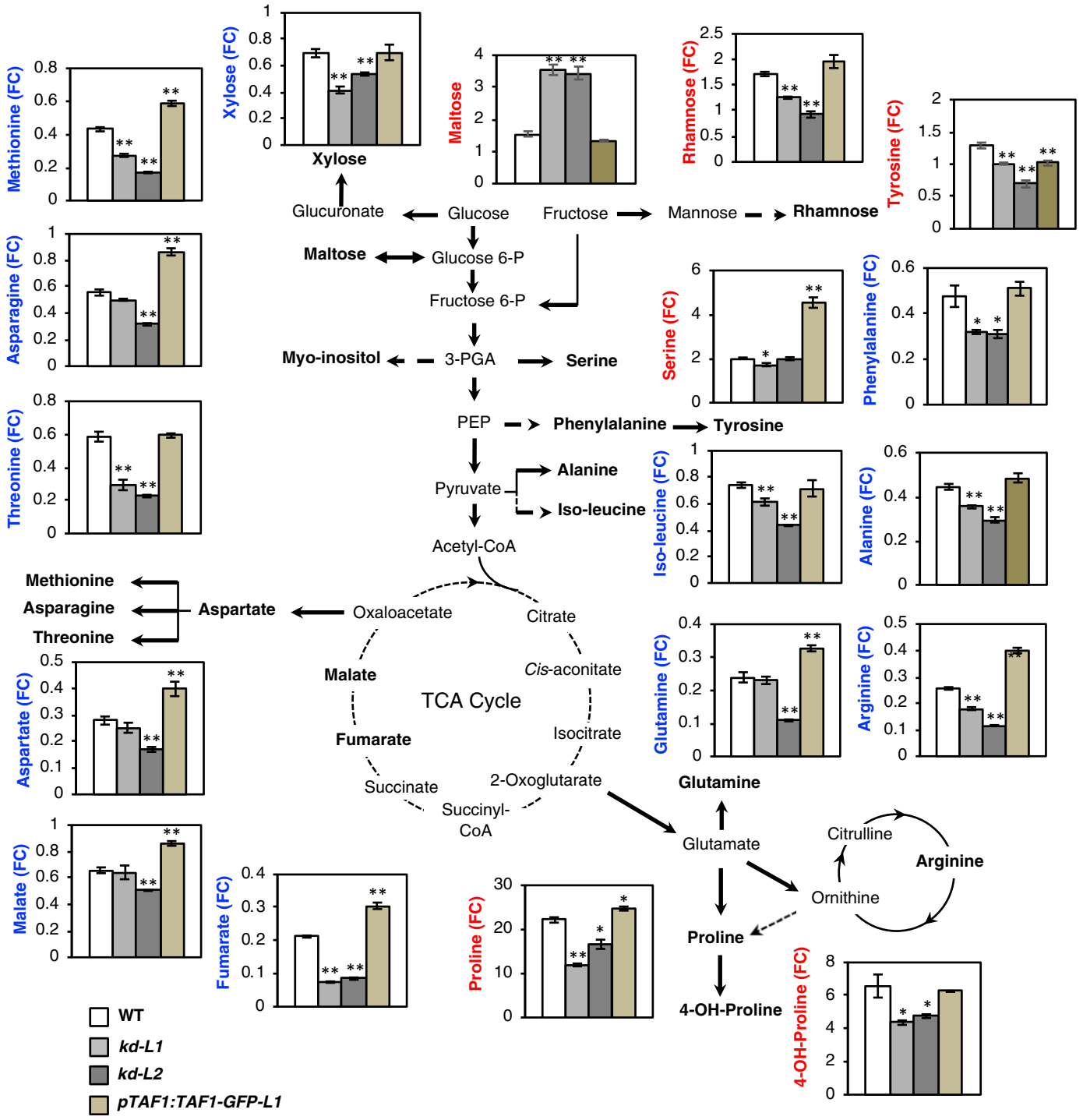

Fig. 5 Schematic representation of primary metabolites and linked TCA cycle intermediates in tomato leaves upon salinity stress. Primary metabolite content (analysed by GC-MS) in wild-type and *SlTAF1* transgenic plants (leaf no. 6 counted from the bottom of the stem) after 5 d of salt stress (120 mM NaCl). Fold change (FC) values are relative to control (no salt added). Values are means of four biological replicates ± SE. Red and blue colours indicate upregulation or downregulation in wild-type, respectively. Asterisks indicate a significant difference between wild-type and *SlTAF1* transgenic lines (Student's *t*-test: **, $P < 0.01$; *, $P < 0.05$).

SlTAF1 regulates salt-responsive genes in tomato

To acquire further insight into salt-tolerance mechanisms regulated by SlTAF1 and to identify the early responses at the gene expression level, we generated estradiol-inducible *SlTAF1* overexpression lines (hereafter, *SlTAF1-IOE;* Fig. S2e) and checked the expression of 23 stress-relevant genes in *SlTAF1-IOE* after 6 h of estradiol (EST) or EST in combination with salt (200 mM NaCl) treatments. The examined genes include genes that encoded TFs whose expression is strongly induced by salt (Table S3) and genes involved in ABA metabolism. Moreover, genes encoding alternative oxidases (AOX) were included in our study as manipulation

of alternative oxidases has been reported to influence salt and drought tolerance in different species (Smith *et al.*, 2009; Hu *et al.*, 2018; Zhu *et al.*, 2018).

Based on the expression profile, genes were categorised into two groups. The first group corresponds to salt-independent SlTAF1 early induced/responsive genes (nine genes); and the second group compiles salt-dependent early induced/responsive genes (seven genes) whose significant rapid induction by SlTAF1 required salt treatment (Fig. 6a). Overall, transcript levels of several genes encoding TFs such as *SlJUB1* (*S. lycopersicum JUNGBRUNNEN 1*), *SlJUB2*, *SlHB7* (*S. lycopersicum HOMEOBOX 7*), *SlJA2* (*S. lycopersicum JASMONIC ACID 2*), *SlDREB2A1* and *SlDREB2A2* as well as ABA-signalling TFs such as, *SlABF1* (*S. lycopersicum ABA-RESPONSIVE ELEMENT-BINDING FACTOR 1*), *SlAREB1/SlABF2* and *SlABF3* were rapidly and significantly induced by SlTAF1 either in a salt-dependent or salt-independent manner. Among ABA biosynthesis genes *SlSDR1A* (*S. lycopersicum SHORT-CHAIN ALCOHOL DEHYDROGENASE/REDUCTASE 1A*), *SlSDR1B* and *SlSDR1C* were significantly upregulated by SlTAF1. Other ABA synthesis genes, such as *SlNCED1* (*S. lycopersicum 9-CIS-EPOXYCAROTENOID DIOXYGENASE 1*), *SlNCED2* and *SlNCED3* as well as *Sitiens* (which encodes an aldehyde oxidase), were slightly induced by SlTAF1. Finally, we quantified the ABA level in 8-d-old seedlings of wild-type and *SlTAF1* transgenic plants. The ABA level was significantly higher in *pTAF1:TAF1-GFP* (Fig. S8) However, the ABA level remained unchanged between *SlTAF1-kd* and *CR* lines compared with wild-type (data not shown) suggesting that regulation of ABA by SlTAF1 may be redundant with other control mechanisms. Additionally, the expression of *SlAOX1a* (*S. lycopersicum ALTERNATIVE OXIDASE 1a*) was considerably enhanced by SlTAF1.

SlTAF1 potential direct target genes

To identify potential target genes of SlTAF1, we first attempted to identify its consensus binding motifs. SITAF1 is phylogenetically clustered into a stress-responsive SNAC group (Nuruzzaman *et al.*, 2010), which bind a (C/T)ACG core motif (Fujita *et al.*, 2004; Tran *et al.*, 2004; Olsen *et al.*, 2005; Xue *et al.*, 2006; A. Wu *et al.*, 2012; Garapati *et al.*, 2015). To determine the DNA-binding sequences of SITAF1, a diverse set of the (C/T)ACG motif-containing sequences, including the high-affinity binding sites of TaNAC69 from wheat (*Triticum aestivum*), and AtJUB1, ATAF1 and ANAC019 from Arabidopsis, were used as probes for measuring the potential DNA-binding activity of SITAF1 towards these probes (Table S4). Similar to TaNAC69, SITAF1 has two types of binding sites (BS-I and BS-II). BS-I has a sequence of CGT(A/G)5-6N(T/C)ACG(C/T/G)(A/C/T)(A/T/G)(C/T/G)(T/C), which contains two ((C/T)ACG or CGT(A/G)) core motifs and a spacer of five or six nucleotides. BS-II contains only one (C/T)ACG core motif with a sequence of (C/T)ACGN(C/A/T)(T/A)N(C/T/A). However, the sequence flanking the left side of the core motif appears to be important for its binding activity (Table S4; Xue *et al.*, 2006).

Next, we searched for its consensus binding motifs in the promoters (1 kb) of SlTAF1 early responsive genes. Among those, nine genes (*SlHB7, SlJUB1, SlJUB2, SlERD10* (*S. lycopersicum EARLY RESPONSE TO DEHYDRATION 10*), *SlSDR1A, SlJA2, SlAREB1*, *SlRD29B* (*S. lycopersicum RESPONSIVE TO DESICCATION 29B*) and *SlAOX1a*) harbour an SlTAF1 binding site in their promoters (Fig. 6b). Importantly, after 4 h of NaCl (200 mM) treatment, expression of all potential direct target genes of SlTAF1 was elevated in *pTAF1:TAF1-GFP-L1* seedlings compared with wild-type, but reduced in *CR-taf1-L18* plants (Fig. 6c). As expected, expression of the genes was intermediary in the *SlTAF1-kd* seedlings (Fig. 6c).

To determine binding of SlTAF1 to the promoters of the potential direct target genes we employed an electrophoretic mobility shift assay (EMSA). As depicted in Fig. 6d, SlTAF1 binds and physically interacts with a 40-bp promoter fragment (harbouring SlTAF1 BS) of all the potential direct target genes.

Discussion

We investigated the role of NAC transcription factor SlTAF1 for the response to salt stress in tomato and discovered its involvement in the regulation of key processes underlying the tolerance to salinity stress; to summarise the role of SlTAF1 in this process, we provide a model in Fig. 7. Expression of *SlTAF1* is highly upregulated by dehydration, exposure to hydrogen peroxide (H_2O_2), salt stress and by treatment of plants with ABA, a phytohormone integrating stress signals with growth and developmental programmes (Fig. 1). We observed that *SlTAF1-knockout* (*CR-taf1*) and *SlTAF1-knockdown* (*kd*) lines exhibited enhanced sensitivity to salt stress, while an increased expression of *SlTAF1* in overexpressors conferred increased tolerance to salinity stress. Furthermore, proline, a compatible solute involved in osmotic adjustment, accumulated to higher levels in *pTAF1:TAF1-GFP* plants than wild-type during salinity stress, while a reduction in proline content was observed in *SlTAF1-kd* lines. Proline levels often increase in plants during drought and salt stress, and proline contributes to osmotic adjustment under stress conditions (Verbruggen and Hermans, 2008; Szabados and Savouré, 2010).

The TCA cycle plays a central role in energy metabolism. A decline in the content of the TCA cycle intermediates is frequently observed in glycophytes under salt stress (Gong *et al.*, 2005; Sanchez *et al.*, 2008; Zhao *et al.*, 2014; Richter *et al.*, 2019). In all genotypes tested here (i.e. wild-type, *SlTAF1-kd* and *pTAF1:TAF1-GFP*), we observed a slight increase in citric acid after salt stress (Table S2). Malate and fumarate decreased in all genotypes, however the level of reduction was more pronounced in *SlTAF1-kd*, but less prominent in *pTAF1:TAF1-GFP* plants (Fig. 5). While a change in the flux mode with a concomitant reduction of oxaloacetate-derived aspartate and 2-oxoglutarate-derived glutamine, indicating a reduction in the flux of carbon skeletons from the TCA cycle to amino acids, appears to be common in all lines, the negative effect of salt stress appeared to be less pronounced in *pTAF1:TAF1-GFP* plants, in accordance with their higher salinity tolerance compared with wild-type.

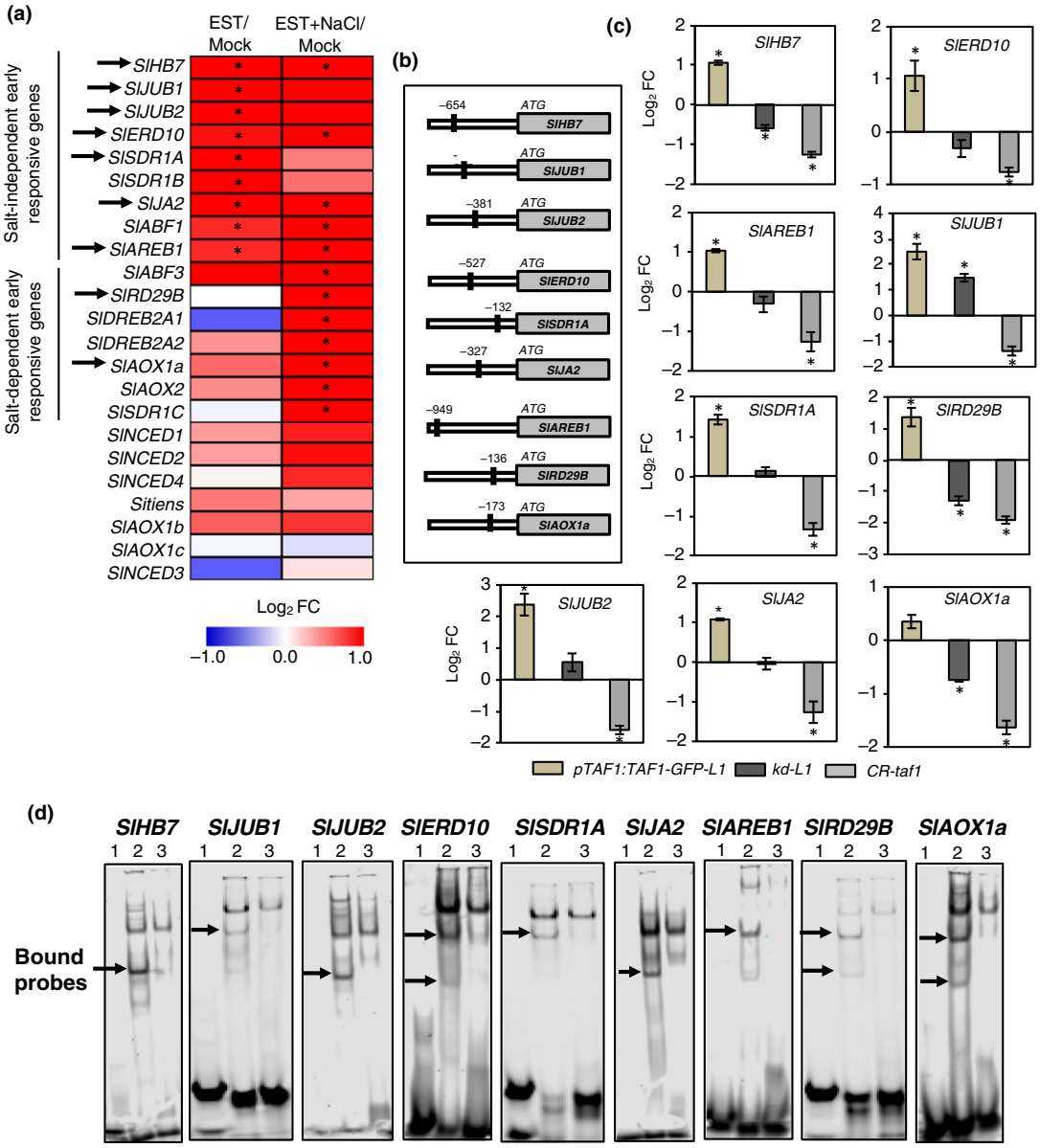

Fig. 6 SlTAF1 regulates salt-responsive genes. (a) Heat map showing the fold change (FC; $\log_2$ basis) of the expression ratio of salt-responsive genes in the following samples: *SlTAF1-IOE* seedlings treated with 15 μM estradiol (EST) compared with control seedlings treated with 0.15% (v/v) ethanol (Mock); *SlTAF1-IOE* seedlings treated with 15 μM EST plus 200 mM NaCl (EST + NaCl) compared with seedlings treated with 200 mM NaCl in the absence of EST (0.15% (v/v) ethanol; Mock). Treatment times were 6 h. Blue, downregulated; red, upregulated (as indicated by the colour bar). Arrows indicate genes harbouring a SlTAF1 binding site (BS) in their 1-kb promoters (upstream of translational ATG codon). Asterisks indicate significant differences between EST and Mock treatment (left column), or between EST + NaCl and NaCl treatment only (right; Student's *t*-test; *, $P < 0.05$). (b) Schematic presentation of the position of SlTAF1 BSs in the promoters of *SlHB7* (*Solanum lycopersicum HOMEOBOX 7*), *SlJUB1* (*Solanum lycopersicum JUNGBRUNNEN 1*), *SlJUB2*, *SlERD10* (*Solanum lycopersicum EARLY RESPONSE TO DEHYDRATION 10*), *SlSDR1A* (*Solanum lycopersicum SHORT-CHAIN ALCOHOL DEHYDROGENASE/REDUCTASE 1A*), *SlJA2* (*Solanum lycopersicum JASMONIC ACID 2*), *SlAREB1* (*Solanum lycopersicum ABA-RESPONSIVE ELEMENT-BINDING PROTEIN 1*), *SlRD29B* (*Solanum lycopersicum RESPONSIVE TO DESICCATION 29B*), and *SlAOX1a* (*Solanum lycopersicum ALTERNATIVE OXIDASE 1a*). (c) Transcript abundance of SlTAF1 early responsive genes (harbouring an SlTAF1 BS in their promoter) in *pTAF1:TAF1-GFP-L1, kd-L1* and *CR-taf1* plants compared with wild-type after NaCl (200 mM) treatment for 4 h. Values on *Y*-axis denote fold change ($\log_2$ basis). Data are means of three biological replicates. Asterisks indicate a significant difference between wild-type and *SlTAF1* transgenic lines (Student's *t*-test; *, $P < 0.05$). (d) Electrophoretic mobility shift assay. SlTAF1-6xHis protein binds to double-stranded 5′-DY682-labelled 40-bp promoter fragments (probes) of *SlHB7, SlJUB1, SlJUB2, SlERD10, SlSDR1A, SlJA2, SlAREB1, SlRD29B and SlAOX1a*, respectively. Lane 1, labelled probe only; lane 2, labelled probe plus SlTAF1-CELD-6xHis protein, showing the retardation band (bound probe); lane 3, labelled probe, SlTAF1-CELD-6xHis protein plus 200× nonlabelled probe (competitor, 40-bp promoter fragment containing SlATF1-BS).

In the longer term, salinity leads to ion toxicity. Plants have evolved mechanisms to alleviate the toxic effects of Na^+ by regulating Na^+ transport from root to shoot, exclusion of Na^+ from the cytoplasm, or sequestration of salt ions in vacuoles (Yamaguchi and Blumwald, 2005; Munns and Tester, 2008; Deinlein *et al.*, 2014). We observed a higher accumulation of Na^+ in leaves

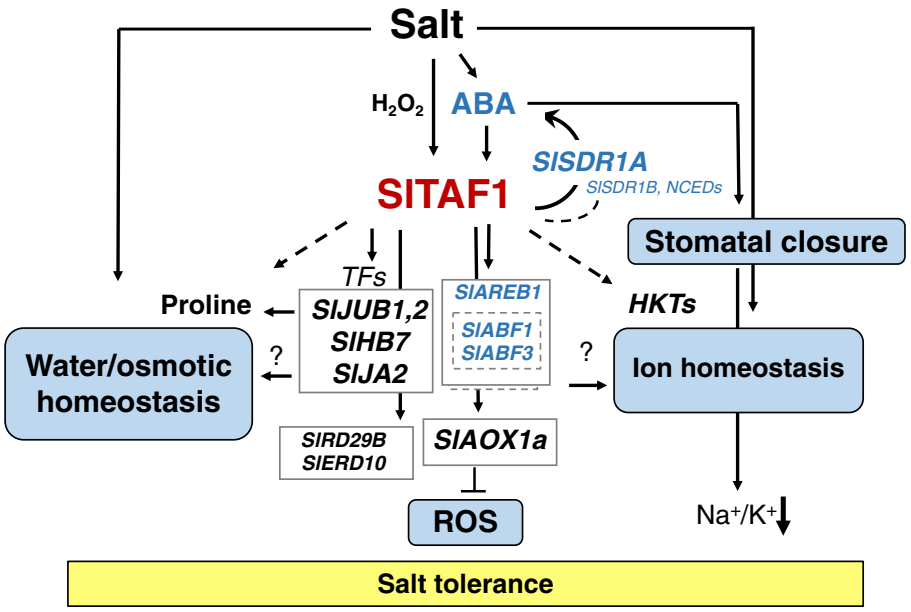

Fig. 7 Model of SlTAF1 action in the regulation of salt stress tolerance. Salt stress enhances H_2O_2 and abscisic acid (ABA) levels that trigger induction of *SlTAF1* in tomato (*Solanum lycopersicum* cv Moneymaker). SlTAF1 significantly activates the expression of the ABA biosynthesis genes *SlSDR1A* (*S. lycopersicum SHORT-CHAIN ALCOHOL DEHYDROGENASE/REDUCTASE 1A*) and *SlSDR1B*, and slightly *NCED* (*9-CIS-EPOXYCAROTENOID DIOXYGENASE*) genes, and enhances ABA level, boosting its own transcriptional activation. Furthermore, SlTAF1 affects ABA signalling by regulating the expression of ABA-signalling TFs such as *SlAREB1* (*S. lycopersicum ABA-RESPONSIVE ELEMENT-BINDING PROTEIN 1*), *SlABF1* (*S. lycopersicum ABA-RESPONSIVE ELEMENT-BINDING FACTOR 1*) and *SlABF3*. SlTAF1 fine tunes the response to salt stress by controlling and integrating complex regulatory networks that are involved in both, osmotic and ion homeostasis. Under salt stress, SlTAF1 triggers the accumulation of proline, an osmolyte with a critical role in maintaining cellular osmotic balance. Furthermore, it regulates transcriptional activation of salt stress-responsive TFs, namely *SlJUB1* (*S. lycopersicum JUNGBRUNNEN 1*), *SlJUB2*, *SlHB7* (*S. lycopersicum HOMEOBOX 7*), and *SlJA2* (*S. lycopersicum JASMONIC ACID 2*), stress-responsive genes such as *SlRD29B* (*S. lycopersicum RESPONSIVE TO DESICCATION 29B*) and *SlERD10* (*S. lycopersicum EARLY RESPONSE TO DEHYDRATION 10*), and the expression of *SlAOX1a* (*S. lycopersicum ALTERNATIVE OXIDASE 1a*) encoding a reactive oxygen species (ROS) scavenging enzyme which collectively enhances the plant's salt stress tolerance. Furthermore, SlTAF1 depletes the accumulation of harmful Na^+ ions in shoots by reducing stomatal aperture and stomatal conductance and by enhancing the expression of Na^+ transporter genes *SlHKT1;1* (*S. lycopersicum HIGH-AFFINITY K(+) TRANSPORTER 1;1*) and *SlHKT1;2* during salinity stress.

of *SlTAF1-kd* than wild-type plants while, by contrast, *pTAF1:TAF1-GFP* plants accumulated significantly less Na^+ in leaves. These data suggested that SlTAF1 contributes to the lower accumulation of Na^+ in the plant's aerial parts, consistent with its role in improving salt tolerance.

In Arabidopsis, the class I HKT sodium transporter AtHKT1;1 retrieves Na^+ ions from the xylem transpiration stream, thereby preventing its transport to shoots (Davenport *et al.*, 2007; Moller *et al.*, 2009). The function of HKT1 appears to be conserved in dicotyledonous plants such as tomato. Asins *et al.* (2013) identified quantitative trait loci (QTL) involved in the regulation of shoot Na^+ homeostasis, harbouring closely linked *SlHKT1;1* and *SlHKT1;2* sodium transporter genes. These two genes are expressed in xylem parenchyma and phloem cells suggesting a role for retrieving Na^+ from the xylem transpiration stream and possibly loading it into phloem sieves. Moreover, silencing of *SlHKT1;2* led to a higher Na^+/K^+ ratio in leaves and salt-hypersensitivity (Jaime-Perez *et al.*, 2017). Here, shoot and root tissues of *pTAF1:TAF1-GFP* plants showed higher transcript abundance of *SlHKT1;1* and *SlHKT1;2* after 2 d of salt treatment; conversely, transcript levels of both genes were significantly reduced in *SlTAF1-kd* plants. Taken together, the above data suggest that SlTAF1 is involved in controlling the retrieval of Na^+ from the xylem transpiration stream via regulating the expression of *SlHKT1;1* and *SlHKT1;2* under saline conditions.

Recently, Shkolnik *et al.* (2019) showed that an ABA-responsive element (ABRE) in the promoter of *AtHKT1;1* is required for its enhanced expression in response to salt and ABA treatment. *SlHKT1;1* and *SlHKT1;2* promoters do not contain SlTAF1 binding sites and can, therefore, not be directly bound by SlTAF1. It is therefore likely that SlTAF1 enhances the expression of *SlHKTs* in cooperation with ABRE-binding TFs in an ABA-dependent manner during salinity stress.

During salinity stress, Na^+ moves from roots to shoots through the xylem transpiration stream and accumulates in aerial parts of the plant which leads to toxicity. Optimising transpiration by controlling stomatal aperture is amongst the main determinants for reducing the rate of Na^+ transport to shoots, thereby leading to salt acclimation over time (Shabala, 2013; Campos *et al.*, 2016). Here, we observed higher stomatal conductance and stomatal pore area in response to salt stress in *SlTAF1-kd* than wild-type plants, while the opposite trend was detected in *pTAF1:TAF1-GFP-L1* (Fig. 4). ABA plays a vital role for stomatal closure and regulating water loss via transpiration (Raghavendra *et al.*, 2010). Expression of *SlTAF1* is induced by ABA treatment (Fig. 1e). Moreover, SlTAF1 is involved in ABA

biosynthesis by regulating the expression of *SlSDR1A*. Upon treatment with external ABA, stomatal pore area was significantly less reduced in *SlTAF1-kd* than in the wild-type, similar to the effect observed after salt stress treatment. By contrast, ABA treatment caused stomates to close more in *pTAF1:TAF1-GFP-L1* than in wild-type plants. These results clearly demonstrated that SlTAF1 is involved in stomatal closure in the response to salinity stress, partly through an ABA-mediated pathway, thereby contributing to enhanced salinity stress tolerance. In summary, SlTAF1 inhibits the transport of Na^+ to the shoots by promoting stomatal closure in leaves and enhancing the expression of *SlHKTs* in roots.

To acquire further molecular insights into the salt tolerance mechanism of SlTAF1, we tested expression of several stress-responsive genes encoding TFs, ABA biosynthesis enzymes and signalling and defence-related proteins in *SlTAF1-IOE* seedlings, shortly after induction of *SlTAF1* either by EST or by EST in combination with salt stress (Fig. 6a). The expression level of most genes was significantly upregulated by SlTAF1. Among these, expression of *SlJUB1*, *SlJUB2*, *SlHB7*, *SlJA2*, *SlABF1*, *SlAREB1*, *SlERD10*, *SlSDR1A* and *SlSDR1B* increased when SlTAF1 was induced already in the absence of salt treatment, indicating that SlTAF1 is sufficient for their induction. However, transcript induction of other genes (such as *SlRD29B*, *SlAOX1a*, *SlAOX2*, *SlSDR1C*, *SlDREB2A1* and *SlDREB2A2*) by SlTAF1 required salt, suggesting an involvement of a yet unknown salt activated factor(s) in the regulation of those genes by SlTAF1. Several of the SlTAF1 early responsive genes are functionally involved in the regulation of stress responses in different species. For example, JUB1 appears to enhance the tolerance towards drought and salt stress in part via an accumulation of proline in Arabidopsis, banana and tomato (A. Wu *et al.*, 2012; Ebrahimian-Motlagh *et al.*, 2017; Tak *et al.*, 2017). Recently, it has been shown that overexpression of Arabidopsis *JUB1* in tomato enhances drought stress tolerance by directly regulating *SlDREB1* and *SlDREB2* expression. VIGS-mediated transient silencing of the tomato *JUB1* gene (*SlJUB1*) resulted in drought sensitivity and increased oxidative damage (Thirumalaikumar *et al.*, 2018). Therefore, the increase of proline content and stress tolerance conferred by SlTAF1 can in part be explained by the transcriptional upregulation of *JUB1*. *SlHB7* is a tomato homologue of Arabidopsis *AtHB7*, which encodes a homeodomain-leucine zipper class I (HD-Zip I) transcription factor; HD-Zip I regulators are known for their roles in abiotic stress responses (Romani *et al.*, 2016). In tomato, ectopic expression of *AtHB7* elevated drought tolerance (Mishra *et al.*, 2012). *SlJA2* is a NAC TF and tomato homologue of *RD26* (*ANAC072*) from Arabidopsis. RD26 was reported to regulate salt and drought stress (Tran *et al.*, 2004; Li *et al.*, 2014; Ye *et al.*, 2017). In tomato, SlJA2 is involved in the direct regulation of ABA-dependent stomatal closure upon pathogen infection via activation of ABA biosynthesis gene *SlNCED1* (Du *et al.*, 2014). SlTAF1 regulates expression of *SlJA2* and the observed induction of *SlNCED1* by SlTAF1 (Fig. 6) might be mediated through SlJA2. Furthermore, SlTAF1 enhances expression of *SlERD10*, a homologue of Arabidopsis *ERD10*, which encodes a subgroup 2 LEA (late embryogenesis abundant) protein. LEA proteins are highly hydrophilic and involved in the protection of cells during stress conditions (Graether & Boddington 2014). In rice, overexpression of *ATAF1* leads to a strong induction of the LEA gene *OsLEA3* (Liu *et al.*, 2016). Another stress-responsive gene whose expression is rapidly induced by SlTAF1 is *SlAOX1a*, which encodes an alternative oxidase. AOXs play a role in the detoxification of ROS generated during osmotic stress, particularly by decreasing production of O_2^- and preventing oxidative damage in mitochondria (Mittler *et al.*, 2004). In Arabidopsis, constitutive overexpression of *AtAOX1a* reduces H_2O_2 levels and shoot Na^+ content after salt stress and promotes salt stress tolerance (Smith *et al.*, 2009). Recently, Zhu *et al.* (2018) revealed that *SlAOX1*a is a positive regulator of drought stress tolerance in tomato. *SlAOX1a* overexpression reduces H_2O_2 levels, while formation of H_2O_2 is enhanced in *SlAOX1a-RNAi* plants. Indeed, H_2O_2 accumulation was higher in *SlTAF1-kd* but lower in *pTAF1:TAF1-GFP* plants, compared with wild-type, indicating that SlTAF1 is involved in regulating ROS signalling during salt stress, in part through regulation of *SlAOX1a*.

Among ABA biosynthesis genes whose transcript levels were significantly upregulated by SlTAF1 are *SlSDR1A* and *SlSDR1B*. Among SlSDR1s, however, only *SlSDR1A* expression is dramatically induced by salt stress, indicating that it has a pivotal role in the stress response. *SlSDR1A* is a homologue of Arabidopsis *SDR1*/*ABA2* (*ABSCISIC ACID DEFICIENT 2*) and the enzyme it encodes performs the second last step in ABA biosynthesis, that is the conversion of xanthoxin to abscisic aldehyde (Gonzalez-Guzman *et al.*, 2002). In Arabidopsis, *SDR1* positively regulates salt tolerance which is correlated with elevated ABA levels (Lin *et al.*, 2007). Indeed, the ABA level was higher in *pTAF1:TAF1-GFP* plants compared with wild-type (Fig. S8) suggesting that SlTAF1 is an activator of its own expression (as expression of *SlTAF1* is positively affected by ABA; Fig. 1e). The *SlTAF1* promoter contains at least four core *cis*-acting ABREs (Fig. S9), suggesting that the induction of *SlTAF1* by ABA occurs via ABRE-binding TFs; this, however, remains to be demonstrated.

Among the genes induced by EST treatment in *SlTAF1-IOE* seedlings, *SlRD29B*, *SlJUB1*, *SlJUB2*, *SlHB7*, *SlJA2*, *SlAREB1*, *SlERD10*, *SlAOX1a* and *SlSDR1A* harbour an SlTAF1 binding site in their promoter and their expression was strongly enhanced during salt stress (Table S3). Electrophoretic mobility shift assays (EMSA) revealed that SlTAF1 physically interacts with the promoters of these genes *in vitro* (Fig. 6d) suggesting that it promotes their transcriptional regulation through direct interaction *in vivo*. In accordance with this, expression of most of them was significantly induced in *pTAF1:TAF1-GFP* during salinity stress, but reduced in *CR-taf1* seedlings. Collectively, SlTAF1 controls a gene network consisting of key stress regulatory elements such as TFs, ABA biosynthesis genes and signalling, and defence-related components (Fig. 7).

In summary, our study demonstrates that SlTAF1 regulates various responses to salt stress at the molecular, metabolic and physiological levels and plays an important role for salinity tolerance.

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Author contributions

SB and BM-R conceived the study; SB designed the research and supervised the work; VD generated the transgenic lines, performed salt treatment experiments, determined plant phenotypes, performed qRT-PCR analyses and contributed to the design of the research; VD and VPT jointly performed the EMSA experiments and confocal microscopy studies; G-PX performed the binding site selection assays; VT and MS performed the ABA measurements; JV performed primary metabolite profiling under the supervision of ARF; RH performed the ion measurements; SB wrote the manuscript with contributions from VD and BM-R; all authors read and commented on the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 *SlTAF1* expression and phenotypes of constitutive *SlTAF1* overexpressors.

Fig. S2 Expression of *SlTAF1* in *SlTAF1 knockdown* and *pTAF1:TAF1-GFP* lines.

Fig. S3 SlTAF1 regulates salt stress tolerance in tomato.

Fig. S4 Effect of salinity stress on soil-grown tomato plants.

Fig. S5 CRISPR/Cas9-mediated knockout of *SlTAF1* results in salt sensitivity.

Fig. S6 Effect of salt stress on Na^+ and K^+ ions in tomato roots.

Fig. S7 Involvement of SlTAF1 in ABA-mediated stomatal closure.

Fig. S8 Higher accumulation of ABA in *pTAF1:TAF1-GFP-L1* than wild-type plants.

Fig. S9 Schematic presentation of the positions of ABRE elements in the promoter of *SlTAF1*.

Methods S1 Supporting methods.

Table S1 Oligonucleotide sequences.

Table S2 Primary metabolite profiles of wild-type and *SlTAF1* transgenic plants.

Table S3 Expression of *SlTAF1* and stress-associated genes in tomato seedlings.

Table S4 DNA-binding specificity of SlTAF1.

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