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Research article

Overexpression of the NAC transcription factor *JUNGBRUNNEN1* (*JUB1*) increases salinity tolerance in tomato

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ABSTRACT

Soil salinity is a major abiotic stress affecting plant growth and yield, due to both osmotic and ionic stresses. *JUBGBRUNNEN1* (*JUB1*) is a NAC family transcription factor that has been shown to be involved in responses to abiotic stresses, such as water deficit, osmotic, salinity, heat and oxidative stress. In *Arabidopsis thaliana* (Arabidopsis), JUB1 has been shown to improve plant stress tolerance by regulating H_2O_2 levels. In the horticultural crop, *Solanum lycopersicum* cv. Moneymaker (tomato), overexpression of *AtJUB1* has been shown to partially alleviate water deficit stress at the vegetative stage. In this study, we investigated the effect of Arabidopsis *JUB1* overexpression in salinity tolerance in tomato. In hydroponically grown tomato seedlings, *AtJUB1* overexpression results in higher prolines levels and improves the maintenance of water content in the plant under salinity stress. The transgenic tomato plants are more tolerant to salinity stress compared to control lines based on plant biomass. However, at the reproductive stage, we found that overexpression of *AtJUB1* only provided marginal improvements in yield-related parameters, in the conditions used for the current work. The combination of improved water deficit and salinity stress tolerance conferred by *AtJUB1* overexpression may be beneficial when tomato plants are grown in the field under marginal environments.

1. Introduction

Soil salinity is a major abiotic stress affecting plant growth and yield. The adverse effects of salinity occur as a result of osmotic and ionic stresses. Osmotic stress occurs immediately after salt imposition and continues for the duration of salt exposure, involving rapid signaling from root to shoot upon salt exposure and resulting in reduced cell expansion in growing tissues (Roy et al., 2014). Plants respond to osmotic stress using mechanisms involving processes such as maximizing water uptake and reducing water loss through stomatal closure (Munns and Tester, 2008). After several days of salinity stress, ions build up in photosynthetically active tissues and affect major processes such as photosynthesis, protein synthesis and energy production (Parida and Das, 2005). In addition, ionic stress results in premature leaf senescence, which reduces photosynthetically active areas available to support plant growth. Plants have evolved several mechanisms to limit the toxic effects of ionic stress; mechanisms include reducing sodium transport to the shoot and compartmentalizing it into the cell vacuole. Accumulation of compatible solutes, such as proline, sugars and amino acids is another adaptive mechanism to tolerate salinity stress (Roy et al., 2014). These solutes help the plant to adjust the osmotic pressures resulting from ion accumulation in the shoot (Munns and Tester, 2008).

Transcription factors (TFs) are key regulators in stress responses. They link stress sensing with many tolerance mechanisms by translating stress signals into changes in gene expression, that ultimately contribute to stress tolerance (Lodeyro and Carrillo, 2015). In salinity stress, for example, plants are thought to transmit the salt signal through various mechanisms, such as elevated cytosolic free Ca^{2+}, ROS and hormonal signaling, that affect the expression of stress related TFs (Choi et al., 2017). These TFs, subsequently, target many genes involved in stress responses to orchestrate biochemical and physiological processes critical for the stress tolerance (Lodeyro and Carrillo, 2015).

The widespread plant specific TF family, NAC (NAM, ATAF and CUC), regulates multiple processes related to plant growth, development and senescence (Souer et al., 1996; Xie et al., 2000; Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2011; Shahnejat-Bushehri et al., 2016). NACs have also been related to stress responses; several

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NAC genes were found to be induced by biotic and abiotic stresses (Nakashima et al., 2012; Nuruzzaman et al., 2013; Shao et al., 2015; Hong et al., 2016; Wang et al., 2017). Functional characterization of NAC TFs in response to salt stress have been conducted in different plant species. For example, overexpression of wheat *TaNAC29* in Arabidopsis plants improved salinity tolerance by reducing H_2O_2 accumulation and increasing cell membrane stability through enhancing expression of several genes encoding antioxidant enzymes (Xu et al., 2015). In rice, overexpression of *SNAC1* improved the survival rate of rice seedlings grown in hydroponics and in soil (Hu et al., 2006). SNAC1 has also been shown to contribute to drought tolerance of rice at both seedling and reproductive stages by controlling stomatal closure and thus reducing the rate of water loss through transpiration (Hu et al., 2006). *SNAC2* is another rice NAC TF whose expression is induced by drought, salt, cold, wounding and abscisic acid (ABA) (Hu et al., 2008). Overexpression of *SNAC2* enhanced plant germination rate and root length under high salinity (Hu et al., 2008).

JUBGBRUNNEN1 (JUB1, also known as NAC042) acts as a central regulator of plant growth (Shahnejat-Bushehri et al., 2016), longevity and stress response (Wu et al., 2012). Expression of Arabidopsis *JUB1* is upregulated by abiotic stresses and confers tolerance to osmotic, salt, heat, drought and oxidative stresses (Shahnejat-Bushehri et al., 2012; Wu et al., 2012). The effect of JUB1 in stress tolerance is thought to occur through controlling H_2O_2 levels (Wu et al., 2012). JUB1 directly activates the expression of the transcription factor Dehydration-Responsive Element-Binding Protein 2A (*DREB2A*), which is an important regulator of drought and heat responses (Sakuma et al., 2006; Kant et al., 2008). DREB2A then regulates Heat-shock factor A2 (*HsfA2*) and thereby several Heat-Shock Protein (*HSP*) genes and genes encoding H_2O_2-scavenging enzymes (Schramm et al., 2008; Yoshida et al., 2008). With respect to growth regulation, JUB1 mediates the reduction of two growth hormones: gibberellic acid (GA) and brassinosteroids (BR). JUB1 represses the expression of several key enzymes involved in the GA and BR biosynthesis pathways (Shahnejat-Bushehri et al., 2016). In addition, JUB1 activates the expression of *DELLA* genes (*GAI* and *RGL1*). DELLA proteins are transcriptional regulator proteins and regulate plant growth and stress responses. High levels of DELLA proteins mediate stress tolerance by limiting the accumulation of stress-induced reactive oxygen species (ROS) (Shahnejat-Bushehri et al., 2016).

The tomato homolog of Arabidopsis *JUB1*, *SlJUB1*, was found to be induced by different abiotic stresses, including salinity. Downregulation of *SlJUB1* by virus-induced gene silencing (VIGS) in *Solanum lycopersicum* cv. Moneymaker (tomato) substantially decreased drought tolerance of tomato plants and, congruently, ectopic overexpression of *AtJUB1* in tomato improved its tolerance to water deficit by maintaining high relative leaf water content and reducing H_2O_2 levels (Thirumalaikumar et al., 2018). Arabidopsis JUB1 was able to bind and directly regulate the tomato homolog of Arabidopsis *DREB2A* and *DELLA* (*SlDREB1*, *SlDREB2* and *SlDELLA*). This suggests a considerable conservation in the gene regulatory networks controlled by JUB1 between Arabidopsis and tomato (Thirumalaikumar et al., 2018).

In this study we investigate the effect of *AtJUB1* in salinity tolerance in tomato. Hydroponically grown tomato plants overexpressing *AtJUB1* display improved maintenance of biomass in response to salt stress compared to wild type plants, an effect associated with a higher maintenance of water within the plant. However, plants grown to maturity in soil overexpressing *AtJUB1* only display marginal advantages compared to wild type plants, in the conditions used in these experiments.

2. Material and methods

2.1. Plant material

Plants of *Solanum lycopersicum* cv. Moneymaker (tomato) were used as wild type controls in salinity experiments. Tomato lines overexpressing *AtJUB1* were previously described in (Shahnejat-Bushehri et al., 2017). In brief, tomato plants (*Solanum lycopersicum*, cv Moneymaker) were transformed with the 35S:*AtJUB1-GFP* overexpression construct described in (Wu et al., 2012).

2.2. Salinity stress in a supported hydroponic system

The salinity stress experiment was performed in a supported hydroponic system. Plants were grown in the greenhouse located at KAUST (Thuwal, Saudi Arabia) at 25 °C/23 °C day/night with a day length of approximately 13.5 h. Tomato seeds were surface sterilized with 20% bleach and washed thoroughly eight times with sterilized milliQ water. Seeds were stratified at 4 °C for three days then germinated on agar plugs consisting of ¼ Murashige and Skoog medium, 1% phyto-agar, pH 5.8. The agar plugs were placed in square pots (7 cm) filled with plastic beads and covered with water. This plant nursery was kept covered with transparent plastic covers until seeds started germinating. When the cotyledons emerged, plants were transferred from the nursery to the ebb-and-flow supported hydroponic system (Fig. S1) filled with growth medium. Growth medium was prepared by mixing equal amounts of three commercial media: FloraMicro, FloraGro and FloraBloom (General Hydroponics, USA). The pH was monitored throughout the experiment and maintained at 5.8–6.0 by supplementing with buffer solutions called “pH up” or “pH down” for hydroponics, supplied by Botanicare (USA). Growth solution was pumped up to the roots for 30 min, then allowed to drain for 30 min, giving an ebb-and-flow cycle of 60 min. This allowed the system to fully drain and roots to be well aerated before refilling. Uniformly sized seedlings at the five-leaf growth stage were selected for the salinity experiment.

Three salt concentrations were used: 0 mM NaCl (as control), 125 mM NaCl (as mild salt stress) and 200 mM NaCl (as high salt stress). To avoid osmotic shock, salt (NaCl) was applied in the following increments every 24 h: 75 mM, 125 mM, 200 mM NaCl. Calcium (in the form of $CaCl_2$) was supplemented with the salt treatments to compensate for the reduction of Ca^{2+} activity due to NaCl addition (Tester & Davenport, 2003). Calcium activity and the amount of required $CaCl_2$ was calculated using GeochemEZ (Shaff et al., 2010). After 10 days of salt stress, the following measurements were made: plant biomass (fresh and dry), stem height and root length. Leaf area measurements were analyzed using the scanning software WinFOLIA (Régent Instruments Inc.). For the biochemical measurements; leaf three (as an older leaf) and leaf five (as a younger leaf) were collected. Salinity tolerance traits were assessed and analyzed according to Negrão et al. (2017).

2.3. Salinity stress in soil

To test the tomato plants under soil conditions a pot trial was performed. Tomato seeds were germinated in jiffy pellets and transferred to soil filled pots (20 cm) at the five-leaf growth stage. Salt (NaCl) was applied at the eight-leaf growth stage by adding a total volume of 1.3 L of 450 mM NaCl to the bottom of the outer pot (distributed as three doses of 400 mL, 400 mL and 500 mL over two weeks). The final concentration of NaCl in the soil was approximately 365 mM NaCl (after the soil had dried down to contain 1.6 L of water per pot). Plants were then assessed after 17 days of salt treatment.

2.4. Na and K measurements

Na and K content was measured in the roots and leaves using flame photometry. The leaf that developed during salt stress imposition (fully expanded leaf, here leaf 5) was used for Na and K measurements. Roots were rinsed twice in 10 mM $MgSO_4$ (to remove any excess NaCl from the hydroponic medium) and dried between tissue paper. Oven-dried roots and leaves were digested in 20 mL of 1% nitric acid at 85 °C overnight and the concentration of Na and K were measured with the flame photometer (Sherwood Scientific Ltd., Cambridge, UK, model

420).

2.5. Proline quantification

Proline content was determined according to the method of Bates et al. (1973) with some modifications (Vicente et al., 2004). Briefly, frozen plant material (around 100 mg) was ground to a fine powder. Sulfosalicylic acid (3%) was used for the extraction and cell debris were removed by centrifugation at 13000 rpm for 10 min. One volume of the supernatant was mixed first with one volume of freshly prepared acid ninhydrin (25 mg/mL ninhydrin in 10.44 M acetic acid and 2.4 M phosphoric acid); and then with one volume of glacial acetic acid. Samples were mixed and incubated at 99 °C for 1 h, and then the reaction was stopped by cooling the samples on ice. The proline-ninhydrin complex was extracted with two volumes of toluene. The absorbance of the organic phase was determined by UV-Spectrometer (model U-2910, Hitachi, Japan) at 520 nm, using toluene as a blank. A calibration curve was used to calculate the concentration of proline.

2.6. Determining the osmotic potential

Osmotic potential was measured on leaf 3 (old leaf) and leaf 5 (young leaf) using a vapor pressure osmometer (model 5600, Vapro, Wescor, Inc. USA). Leaves were collected in a 2 mL syringe and stored at −80 °C. Leaf sap was prepared by thawing the samples on ice and pressing the syringe pestle to collect leaf sap. Only 10 µL of the leaf sap was used for analysis.

2.7. Chlorophyll quantification

Chlorophyll was extracted from leaf four according to Nayek et al. (2014) with a slight modification. Briefly, frozen plant material (approximately 50 mg) was ground to a fine powder. One mL of DMSO was added to the ground plant tissue and the mixture was sonicated for 15 min in an ultrasonic bath (model 3510, Branson, USA). The extracted mixture was centrifuged at 12,000 rpm, 4 °C for 10 min and the supernatant was collected. The same extraction procedure was repeated on the residue for a second time. Thereafter, each 2 mL supernatant was mixed with another 1 mL DMSO. The absorbance of Chlorophyll-a and Chlorophyll-b was determined by UV-spectrophotometry at 645 and 663 nm. Chlorophyll-a, Chlorophyll-b and total chlorophyll was calculated using the equations described in (Richardson et al., 2002):

Chlorophyll a (mg/g fresh mass) = $[(12.7*A_{663})-(2.69*A_{645})]*V/(M*1000)$

Chlorophyll b (mg/g fresh mass) = $[(22.9*A_{645})-(4.68*A_{663})]*V/(M*1000)$

Total Chlorophyll s = $(20.08*A_{645}+8.02*A_{663})*V/(M*1000)$

With V: final volume of chlorophyll extracted; M: fresh mass of leaf material.

2.8. ABA quantification

ABA was quantified according to Almeida Trapp et al. (2014) with modifications. Approximately 15 mg of freeze dried ground leaf (leaf number four) was used to measure the ABA level. The internal standard D_6-ABA (1.05 ng) was added to the ground tissue along with 1 mL of ethyl acetate. The mixture was sonicated for 15 min in an ultrasonic bath (Branson 3510 ultrasonic bath). The mixture was then centrifuged at 13000 rpm, 4 °C for 5 min. The ABA-rich organic phase was collected from each sample. The collection was repeated a second time. The organic phase was dried using a vacuum pump. The residue was dissolved with 50 µL ethyl acetate and 2 mL of hexane for purification. The sample was run through a preconditioned (3 mL of ethyl acetate, and then 3 mL hexane) silica gel SPE (500 mg/3 mL). After washing with 3 mL hexane, ABA was eluted with 3 mL methanol and evaporated to dryness. The sample was re-dissolved in 200 µL of acetonitrile:water (25:75, v:v) and filtered through a 0.22 µm filter for LC-MS analysis. ABA was analyzed using HPLC-Q-Trap-MS/MS with MRM mode. Chromatographic separation was achieved on a ZORBAX Eclipse plus C18 column (150 X 2.1 mm; 3.5 µm; Agilent). Mobile phases consisted of water:acetonitrile (95:5, v:v) and acetonitrile, both containing 0.1% formic acid. A linear gradient was optimized as follows (flow rate, 0.2 mL/min): 0–15 min, 5%–100% B, followed by washing with 100% B and equilibration with 5% B. The injection volume was 5 µL and the column temperature was maintained at 35 °C for each run. The MS parameters were listed as follows: negative ion mode, ion source of turbo spray, ionspray voltage of −4500 V, curtain gas of 20 psi, collision gas of medium, gas 1 of 50 psi, gas 2 of 60 psi, turbo gas temperature of 500 °C, declustering potential of −25 V, entrance potential of −9 V, collision energy of −17 eV, collision cell exit potential of −20 V. The characteristic MRM transitions (precursor ion → product ion) were 263.2 → 219.1, 263.2 → 153.1 for ABA; 269.2 → 225.2, 269.2 → 159.1 for D_6-ABA.

2.9. Statistical analyses

The salinity tolerance index (ST) and relative root mass ratio were calculated according to Negrão et al. (2017), using the following equations (1)–(4):

For salinity tolerance index:

$$ST = \frac{FM_{\text{after treatment}} - FM_{\text{before treatment}}}{FM_{\text{control}} - FM_{\text{before treatment}}} \quad (1)$$

Where FM is total plant fresh mass.

For relative root mass ratio, root mass ratio (RMR) was first calculated in salt and control, and then the ratio of RMR in salt to RMR in control conditions was calculated according to the following equations:

$$RMR_{\text{control}} = \frac{DM_{\text{root, control}}}{DM_{\text{(root+shoot)control}}} \quad (2)$$

$$RMR_{\text{salt}} = \frac{DM_{\text{root, salt}}}{DM_{\text{(root+shoot) salt}}} \quad (3)$$

$$RRMR = \frac{RMR_{\text{salt}}}{RMR_{\text{control}}} \quad (4)$$

where DM is root dry mass.

A Student T-test was used for treatment comparisons. To obtain an estimate for significance of ratios, each value of the treatment was divided by a mean of the control value; * indicates P-value < 0.05, ** P-value < 0.01.

3. Results

Overexpression of AtJUB1 in tomato results in increased maintenance of biomass in response to salt stress compared with wild type.

We used tomato plants overexpressing *AtJUB1* (referred to as *AtJUB1* OE) to investigate the effect of JUB1 in salinity tolerance in tomato. These plants have been described previously by (Shahnejat-Bushehri et al., 2017) for their improved drought tolerance. After ten days of salt treatment, several physiological parameters related to salinity tolerance were assessed, including shoot and root biomass (fresh mass and dry mass), leaf area, stem height, root length, and Na and K content in the shoot and root. *AtJUB1* OE plants displayed less wilting of the leaves and plants were greener than WT plants (Fig. 1A). At high salinity, the tolerance of *AtJUB1* OE plants was significantly higher compared with WT plants, indicating that the *AtJUB1* OE plants were better able to maintain biomass than the WT plants at high salinity levels (Fig. 1B, Supplementary Table S1). It should also be noted that it

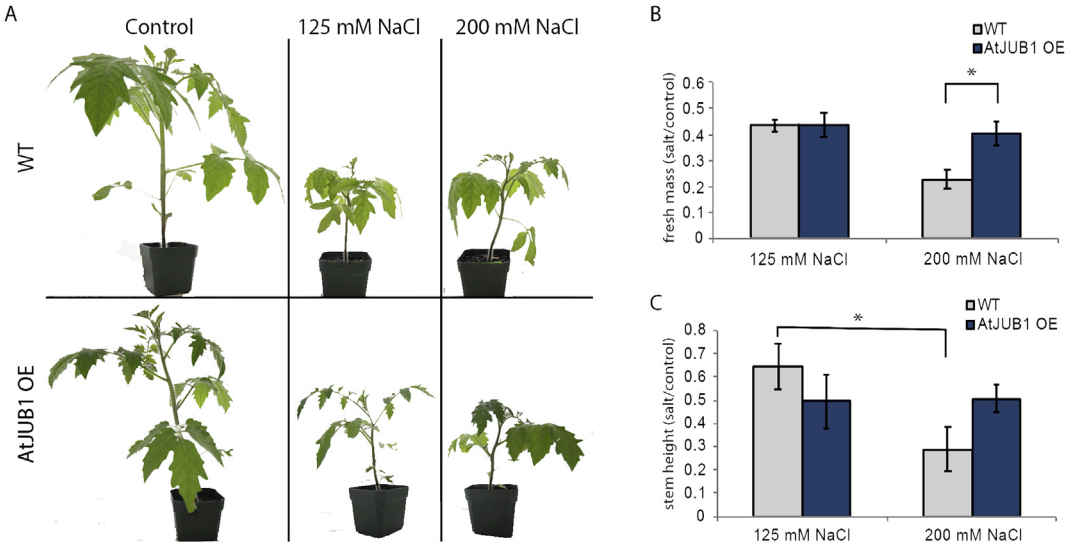

Fig. 1. Phenotypic and physiological analysis of *AtJUB1* OE and WT tomato seedlings hydroponically grown under salt and control conditions. (A) Phenotypes of wild-type cv. Moneymaker (WT) and transgenic (35S:AtJUB1-OE) tomato seedlings after 10 days of salt stress. Note that the background of images was removed using Photoshop, no other changes were made to the images; **(B)** Salinity tolerance (ST) index of WT and *AtJUB1* OE; measured as total fresh mass in salt relative to control; **(C)** Relative stem height of WT and *AtJUB1* OE plants in salt relative to control. n = at least 5 plants, except WT under control conditions, where n = 4; error bars represent standard error of the mean; significant differences were calculated using Student T-test, * indicates *P*-value < 0.05.

appears that *AtJUB1* OE plants do not have a further reduction in stem height as NaCl is increased from 125 to 200 mM, while there is a significant reduction in WT plants (Fig. 1C).

Under mild salinity stress, *AtJUB1* OE plants displayed a higher relative root mass ratio compared to WT plants (root mass compared to total plant mass, salt relative to control) (Fig. 2A), suggesting that the *AtJUB1* OE plants increase the relative allocation of biomass to the roots upon salt stress to a greater extent than under control conditions compared to the WT plants. This effect was not significant under high saline conditions. Notably, root length is decreased for both WT and *AtJUB1* OE plants under salinity stress compared with control; however, there does not appear to be a significant difference between *AtJUB1* OE plant and WT (Fig. 2B).

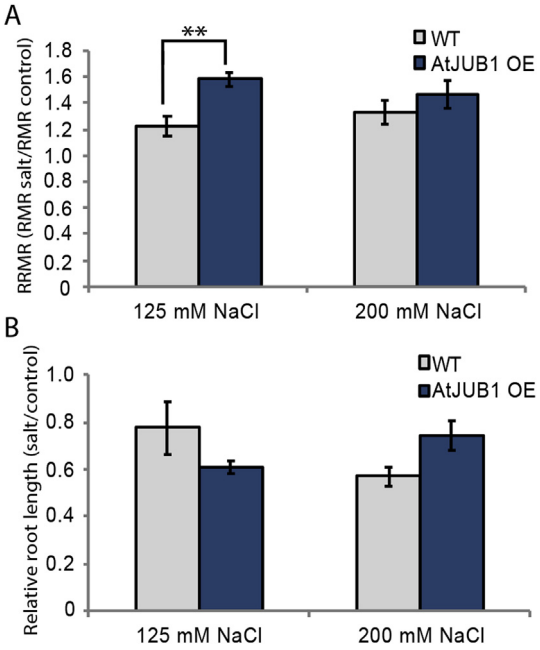

Fig. 2. Effect of salinity on the root biomass and length of *AtJUB1* OE and WT plants. (A) Relative root mass ratio (RRMR); **(B)** Relative root length. n = at least 5 plants, except WT in control conditions, where n = 4; error bar represents standard error of the mean, significant differences were calculated using Student T-test, * indicates *P*-value < 0.05.

The effect of salt on parameters such as leaf area, leaf thickness and leaf elongation factor were analyzed using Winfolia software. All leaf measurements were performed on leaf five as it was the leaf that developed during salt imposition. Leaf area was marginally affected by salt treatment in both WT and *AtJUB1* OE plants. Leaf thickness was not significantly increased in *AtJUB1* OE plants under salt stress. *AtJUB1* OE plants maintained leaf width to length ratio, while this ratio significantly decreased in WT plants under high salt treatment (Fig. S2).

3.1. AtJUB1 OE plants show altered water relations and higher proline levels during salt treatment compared with WT

Water relations are important for plant growth, including the maintenance of cellular water content and maintenance of transpiration. It has been argued in the literature that the hydraulic conductivity in the root decreases under salinity stress, thereby making it less easy for the plant to take up water and, consequently, a reduction in the water fraction can be observed (Negrão et al., 2017). Here we determined shoot water fraction in control and salt stress conditions. Under control conditions, shoot water fraction was comparable in WT and *AtJUB1* OE plants and only a small reduction in water fraction was observed in response to mild salinity levels (Fig. 3A). During high salinity stress, it became apparent that *AtJUB1* OE plants are better able to maintain water fraction, indicating it is better able to maintain water relations in the plant (Fig. 3A and B).

Plants synthesize compatible solutes to maintain plant water potential when salt is accumulated (Lodeyro and Carrillo, 2015). Proline is a compatible solute and its accumulation has been linked to salinity tolerance (Zhu, 2002; Hmida-Sayari et al., 2005). Under control conditions, WT and *AtJUB1* OE plants had similar proline levels in young and old leaves (Fig. 4A). However, during mild and high salinity treatment, an increase in proline levels can be observed in *AtJUB1* OE plants compared with WT, particularly in young leaves (Fig. 4A). This suggests that *AtJUB1* OE plants are better able to maintain water relations by regulating proline accumulation. The overall osmotic potential of leaves (as measured from total leaf fluids from crushed leaves) increased with salinity stress in old and young leaves, as expected;

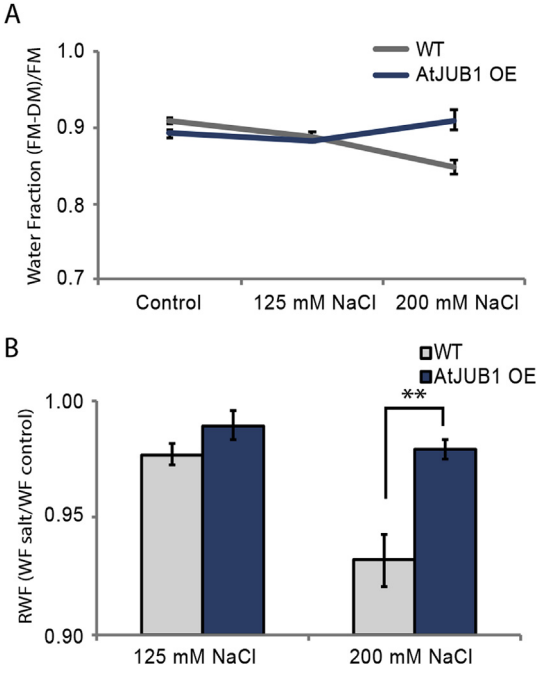

Fig. 3. Water content of *AtJUB1* OE and WT plants. (A) Shoot water fraction of WT and *AtJUB1* OE under control and salt stress conditions; **(B)** relative water fraction of *AtJUB1* OE and wild type plants in salt relative to control. n = at least 5 plants, except WT under control conditions, where n = 4; significant differences were calculated using Student T-test, ** indicates *P*-value < 0.01.

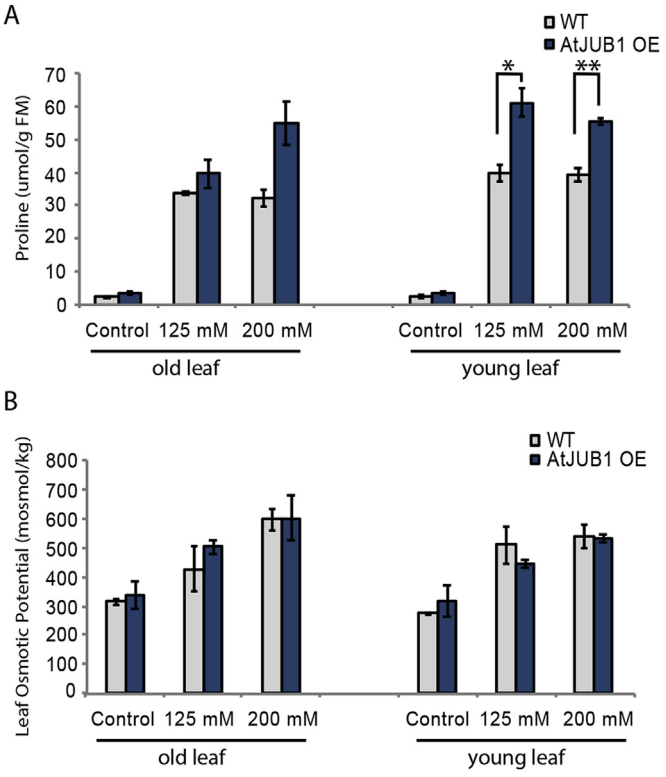

Fig. 4. Proline content and osmotic potential of *AtJUB1* OE and WT plants. (A) Proline level of old leaf and young leaf WT and *AtJUB1* OE plants in control and salt stress conditions; **(B)** Osmotic potential of old leaf and young leaf WT and *AtJUB1* OE plants in control and salt stress conditions. n = 3; significant differences were calculated using Student T-test, * indicates *P*-value < 0.05, ** *P*-value < 0.01.

however, no difference was observed between *AtJUB1* OE and WT plants (Fig. 4B). This could be because the osmotic potential is also driven by ion contents in the leaves (i.e. Na^+, K^+ and Cl^- content). This is consistent with the observation that Na and K contents did not vary between *AtJUB1* OE and WT plants in response to salinity stress in the last fully expanded leaf (Fig. 5A–D). There is a trend for lower Na^+ levels in the roots of *AtJUB1* OE plants during salt stress compared with WT; however, this difference in root Na^+ was not significant. The total chlorophyll content is significantly higher in *AtJUB1* OE plants under salinity stress compared with WT plants (Fig. 6A), suggesting that *AtJUB1* OE plants are better able to maintain photosynthetic activity. The ABA content increased in response to salinity stress; however, it was significantly lower in *AtJUB1* OE plants compared with WT at high salinity (Fig. 6B).

In summary, it appears that JUB1 is involved in maintaining favorable water relations and photosynthetic activity when overexpressed in tomato plants during salinity stress in a hydroponics environment. In the following, we examined if *AtJUB1* overexpression also positively affects yield related parameters.

3.2. *AtJUB1 promotes plant growth under salt stress of soil grown tomatoes*

To assess whether AtJUB1 also affects salinity tolerance of tomato in soil grown plants, WT and *AtJUB1* OE plants were grown in pots containing soil in the greenhouse and subjected to 350 mM NaCl treatment. Salt was imposed at the 8th leaf stage for a total of four weeks and the effect of salinity on plant growth (fresh mass and height) and fruit yield was investigated. The growth of WT plants was visibly reduced after salt stress application, while *AtJUB1* OE plants maintained growth (Fig. 7A). The salinity tolerance index based on plant height and biomass showed that *AtJUB1* OE plants are better able to maintain growth compared with WT (Fig. 7B and C). However, in contrast to hydroponically grown plants, the water fraction was not different between *AtJUB1* OE and WT plants (Fig. 7D). All yield related parameters measured, such as total fruit number, number of flowers, and number of trusses decreased with salinity treatment in both WT and *AtJUB1* OE. This decrease tends to be slightly more prominent in WT plants; however, no statistically significant differences were observed when comparing WT and *AtJUB1* OE plants (Fig. 7E). In summary, overexpression of *AtJUB1* in tomato plants appears to improve plant growth under salinity stress compared with WT plants, and there is a trend for positive effects on yield related parameters (however, these were not significant, at least under the conditions tested).

4. Discussion

To investigate if JUB1 is involved in salinity tolerance in tomato, we performed salt stress experiments in a hydroponics and soil setup with tomato plants overexpressing *AtJUB1* (described previously by (Thirumalaikumar et al., 2018). It has been shown previously that Arabidopsis overexpressing *AtJUB1* had fewer senescence symptoms in response to salinity stress and was therefore more tolerant compared with WT (Wu et al., 2012). Here, we investigated if *AtJUB1* overexpression also reduces senescence symptoms in tomato and if it influences biomass and fruit production in response to salinity stress. We found that *AtJUB1* overexpression indeed improved the plants' salinity tolerance based on maintenance of biomass compared with WT controls. It appears *AtJUB1* overexpression also improved yield related parameters in tomato plants, although this effect was moderate and not statistically significant under the conditions tested.

Several NAC transcription factors have previously been shown to be involved in the response to salinity stress and that their overexpression affects salinity tolerance (Nuruzzaman et al., 2013). In tomatoes, for instance, it has been shown that knock-down of *SlNac4* reduced the salinity tolerance of transgenic tomato plants in response to salinity stress (Zhu et al., 2014), while overexpression of the tomato gene,

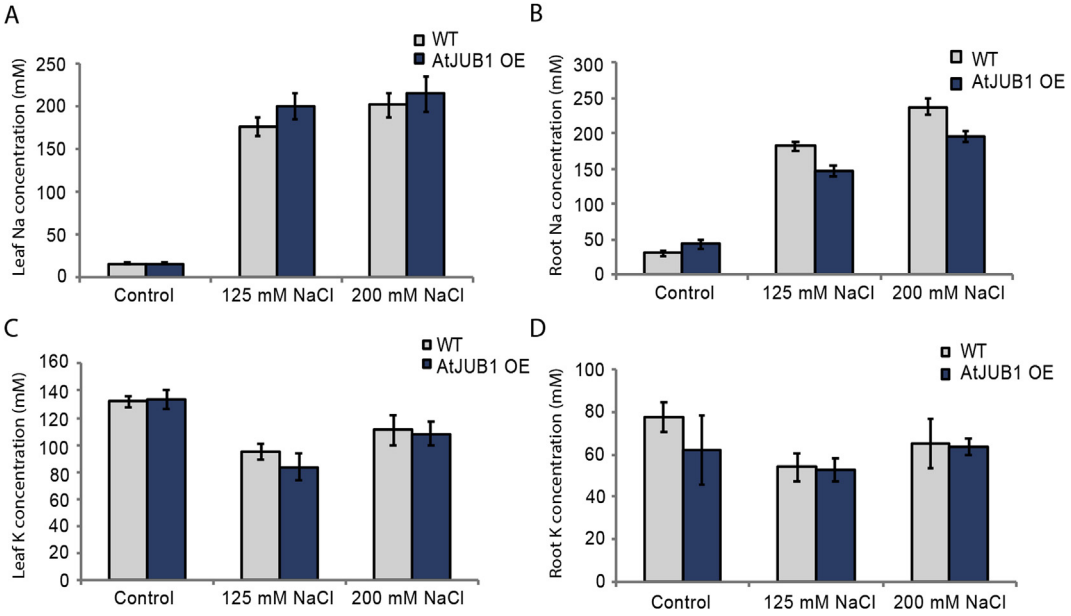

Fig. 5. The ion content of the last fully expanded leaf (leaf five) and root of *AtJUB1* OE and WT plants. (A) Sodium concentration in leaf five; **(B)** Sodium concentration in the roots; **(C)** Potassium concentration in leaf five; **(D)** Potassium concentration in the roots. n = at least 5 plants, except WT under control conditions, where n = 4; error bars represent standard error of the mean; significant differences were calculated using Student T-test, no significant differences were detected.

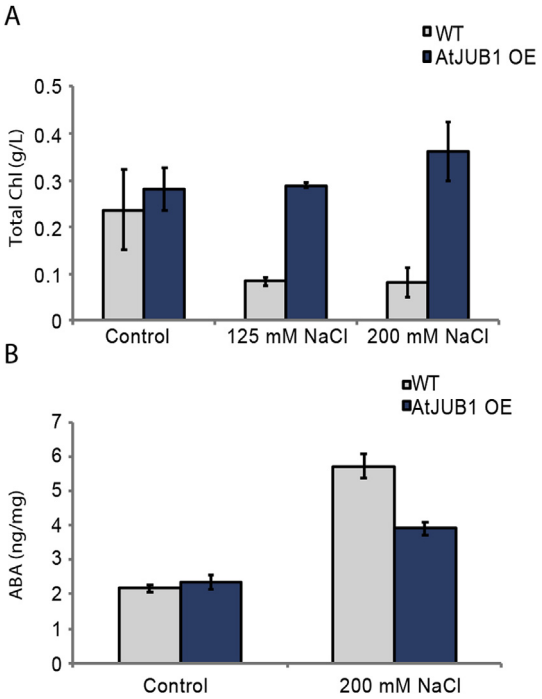

Fig. 6. Chlorophyll and ABA levels of *AtJUB1* OE and WT plants. (A) Total chlorophyll content of leaf 4; **(B)** ABA content of WT and *AtJUB1* OE plants. The ABA concentration represents the mean of two technical replicates and three biological replicates. n = 3; error bars represent standard error of the mean; significant differences were calculated using Student T-test, * indicates *P*-value < 0.05 and ** *P*-value < 0.01.

SlNac35, in Arabidopsis and *Nicotiana benthamiana* (tobacco) appears to improve salinity tolerance based on germination and root growth compared with WT controls (Wang et al., 2016). Although many stress responsive NACs have been identified, the underlying molecular mechanisms and stress-related genes directly regulated remain to be identified.

Here we found that *AtJUB1* OE plants showed less reduction in biomass, stem height and root length under high salinity stress, while the effects of mild salinity stress were not significantly different between *AtJUB1* OE and WT plants. It is notable that the effect appears to be stronger under high salinity conditions, as opposed to mild salinity stress. A similar effect has been observed before in a durum wheat introgression line carrying a locus for the Na^+ transporter HKT, where benefits of the HKT gene were substantial at high salinity stress, but less so at moderate stress (Munns et al., 2012). The effect of yield improvement in those durum wheat introgression lines was linked to enhanced Na^+ exclusion from the shoot, thereby reducing the accumulation of toxic amounts of Na^+ in the photosynthetically active tissues. To tolerate salinity stress, plants have evolved several mechanisms, such as exclusion of Na^+ from the shoot, the compartmentalization of Na^+ into vacuoles and stress signaling (Munns and Tester, 2008; Roy et al., 2014). We did not find differences in Na^+ accumulation in the last fully developed leaf (leaf five) of our *AtJUB1* OE tomato plants, suggesting that Na^+ exclusion or compartmentalization in the young leaves are not the main mechanism of salt tolerance conferred by AtJUB1. However, there is a possibility that Na^+ may accumulate in the old leaves or in the stem of tomato plants.

The regulatory pathway through which JUB1 is involved in salinity tolerance is not fully understood. A recent study by Sakuraba et al. (2017) identified PIF-4 as an upstream negative regulator of JUB1. PIF-4 directly binds to the JUB1 promoter and suppresses its expression. Plants overexpressing PIF-4 were more salt sensitive, likely in part due to JUB1 suppression by PIF-4 (Sakuraba et al., 2017). Homeodomain-leucine zipper 13 (AtHB13) was also identified as another upstream regulator of JUB1 (Ebrahimian-Motlagh et al., 2017). AtHB13 binds to the *JUB1* promoter and induces its expression under drought stress, conferring tolerance to water deficit stress likely through the DREB2A pathway. DREB2A is a key regulator in response to water deficit, heat and salt stresses (Dubouzet et al., 2003).

It has previously been described that AtJUB1 directly binds to AtDREB2A (Wu et al., 2012), and therefore is involved in the ABA-independent pathway. Interestingly, we found that *AtJUB1* OE plants had lower amounts of ABA under salt stress compared with WT. ABA is a common stress hormone, which increases in concentration upon stress

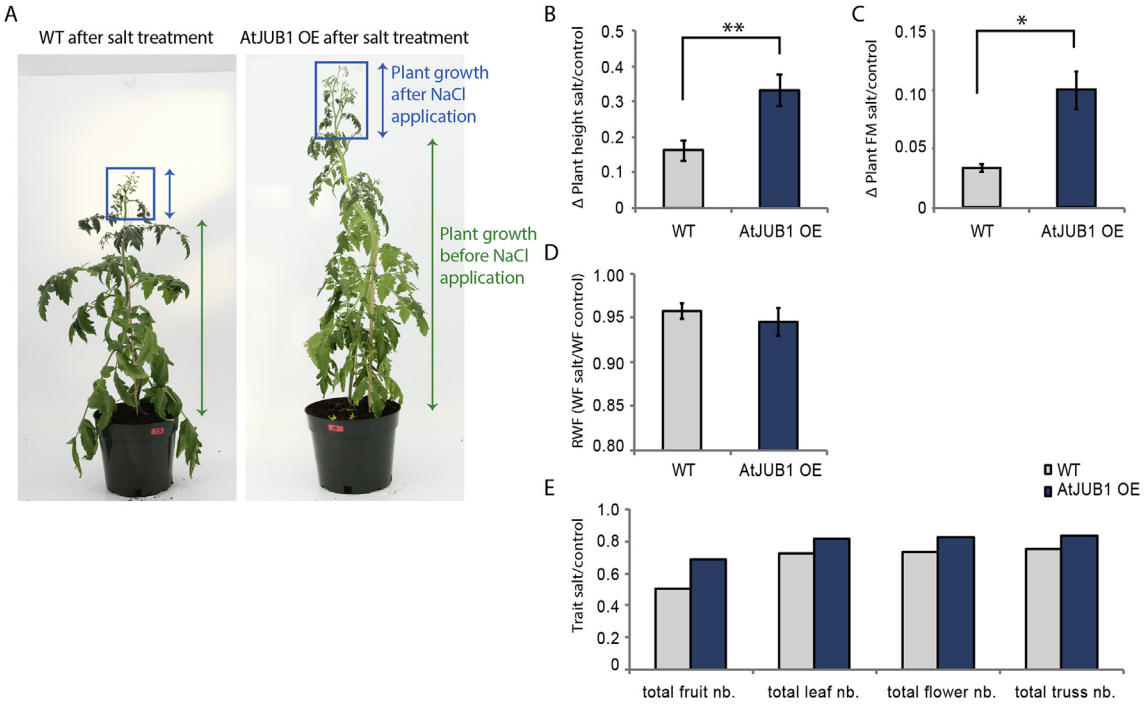

Fig. 7. Assessing JUB1 in salinity tolerance under soil conditions. (A) Phenotypes of wild-type cv. Moneymaker (WT) and transgenic (35S:AtJUB1-OE) tomato plants after 17 days of salt stress; **(B)** Salinity tolerance index "i.e Δ Plant height Salt/control" calculated as the increase in plant height after salt treatment relative to the increase in plant height in control plants over the same time period; **(C)** Salinity tolerance index "Δ Plant height salt/control" calculated as the increase in plant fresh mass after salt treatment relative to the increase in plant fresh mass in control plants over the same time period; **(D)** Relative water fraction of *AtJUB1* OE and wild type plants in salt relative to control "based on total shoot mass"; **(E)** Relative ratio of some yield-related parameters. n = at least 5 plants, except WT under control conditions, where n = 3; error bars represent standard error of the mean; significant differences were calculated using Student T-test, * indicates *P*-value < 0.05.

imposition to signal responses such as stomatal closure (Vishwakarma et al., 2017). The closure of stomata reduces transpiration and therefore allows plants to conserve water. In *AtJUB1* OE plants, we found lower ABA levels, perhaps reflecting the higher water status (and thus lower stress levels) of the *AtJUB1* OE plants. The lower ABA levels may also enable *AtJUB1* OE plants to maintain transpiration and gas exchange, and, therefore, growth is better maintained compared with WT plants. It has been hypothesized that there is an interaction between the ABA-dependent signaling and ABA-independent signaling though DREB2A and AREB/ABFs (Yoshida et al., 2014). It appears there is no JUB1 binding motif in the promoter region 1 kb upstream of genes encoding for the ABA-responsive element binding factor (ABF1/2/3/4).

We found that *AtJUB1* OE plants had higher proline levels under salt stress compared with WT plants. This is congruent with recent findings showing that transgenic banana overexpressing *MusaNAC042* (the closest homolog to *AtJUB1* in banana) had higher proline levels and are more tolerant to salinity stress (Tak et al., 2017). These transgenic banana plants also exhibited a more favorable water balance compared with WT plants (Tak et al., 2017). This is consistent with our findings and previous studies investigating drought tolerance of tomato over-expressing *AtJUB1* (Thirumalaikumar et al., 2018). JUB1 binds directly to the promoters of SlDREB1, SlDREB2 and SlDELLA in tomato (Thirumalaikumar et al., 2018), which are important TFs regulating drought responses (Yamaguchi-Shinozaki and Shinozaki, 2006). However, the mechanisms through which JUB1 directly or indirectly regulates proline metabolism (e.g. activation of proline biosynthesis or reduction of degradation) remain to be investigated.

Some components of salt stress and water deficit stress are similar. For instance, both stresses can cause cellular dehydration. Several tolerance mechanisms have been identified to be important in response to both salt and/or water deficit stress, such as osmotic adjustment and maintenance of relative water content (Bartels and Sunkar, 2005). We confirmed altered water relations of plants overexpressing *JUB1* and observed higher proline levels in the transgenic compared with the WT in response to salinty stress. This is congruent with previous findings that JUB1 is involved in tolerance to water deficit (Thirumalaikumar et al., 2018). The mechanism of salt tolerance in *AtJUB1* OE tomatoes might be related to avoidance of water stress imposed by NaCl.

Importantly, we investigated if the improvement of salinity tolerance related to biomass in a hydroponics experiment also translates to an increase in yield and yield related parameters in soil grown plants through to maturity. It appears *AtJUB1* OE has only moderate effects on improving yield parameters such as fruit number and number of trusses under salinity stress. However, it has been shown that *AtJUB1* OE plants are delayed in flowering and fruit ripening compared with WT tomato plants and that several ripening-related genes are differentially regulated and (Shahnejat-Bushehri et al., 2017). It also remains to be investigated if fruit quality remains comparable to wild type plants during salinity stress. Salinity has known to increase sugar levels in tomato fruit, which may be considered a positive or negative sensation to consumers. A higher sugar content, together with delayed fruit ripening may provide desirable traits for commercial growers. The trend that *AtJUB1 OE* tomatoes have improved yield and yield related parameters needs to be verified in future field studies.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

NA performed most experiments and analyzed the data. JW and SAB

performed proline, chlorophyll and ABA analyses. NA, SA, MT and SMS conceived the project and designed experiments. NA and SMS drafted the manuscript, with contributions from all authors.

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Declarations of interest

none.

Data availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.038.

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