Overexpression of dehydrin *tas14* gene improves the osmotic stress imposed by drought and salinity in tomato

Alicia Muñoz-Mayor[1] · Benito Pineda[2] · Jose O. Garcia-Abellán[1] · Teresa Antón[1] · Begoña Garcia-Sogo[2] · Paloma Sanchez-Bel[1] · Francisco B. Flores[1] · Trinidad Angosto[3] · Jose A. Pintor-Toro[4] · Vicente Moreno[2] · Maria C. Bolarin[1]

A. Muñoz-Mayor and Benito Pineda contributed equally to this work

[1]CEBAS-CSIC, Department of Stress Biology and Plant Pathology, Campus de Espinardo, P.O. box 164, 30100 Espinardo-Murcia, Spain

[2]IBMCP-UPV/CSIC, Laboratory of Biotechnological Breeding, Camino de Vera 14, 46022 Valencia, Spain

[3]University of Almeria, Department of Applied Biology, Carretera de Sacramento s/n, 04120 Almeria, Spain

[4]CABIMER**,** Parque Científico y Tecnológico de la Cartuja, 41092 Sevilla, Spain

Abstract One strategy to increase the level of drought and salinity tolerance is the transfer of genes codifying different types of proteins functionally related to macromolecule protection, such as group 2 of late embryogenesis abundant (LEA) proteins or dehydrins. The TAS14 dehydrin was isolated and characterized in tomato and its expression was induced by osmotic stress (NaCl and mannitol) and abscisic acid (ABA) [Godoy et al., Plant Mol Biol 1994;26:1921-1934], yet its function in drought and salinity tolerance of tomato remains elusive. In this study, transgenic tomato plants overexpressing *tas14* gene under the control of the 35SCaMV promoter were generated in order to assess the function of *tas14* gene in drought and salinity tolerance. The plants overexpressing *tas14* gene achieved improved long-term drought and salinity tolerance without affecting plant growth under non-stress conditions. A mechanism of osmotic stress tolerance via osmotic potential reduction and solutes accumulation, such as sugars and K^+ is operating in *tas14* overexpressing plants in drought conditions. A similar mechanism of osmotic stress tolerance was observed under salinity. Moreover, the overexpression of *tas14* gene increased Na^+ accumulation only in adult leaves whereas in young leaves the accumulated solutes were K^+ and sugars, which suggests that plants overexpressing *tas14* gene are able to distribute the Na^+ accumulation between young and adult leaves over a prolonged period in stressful conditions. Measurement of ABA shows that the action mechanism of *tas14* gene is associated to an earlier and higher accumulation of ABA in leaves in short-term periods. A good feature for the application of this gene in improving drought and salt stress tolerance is the fact that its constitutive expression does not affect plant growth under non-stress conditions and tolerance induced by overexpression of *tas14* gene was observed at the different stress degrees applied to long-term.

Keywords: Drought tolerance · Salinity tolerance · Tomato · *Solanum lycopersicum* · *tas14* gene · Osmotic stress

Abbreviations

ABA	Abscisic acid
DW	Dry weight
FW	Fresh weight
IAA	Indole acetic acid

LEA	Late embryogenesis abundant
RWC	Relative water content
TW	Turgent weight
WT	Wild type
35SCaMV	**P**romoter 35S from Cauliflower Mosaic Virus (35SCaMV)
Ψs	Osmotic potential
Ψ_w	Water potential

Introduction

Abiotic stresses such as drought and salinity impose severe production constrains on food production. Drought is a major abiotic stress that affects agriculture in 45% of the world (Foolad, 2007) and the potential yield losses by salinity are estimated at 20% (Ashraf et al., 2008). The problem is growing, as apart from natural salinity a significant proportion of recently cultivated agricultural land has become saline. Although tolerance to drought and salt stresses is a very complex trait, development of crop plants tolerant to stress is vital to meet the growing food demand through sustainable agriculture (Cuartero et al., 2010; Hirayama and Shinozaki, 2010). Drought and salt stresses share a common physiological osmotic stress, as decrease in soil water availability under drought or decrease in water potential of soil solution under salinity cause osmotic stress, which leads to a decreased water uptake and loss of turgor. The differential effect induced by salinity is the toxic effect induced by the root uptake and shoot transport of saline ions (Munns and Tester, 2008). Despite the economic relevance of tomato, the mechanisms that govern responses to these abiotic stresses in this horticultural species are not well characterized, and a very small number of genes playing a role in tomato tolerance to salinity and drought have so far been identified (Atares et al., 2011; Pineda et al., 2011). However, in spite of numerous reports of improved tolerance by the overexpression of different genes, the mechanisms underlying the enhancement of tolerance remain unclear in most of the cases. Thus, in order to elucidate the role of AtNHX1 antiporter, Leidi et al. (2010) carried out a very important work, finally demonstrating that tomato plants overexpressing *AtNHX1* had larger K^+ accumulations in vacuole in all growth conditions tested but no consistent enhancement of Na^+ accumulation, as previously suggested (Pardo et al., 2006).

A strategy to increase the level of drought and salinity tolerance is the transfer of genes codifying different types of proteins involved in the molecular responses to abiotic stress, such as osmoprotectants, chaperones, detoxification enzymes, transcription factors, signal transduction proteins (kinases and phosphatases), heat-shock proteins (HSPs), and late-embryogenesis-abundant (LEA) proteins (Campalans et al., 1999; Capiati et al., 2006; Khurana et al., 2008; Orsini et al., 2010; Amudha and Balasubramani, 2011).

LEA proteins constitute a superfamily of proteins that were detected for the first time during the maturation phase of cotton embryogenesis, which is the stage when

acquisition of desiccation tolerance occurs in the embryo, when they accumulate to high concentrations, a characteristic that gave rise to their name (Dure and Chlan, 1981; Dure and Galau, 1981). This group of very hydrophilic proteins markedly increase their levels during water deficit and/or low-temperature stress in vegetative organs, suggesting a protective role during water limitation (Bies-Etheve et al., 2008; Popelka et al., 2010), although their precise functions and mechanisms of action are still hidden even after twenty years of their discovery (Battaglia et al., 2008; Khurana et al., 2008).

Some of the most studied LEA proteins in higher plants are the group 2 or dehydrins (Zhang et al., 2007; Veeranagamalliah et al., 2011). There are several studies of specific members of this group 2 of LEA proteins that confirm their accumulation during seed desiccation and in response to water deficit induced by drought, low temperature, or salinity (Ismail et al., 1999; Nylander et al., 2001). Since the expression of dehydrins is significantly induced by abiotic stresses such as drought, cold and high salinity, it has been postulated that a positive correlation exists between dehydrin expression and abiotic stress tolerance in plants (Saavedra et al., 2006; Brini et al., 2007).

The TAS14 dehydrin was isolated and characterized in tomato (Godoy et al., 1990). This gene was induced in tomato seedlings and adult plants under osmotic stress (NaCl and mannitol) and abscisic acid (ABA) (Godoy et al., 1994), but the physiological role played by this gene during drought and salt stress in tomato still remains elusive.

In order to study the role of *tas14* gene in tomato and determine whether its overexpression increases drought and salinity tolerance, the *tas14* gene was introduced in tomato under the control of the constitutive promoter 35S from Cauliflower Mosaic Virus (35SCaMV), and growth and physiological responses to drought and salinity were studied in the resulting transgenic tomato plants. Results from different experiments described in this paper show that *tas14* gene plays an essential role during drought and salt stress in tomato by means of improving its tolerance towards the osmotic stress imposed by both abiotic stresses.

Several studies applying overexpression and ectopic expression of dehydrins have already been published. For instance, the overexpression of multiple Arabidopsis dehydrins led to plants showing increased freezing tolerance and improved survival when subjected to low-temperature stress conditions (Puhakainen et al., 2004). Also the ectopic expression of a wheat dehydrin (DHN-5) in Arabidopsis plants improved their tolerance to high salinity and water deficit (Brini et al., 2007). With regard to tomato dehydrins, a study of the ectopic expression in yeast of one of them (Le4) has been

performed (Zhang et al., 2000). It showed that the transformed yeast partially overcame the detrimental effects of ionic and freezing stress by conferring tolerance to high concentration of KCl but not to NaCl or sorbitol. But to our knowledge, the research work described in this paper is the first study to apply overexpression of a dehydrin in a plant species of such an agronomic interest as tomato and where the effects of the accumulation of this type of LEA protein in tomato plants when subjected to water and salt stress conditions, in short and long terms assays, have been investigated.

Material and Methods

Transformation and molecular characterisation of the transgenic tomato plants

The tomato 746-bp *tas14* cDNA (X51904) was introduced into a tomato cultivar of determined growth (*Solanum lycopersicum* L. cv. UC82B) by *Agrobacterium*-mediated transformation using the protocol previously described (Gisbert et al., 2000). Cotyledonary explants were infected with *A. tumefaciens* strain LBA4404 carrying the *tas14* and kanamycin resistance gene *npt*II sequences in the plasmid pPM7 vector containing the 35SCaMV promoter. Transformed shoots were transferred to a rooting culture medium consisting of Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.1 mg L^{-1} IAA and 50 mg L^{-1} kanamycin. Only one regenerated plant from a single poke was counted as an independent transgenic event.

Twenty independently regenerated kanamycin-resistant plants (T0 plants) were transferred into soil and grown under standardized greenhouse conditions (Estañ et al., 2005) to generate T1 seeds, which were a mixture of azygous (transformed line without transgene), homozygous and hemizygous lines. Progenies were obtained from those transgenic plants by selfing in controlled conditions. These progenies (T2 plants) were analyzed for kanamycin (50 µg mL^{-1}) resistance, and azygous and homozygous lines (T3) were identified according to their kanamycin resistance (0% kanamycin resistance in azygous line and 100% kanamycin resistance in homozygous line). The molecular verification of the transgenic plants was performed by PCR and the number of inserted copies in transgenic plants was determined by DNA gel blot analysis using the methods described in Pineda et al., (2010). The expression of the TAS14 protein was verified by protein gel blot analysis as previously described (Godoy et al., 1994).

Drought and salt treatments and tolerance assays

Homozygous plants from the line with higher expression level of TAS14 protein (L4), named positive plants, and their controls, WT and azygous plants from line L6, named negative plants, were tested applying different drought treatments at the 7-8th leaf stage. Plant culture for drought treatments was carried out in a controlled growth chamber. Seeds were germinated in a 2:1:1 (v/v) mixture of peat:perlite:siliceous sand at 28ºC and 90% relative humidity in darkness. When seedlings had developed 2 true leaves (25 days after sowing), they were transferred to 5-L plastic pots filled with peat and plants were daily irrigated with half-strength Hoagland solution (Hoagland and Arnon, 1950). The environmental conditions were optimised for the growth of tomato seedlings, varying the temperature along the day between 18 and 25ºC, the relative humidity between 50 and 80% and with a photoperiod of 16 h light/8 h dark was imposed. A photosynthetic photon flux (400-700 nm) of 345 μmol m^{-2} s^{-1} at the plant level was provided by fluorescent tubes (Osram Lumilux daily-light 58 W and Fluora 58 W).

Drought response was determined by using two different procedures. In the first, drought stress was imposed by watering plants with 30% nutrient solution, compared with the volume applied to well-irrigated plants, for 50 days. The well-irrigated plants were irrigated daily up to pot capacity, and the nutrient solution volume for irrigation of the drought-stressed plants (30% of well-irrigated plants) was calculated, Drought tolerance was evaluated on the basis of plant biomass after 30 and 50 days of treatment, and physiological response was analysed in leaves and roots of the first harvest. In the second procedure, the drought stress was applied by withholding irrigation, so water stress intensity increased with time, and two successive dehydration-rehydration cycles were applied. Thus, the plants dehydrated for 7 days were rehydrated for 1 day and after this time another similar dehydration cycle was applied. Leaves and roots were taken for analysis at the beginning of the experiment (day 0) and on the 2^{nd}, 4^{th} and 6^{th} day of each dehydration cycle. Experiments were repeated twice and eight plants per treatment were used. The leaf relative water content (RWC) was analysed in all samples and ulterior physiological analysis were undertaken only in certain samples depending on the values of RWC that were determined.

To evaluate salt tolerance at the whole plant level, a first experiment was carried out in controlled conditions, by using negative and positive plants and the same environmental and culture conditions as in drought experiments, although salt

treatments (0, 75 and 150 mM NaCl) were applied at a younger growth stage, when the plants had developed two true leaves. At the end of the experiment (25 days), shoot biomass was measured and young (developing leaves), adult (the third completely developed leaves) and roots were taken for analysis from each of the eight plants per treatment.

A greenhouse experiment was carried out until fruit yield by using WT, negative and positive plants. Plants were grown as previously described (Muñoz-Mayor et al., 2008) and the salt treatments (0, 75 and 100 mM NaCl) were maintained throughout the experiment. In eight plants per treatment, ripe fruits were collected weekly from one month of cultivation and the weight recorded.

The salinity response was also studied at the cellular level by using callus culture. Calli were initiated from leaf explants of negative and positive plants and subcultured as previously described (Rus et al., 2001). The salt treatments (0, 100 and 150 mM NaCl) were applied for 30 days. Thirty replicates per treatment were used (10 Petri dishes with 3 calli each). At the end of the experiment, fresh weight was scored and a part of the callus material was taken for analysis.

Physiological measurements

Leaf water potential was measured by inserting the youngest fully expanded leaf in a Scholander pressure chamber (Model 3000, Soil Moisture Equipment Corp., CA) and determining the minimum pressure needed to extract water from the cut end.

In all experiments and harvests, fresh material was rinsed in deionized water and blotted carefully with tissue paper. A part of the plant material was weighed for fresh weight (FW) determination, oven dried for 48 h at 80 °C, and weighed to determine the dry weight (DW). Another part of the plant material was placed into 5-ml pipette tips containing a glass wool filter in the tip, and immediately frozen with liquid nitrogen until analysis. Subsequently, sap was extracted from the thawed plant material samples by centrifugation and used for analysis.

The leaf relative water content (RWC) was calculated as (FW ─ DW)/(TW ─ DW) x 100, and expressed as a percentage. Leaves were excised, the fresh weight (FW) recorded and incubated in water for 24 h at 4 ºC in the dark. The leaves were blotted and the turgid weight (TW) measured. Osmolality was measured by the freezing point depression method using an osmometer (Osmomat 030, Gonotec, Germany).

Osmolalities (mOsm kg^{-1}) were converted to osmotic potential (1 mOsm = -2.408 kPa). Pressure potential was estimated as the difference between water potential and osmotic potential. The concentrations of inorganic (Na^+ and K^+) and organic solutes (sugars and proline) were determined as previously described (Muñoz-Mayor et al., 2008). The total ABA content from samples was extracted and determined by indirect ELISA, according to the methodology by Gosalbes et al., (2004).

Statistical analysis

Data were statistically analysed using the SPSS 13.0 software package by ANOVA and LSD test ($P \leq 0.05$), using the treatments as a statistical parameter, to determine significant differences between means.

Results

Characterization of tas14 overexpressing tomato plants in control and drought conditions

Twenty independent transgenic plants were generated by introducing the tomato *tas14* cDNA into the processing tomato cultivar UC82B, with determined-growth habitus. Most of them were positive transformants as confirmed by PCR analysis for both genes *tas14* and *nptII*. Four transformants with only one copy of the overexpressing *tas14* genetic construction were identified by DNA gel blot analysis for both *tas14* and *nptII* genes (data not shown). The insertion of one copy was in concordance with the segregation observed in T2 for Kanamycin resistance (3:1). Protein gel blot analyses were performed to test for the presence of TAS14 protein in these 4 primary transformants (Supplementary fig. 1a). A 14-kDa band corresponding to TAS14 protein was detected in the transgenic plants but not in WT nor in a negative transformant (L6), indicating that it was successfully expressed in the genetically modified plants. Although *tas14* gene is found in the tomato genome, the expression of the endogenous gene is not induced in standard culture conditions (Godoy et al., 1994). The phenotypic analysis of the *tas14* overexpressing tomato plants was firstly carried out with

homozygous transgenic lines from the 4 transformants with one copy of the overexpressing *tas14* genetic construction. When these lines were grown under well-irrigated conditions, the growth patterns of the transgenic plants were similar to those of the WT plants and the lines without the overexpressing transgene from the same primary transformant (azygous line), and identified at the same time as the homozygous lines (Supplementary fig. 1b).

A preliminary experiment was carried out with the four homozygous lines (positive plants) and both WT and azygous plants from the line 6 (negative plants) by water withholding. After 6 days, both WT and negative plants showed a dehydrated aspect while plants overexpressing *tas14* gene showed only slight dehydration symptoms, especially line 4 with the highest expression of TAS14 protein (data not shown), which was selected for further experiments. Next, the drought tolerance of the WT, negative and positive (homozygous line 4) plants was tested after 50 days of drought treatment (by irrigating the plants with 30% of the volume applied to well-irrigated plants). The positive effect of the overexpression of *tas14* gene had already been noticed by a higher accumulation of shoot biomass measured as plant fresh weight after 30 days of drought treatment (Fig 1 a). At the end of the experiment (50 days), the vegetative shoot biomass continued to be significantly higher in positive than in WT and negative plants under drought conditions (Fig. 1b). The most interesting characteristic is that the positive plants were able to develop fruits after 50 days of intense drought stress, while the WT and negative plants had hardly any fruit at this time (Fig. 1c), as is observed in the photograph taken at the end of the experiment (Supplementary fig. 1d). Under well-irrigated conditions, no significant differences were observed between the shoot and fruit biomass of WT, negative and positive plants (Supplementary fig. 1c).

Drought tolerance mechanisms induced by the overexpression of tas14 gene

To study the physiological changes induced by the overexpression of *tas14* gene, water potential (Ψ_w) was measured in leaves and osmotic potential (Ψ_s), sugars and K^+ contents, which are among the most important solutes contributing to the osmotic potential, and the contents of the osmolyte proline and of ABA were analyzed in both leaves and roots after 30 days of drought treatment (Table 1). It is interesting to point out that the physiological responses of the WT and negative plants were similar, as no significant differences between them were achieved in any of the parameters analysed.

Sugars were the only solutes increasing in leaves of the positive plants, while K^+ increased in roots. No significant differences were found in the proline content in leaves, whereas the root proline content significantly increased in the positive plants, with respect to the WT and negative plants. ABA contents were similar in roots and leaves of the different types of plants. As regards the changes induced by *tas14* overexpression in Ψ_w and Ψ_s, the positive plants showed a water potential less reduced by drought and an osmotic potential that was more reduced, with respect to the WT and negative plants, so the leaf turgor potential (the difference between Ψ_w and Ψ_s) was higher in leaves of the positive plants (Table 1).

Since plants may respond to drought by using different tolerance mechanisms depending on how the stress is applied as well as on the duration of the treatment, negative and positive plants were submitted to two successive cycles of complete withholding of irrigation solution for 7 days, with 1 day of rewatering and recovery of plants between the two cycles. The RWC was maintained practically constant after 2 and 4 days of withholding irrigation in both negative and positive plants (around 90%), while the reductions induced by drought at the 6^{th} day of each dehydration cycle were significantly lower in the positive plants compared with the negative plants, reaching values between 75-80% in positive and 50-56% in the negative plants. Clear dehydration visual symptoms (leaf wilting) in negative plants were observed after the 6^{th} day of treatment, which was not the case for positive plants (Supplementary fig. 1e). On the basis of these results, a physiological analysis was carried out at the beginning of the experiment and after 4 days of water withholding in the first and second dehydration cycles, as neither the negative nor positive plants showed dehydration symptoms yet at this time. Under well-irrigated conditions, the physiological responses of negative and positive plants were quite similar (data not shown). Under drought, roots of plants overexpressing *tas14* reduced their osmotic potential (Ψ_s) more than those of the negative plants in both dehydration cycles, with similar negative Ψ_s values being achieved in the first and second cycles (Fig. 2a). The stressed-leaf Ψ_s values were also more negative in positive than in negative plants in both dehydration cycles, although the Ψ_s values in both positive and negative plants were more reduced in the second than in the first dehydration cycle (Fig. 2a). Sugars were the solutes that more speedily increased in positive plants with respect to the negative plants, as they significantly rose from the first dehydration cycle in leaves and roots and the differences were maintained in the second cycle (Fig. 2b). However, significant K^+ increases in leaves and roots of

the positive plants were not found until the second dehydration cycle (Fig. 2c). With respect to the osmolite proline, increases are induced by drought stress in roots and leaves of both negative and positive plants, but these increases are significantly higher in the positive plants, especially in the first dehydration cycle (Fig. 2d).

Leaf and root ABA concentrations of the negative and positive plants were analysed at the beginning of the experiment and after 4 days of water withholding in the first and second dehydration cycles. Differences between negative and positive plants were only observed in leaves (Fig. 3a) but not in roots (data not shown). The leaves of the positive plants significantly accumulated more ABA (2.5 nmol g^{-1} FW) than those of the negative ones in the first dehydration cycle (1.4 nmol g^{-1} FW). In the second cycle, where the ABA concentrations were higher than in the first, similar levels were however achieved in the leaves of both types of plants (Fig. 3a).

In order to confirm that the higher increase of endogenous ABA in leaves of the *tas14* overexpressing plants was associated to the action mechanism of *tas14* gene, the time-course of ABA content from the 2nd to 5th days of the first dehydration cycle was compared in leaves of the positive plants (homozygous line for *tas14* gene), and a homozygous line overexpressing other different tomato gene, the *tsw12* gene, which is involved in osmotic stress (Torres-Schumann et al., 1992). From the 2nd day onwards the ABA concentration in leaf increased significantly with drought in all plants (Fig. 3b); the time course of the ABA accumulation under drought stress was similar in the negative and the *tsw12*-overexpressing plants, increasing linearly during the progress of drought stress (between 2 and 5 days of water withholding), but the increases were significantly lower than in the *tas14* overexpressing plants during the whole period of analysis. Interestingly, the highest differences between *tas14* overexpressing and the other plants (negative and *tsw12* plants) were found at the 3rd dehydration day. Thus, the leaf ABA concentration in *tas14* overexpressing plants was 2.4 nmol g^{-1} FW, whereas in negative and *tsw12* overexpressing plants was around 1.4 nmol g^{-1} FW. These results indicate that the mechanism of action of *tas14* is associated to a rapid ABA increase in leaves.

Phenotypic characterization and salt tolerance mechanisms induced by the overexpression of tas14 gene

To evaluate the effects of *tas14* overexpression on plant response to salinity, the same negative and positive plants were grown from the 2nd-leaf stage at different salt stress levels (0, 75 and 150 mM NaCl) for 25 days. The shoot biomass of negative and positive plants were similar under control conditions, while at mild salt stress treatment (75 mM NaCl) the positive plants increased their growth significantly, compared with the negative plants after 25 days of treatment (Fig. 4a); moreover, the shoot biomass of the positive plants grown at 75 mM NaCl was similar to that of the positive plants grown without salt. Although at a lower degree, a positive effect was also observed at 150 mM NaCl for the same period (Fig. 4a). These results show that *tas14* also plays an essential role during salt stress in tomato.

In order to determine whether the higher salt tolerance induced by overexpression of *tas14* was prolonged along the growth cycle, the fruit yield of the WT, negative and positive plants was determined after 75 days at 0, 75 and 100 mM NaCl (Fig. 4b). Fruit yield increased in saline medium in the positive plants compared with WT and negative plants, with the greatest increase being observed at mid salt levels (75 mM NaCl).

The salinity physiological response was studied by separately analyzing young and adult leaves as well as roots in the plants of the first experiment (salt levels of 75 and 150 mM NaCl). As shown in table 2, Ψ_s of the different plant parts analyzed decreased up to significantly lower values in positive plants compared with negative ones at mild salt stress treatment (75 mM NaCl), where the most important mechanism contributing to the salt tolerance may be the osmotic tolerance mechanism. At high stress level (150 mM NaCl), where the toxic effect may be more important than the osmotic effect, there was also significant differences between the Ψ_s of negative and positive plants in young and adult leaves, but similar values were found in roots (Table 2). In young leaves, the K^+ and sugar concentrations were significantly higher in positive than in negative plants for both salt levels, whereas similar increases of young leaf sap Na^+ concentrations were found in both negative and positive plants. However, the situation changed in adult leaves, as the Na^+ concentration increased in positive plants, with respect to the negative plants at 75 and 150 mM NaCl, whereas there were no differences in K^+ and sugars concentrations. In roots, significant Na^+ increases were only found at 75 mM NaCl, which agrees with the significant Ψ_s reduction at this level. Proline was also analyzed in this experiment. The salinity treatments increased the proline levels in the three plant parts studied, but the increases were significantly higher in positive plants, except for

adult leaves of plants subjected to 150 mM NaCl (Table 2). It is interesting to note the very high levels of this osmolite achieved in young leaves of positive plants.

Finally, we attempted to ascertain whether the overexpression of *tas14* enhanced salt tolerance not only at the whole plant level but also at the cellular level. Moreover, we tried to confirm the ability of *tas14* gene to avoid loss of cellular water as well as to determine whether the mechanism of *tas14* gene was or was not associated to a higher Na^+ accumulation within the cells. The calli regenerated from leaves of positive plants showed significantly higher fresh weight and water content gains than the negative ones cultured for 30 days in a medium containing 100 and 150 mM NaCl (Fig. 5a, b). It is interesting to point out the significant increases of water contents in positive calli grown under salt stress, which does not occur under control conditions (Fig. 5b). These results indicate that the overexpression of *tas14* gene also increases the salt tolerance at the cellular level, similarly to the response observed at the whole plant level. Na^+ concentration was also measured at the end of the experiment (Fig. 5c). The Na^+ accumulation was similar at 100 mM NaCl for both types of calli but significantly lower at 150 mM NaCl in the positive calli compared with negative ones.

Discussion

The plants overexpressing *tas14* gene with the constitutive promoter 35S did not exhibit morphological or significant growth differences under unstressed conditions, compared to wild type plants (Supplementary fig. 1b). This is a good feature for the potential use in biotechnology of this gene in improving abiotic stress resistance, since the constitutive overexpression of most stress-related genes generally causes slower growth and, consequently, impacts negatively on the plant growth and yield under non-stressed conditions (Muñoz-Mayor et al., 2008; Ray et al., 2009). The overexpression of *tas14* gene enhanced drought tolerance on the basis of shoot biomass (Fig. 1). It is interesting to point out that the positive effect of the *tas14* gene was shown in spite of the severe drought stress level applied in long-term assays, according to the important reduction induced by drought stress in shoot vegetative biomass and, especially, in fruit biomass, in negative and WT plants, which suggests that this gene has an important role in drought tolerance in tomato. It is especially relevant the fact that overexpression of *tas14* gene increases drought tolerance, since comparatively much less progress has been made in genetics and breeding of tomatoes for drought tolerance (Foolad 2007).

The overexpression of *tas14* gene also increased salt tolerance, as the shoot biomass was significantly greater in positive than in negative plants, with the positive plants achieving similar shoot growth in medium without salt and with 75 mM NaCl (Fig. 4a). Moreover, salt tolerance induced by overexpression of *tas14* was enhanced throughout the growth cycle, as fruit yield was greater in positive than in negative plants grown in saline medium, especially at 75 mM NaCl (Fig. 4b). In this study, the salt response was also studied at the cellular level, as several studies have shown the role of dehydrins in ameliorating the cellular effects of abiotic stress (Battaglia et al., 2008; Bae et al., 2009). *tas14* overexpressing calli increased significantly its salt tolerance compared with negative calli, and this positive effect on the growth was mainly due to the high callus water content (Fig. 5). Considering that tolerance at the cell level in tomato is associated with the ability to avoid dehydration (Rus et al., 1999), these results corroborate that *tas14* gene seems to function by increasing cellular water content. Taken together, *tas14* overexpressing plants improve drought and salinity tolerance without affecting the plant growth under unstressed conditions.

A mechanism of drought tolerance via osmotic potential reduction and solute accumulation may be very important to avoid dehydration (Chaves and Oliveira 2004; Cattivelli et al., 2008). This mechanism was clearly shown in *tas14* overexpressing plants during short periods of drought (after 4 days of water withholding in the first and second dehydration cycles) as higher reductions in root and leaf Ψ_s and an increase in the concentration of solutes (K^+ and sugars) were found in positive plants with respect to negative ones (Fig. 2). Sugars most rapidly increased in the positive plants, since they significantly increased in the first dehydration cycle in leaves and roots. It has been suggested that sugars are the only compounds that can substitute water in severely dehydrated cells so as to preserve the structure and function of macromolecules (Hoekstra et al., 2001). It is therefore possible that sugars could maintain the stability of membranes during cellular dehydration induced by water withholding.

The physiological basis for mid- and long-term osmotic adjustment may respond to different biological and environmental cues, since plants that osmotically are best adjusted to mid-term drought treatments may not necessarily be those that are best adjusted to long-term stress (Maggio et al., 2007). However, the *tas14* overexpressing plants also improved the osmotic tolerance to longer periods of drought by using a similar mechanism, by means of reducing Ψ_s and increasing solute accumulation (Table 1), which enables transgenic plants to maintain cell turgor under drought conditions. In

effect, turgor potential was higher in positive plants than in both WT and negative plants after 30 days of drought due to both the lower reduction of Ψ_w and the higher reduction of Ψ_s. Under salt stress, evidence supporting the greater ability of positive plants to reduce Ψ_s in long-term stress assays (25 days of salt treatment) was also observed in root and leaves, which corroborates the osmotic tolerance mechanism induced by overexpression of the *tas14* gene. Godoy et al (1994) found a similar relative abundance of the TAS14 protein in tomato seedlings treated with equiosmolar concentrations of mannitol or NaCl, suggesting that *tas14* expression under salt stress is driven mainly by its osmotic component.

Taking into account that salt tolerance in tomato is associated to the partitioning and distribution of saline ions in leaves (Cuartero et al 2006, Olias et al 2009), it is interesting to remark the different types of solutes contributing to Ψ_s in young and adult leaves of the salt-treated plants, as the overexpression of *tas14* gene increased the Na^+ accumulation only in adult leaves whereas in young leaves the increased solutes were K^+ and sugars (Table 2). These results indicate that the osmotic balance is achieved mainly by saline ions in adult leaves, which is energetically much less expensive than the use of the organic solutes for osmotic adjustment (Estañ et al., 2005; Muñoz-Mayor et al., 2008). However, Na^+ accumulation did not occur in young leaves, which suggests that plants overexpressing *tas14* gene are able to distribute the Na^+ between young and adult leaves over a prolonged period of time, a trait linked to salt tolerance (Cuartero et al., 2006). Moreover, Na^+ accumulation in calli overexpressing *tas14* gene was similar to that of the negative calli at mid-salt level, but was significantly lower at high-salt level (Fig. 5c). This result reveals that Na^+ homeostasis is similarly maintained or even improved at the cellular level. According to Tester and Davenport (2003), there are two quite distinct mechanisms of tolerance to elevated concentrations of Na^+: (i) the tolerance of single cells to high salinity, and (ii) the tolerance at a higher organism level than that of the single cell, involving, for example, control of long-distance transport. Overexpression of *tas14* does not affect or even reduce Na^+ accumulation at the cellular level, whereas at the whole plant level it may increase the use of saline ions to reduce the osmotic potential. Taken together, the mechanism of osmotic tolerance seems to operate at the whole plant level, where the transport processes from the root to the shoot and the distribution between old and young leaves are the main genetic determinants of salt tolerance.

Accumulation of compatible solutes such as proline may play a role in adaptation to drought and salt stresses by means of increasing the cellular solute content (Hmida-Sayari et al., 2005; Türkan and Demiral 2009). Thus, proline accumulation seems to be involved in the initial response to drought stress of the *tas14* overexpressing plants, as the leaf proline rise in the positive plants with respect to the negative plants is much higher in the first than in the second dehydration cycle (Fig. 2d), and no increases were observed in the leaves of positive plants after 30 days of reduced irrigation (Table 1). Proline could play a different role under salinity, since the ionic homeostasis has to be re-established under salt stress in addition to osmotic homeostasis. This seems to occur in the *tas14* overexpressing plants submitted to salt stress, as the enhanced proline accumulation in roots and leaves, especially in young leaves, was observed after 25 days of salt treatment (Table 2). This proline accumulation could protect protein and membrane structures, regulate redox status or in relation to this last fact, has a role as a scavenger of reactive oxygen species (Hsieh et al., 2002; Türkan and Demiral 2009), or it may even be involved in regulating the Na^+ accumulation in young leaves (Kant et al., 2006). In summary, the role of proline may be different under drought and salinity conditions in *tas14* overexpressing plants, although in both cases the observed proline accumulation is associated to a tolerance response.

The *tas14* gene expression was induced by ABA (Godoy et al., 1994) which tags *tas14* as an ABA-responsive gene, and it has been shown that ABA is involved in responses to dehydration and salinity (Shinozaki and Yamaguchi-Shinozaki 2007; Thompson et al., 2007; Chinnusamy et al., 2008). Therefore, it was considered interesting to study in the *tas14* overexpressing plants the ABA accumulation at different time periods of drought treatment (Fig. 3). The results show that *tas14* overexpression induces earlier and higher ABA accumulation to short-term in positive plants, where the plants did not show any symptoms of dehydration, so as a short period as 2 days of water withholding is sufficient to trigger the cascade of events inducing tolerance to drought. Moreover, the rapid increase of the leaf ABA concentration was observed only in plants overexpressing *tas14* gene but not in plants overexpressing *tsw12* gene, a tomato gene which is also involved in osmotic stress (Torres-Schumann et al., 1992; Pineda et al., 2011). These results suggest that the higher ABA accumulation observed in stressed leaves of the plants overexpressing *tas14* during the first days of dehydration is associated to the action mechanism of *tas14* gene. It is interesting to point out that increased ABA levels were only shown in the first

dehydration cycle, but not over longer time (Fig. 3a and Table 1), which could have induced morphological and developmental alterations, as it has been observed in plants that accumulate very high levels of ABA and exhibit severe detrimental phenotypes (Tung et al 2008). Moreover, ABA accumulation may be valuable for enhancing plant tolerance at short- and mid- term stress treatments, but this strategy often results in reduced productivity. This is a consequence of the decreased stomatal conductance that leads to diminished intercellular CO_2 concentrations which limits photosynthesis (Chaves and Oliveira, 2004).

To sum up, the overexpression of *tas14* gene enhances drought and salinity tolerance in an interesting agronomic species of key importance like tomato. The fact that the *tas14* overexpressing plants are similar to the wild type plants is, moreover, a good feature for the usefulness of this gene in improving abiotic stress. This study shows that a mechanism of osmotic tolerance via osmotic potential reduction and solute accumulation is operating in the *tas14* overexpressing plants under both drought and salinity. This mechanism is observed in a relatively short period after drought imposition and maintained over long time to avoid damage induced by drought and salinity. Moreover, under salinity, the plants overexpressing *tas14* gene are able to distribute Na^+ between young and adult leaves, a trait related to salt tolerance. Finally, it is interesting to highlight that the tolerance induced by overexpression of *tas14* gene is associated to their ability to rapidly increase ABA after they perceive drought stress. In conclusion, the activation and function of *tas14* gene may be useful to grow tomato plants in drought and salinity conditions.

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Legends of figures

Fig. 1. Effects of overexpression of *tas14* gene on plant growth response to drought. Drought tolerance of wild type plants (WT), azygous or negative plants (-) and homozygous or positive plants (+) was tested by irrigating the plants with a 30% of the volume applied to well-irrigated plants. The absolute and relative values of shoot biomass after 30 days of drought treatment (**a**) and shoot biomass (**b**) and fruit biomass (**c)** at the end of the experiment (50 days) are shown. Data are the mean ± SE (n = 8). Significant differences at $P < 0.05$ between lines were indicated with *.

Fig. 2. Effects of *tas14* overexpression on plant physiological response to drought in plants subjected to dehydration cycles. Osmotic potential (**a**), sugar (**b**), K^+ (**c**), and proline (**d**) concentrations were measured in roots (circles) and leaves (squares) of negative (open symbols) and positive (solid symbols) plants at the beginning of the experiment (day 0) and after 4 days water withholding in the first and second dehydration cycles. Data are the mean ± SE (n = 8). Significant differences at $P < 0.05$ between lines were indicated with *.

Fig. 3. Effect of *tas14* overexpression on leaf ABA concentration in plants subjected to dehydration cycles. **a** ABA concentration was measured in leaves of negative (open squares) and positive (solid squares) plants at the beginning of the experiment and after 4 days water withholding in the first and second dehydration cycles (0, 1 and 2 in abscises axis). **b** Time-course of ABA concentration from 2^{nd} to 5^{th} day of first cycle of dehydration in leaves of negative (open squares), positive (solid squares) plants for *tas14* gene overexpression, and a transgenic line overexpressing the tomato *tsw12* gene involved in the osmotic stress (solid triangles). Data are the mean ± SE (n = 5). Significant differences at $P < 0.05$ between lines were indicated with *.

Fig. 4. Effect of *tas14* overexpression on plant growth response to salt stress. **a** Shoot biomass was quantified in negative (open squares) and positive (solid squares) plants grown at different NaCl levels (0, 75 and 150 mM) for 25 days. **b** Fruit yield was quantified in WT (open circles), negative (open squares) and positive (solid squares) plants grown at different NaCl levels (0, 75 and 100 mM) for 75 days. Data are the mean ± SE (n = 8). Significant differences at $P < 0.05$ between lines were indicated with *.

Fig. 5. Effect of *tas14* overexpression on callus growth response to salt stress. Calli were initiated from leaf explants of negative (open squares) and positive (solid squares) seedlings, and further subcultured in medium with 0, 100 and 150 mM NaCl. Fresh weight (**a**), water content (**b**) and Na^+ concentration (**c**) were determined after 30 days of culture. Data are the mean ± SE (n = 30). Significant differences at $P < 0.05$ between lines were indicated with *.

Supplementary fig. 1 Protein gel blot of protein extracts from leaves of transgenic tomato plants grown in control conditions (**a)**: L2 through L9, independent transgenic lines containing one copy of *tas14* gene overexpressing construction, WT, non-transformed tomato plant. The overexpression of *tas14* gene does not affect the plant growth under non-stress conditions (**b**), similar phenotypes for WT and transgenic lines were observed at the 8-leaf stage when plants were grown under unstressed conditions.

Fifty days later similar **s**hoot and fruit biomass values in WT, azygous plants from the L6 and homozygous plants from the L4 (with higher expression of the TAS14 protein) lines were found, data are the mean ± SE (n = 8) (**c**). The drought response of homozygous plants from line L4 was different compared with L6 and WT, as it is observed in photograph taken of plants irrigated with 30% of the volume applied to well-irrigated plants for 50 days, where greater fruit biomass was observed in L4 plants (**d**), as well as in plants submitted to two successive cycles of withholding irrigation, where advanced wilting was observed in leaves of negative plants (L6), but was absent in positive plants (L4) (**e**).

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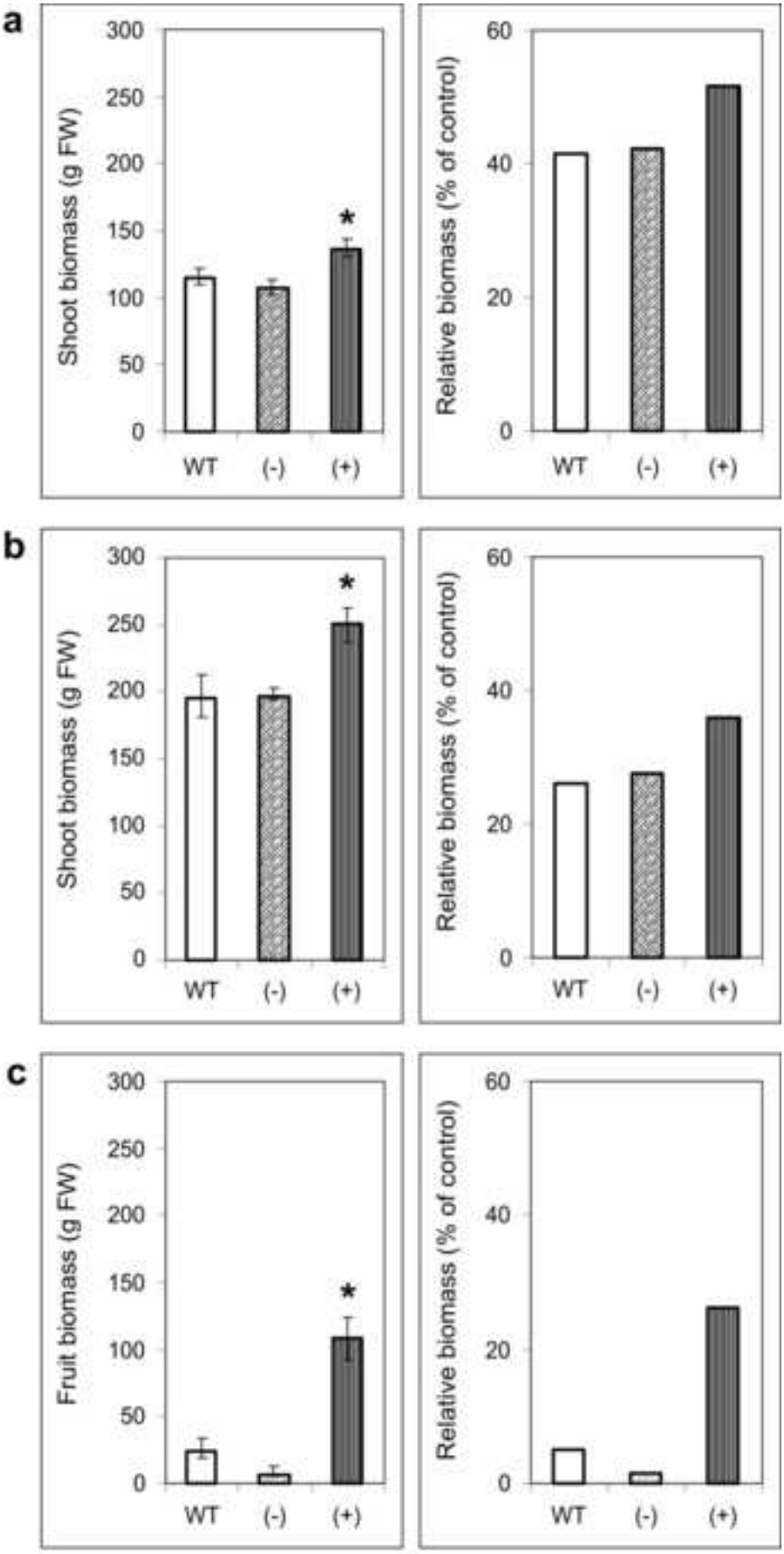

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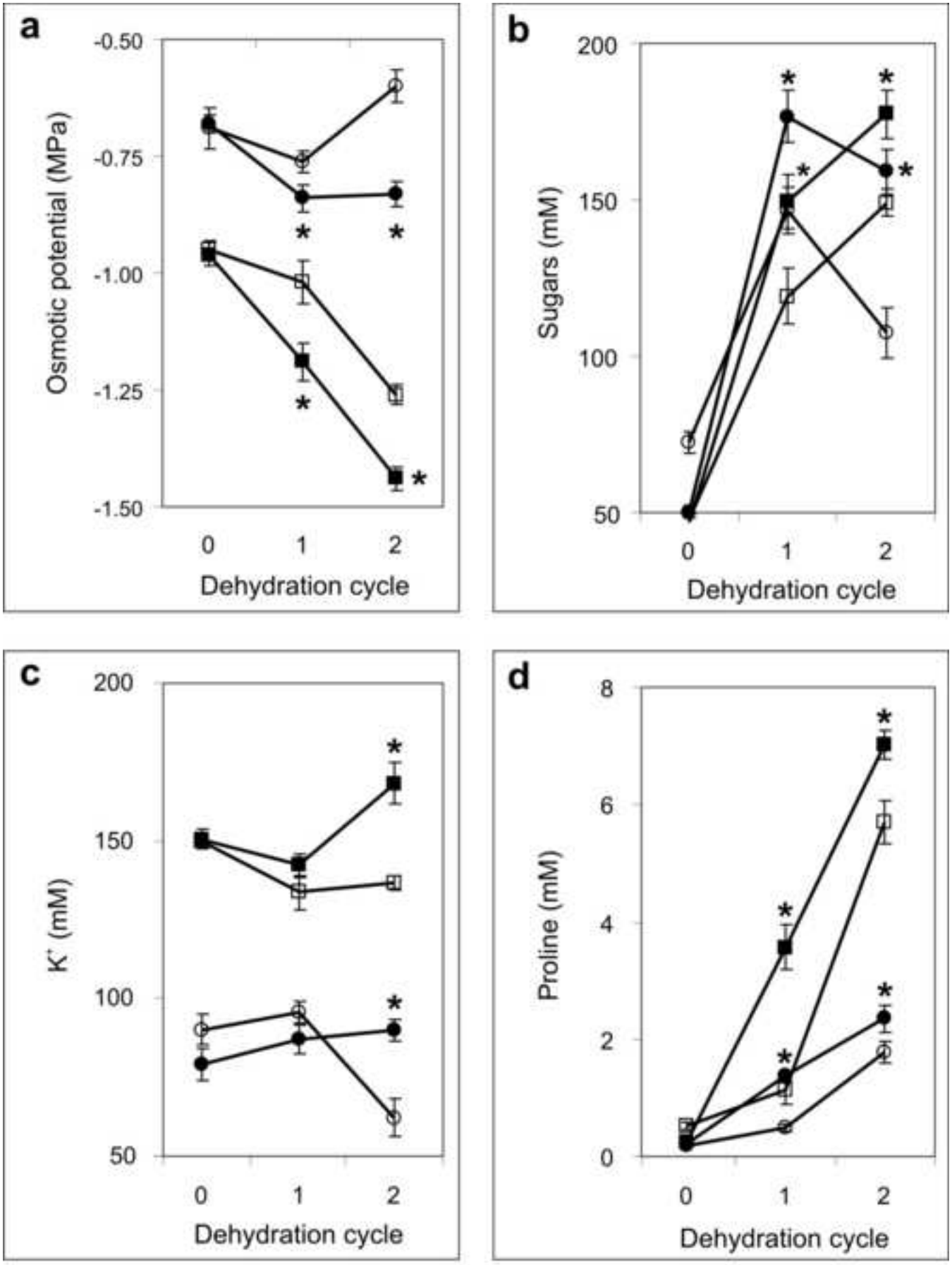

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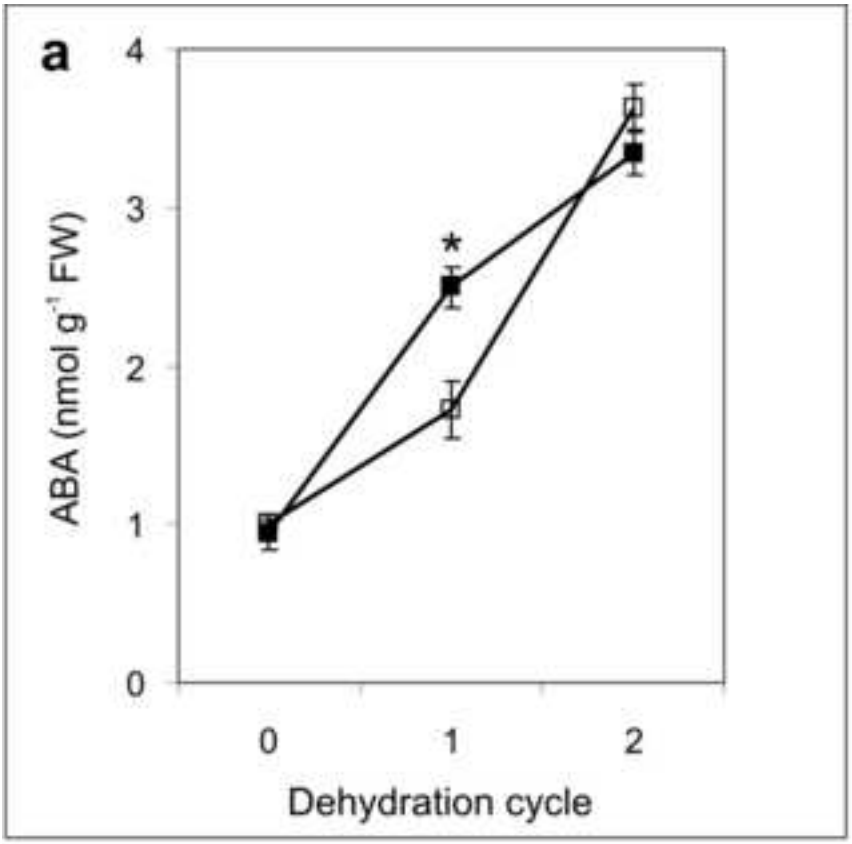

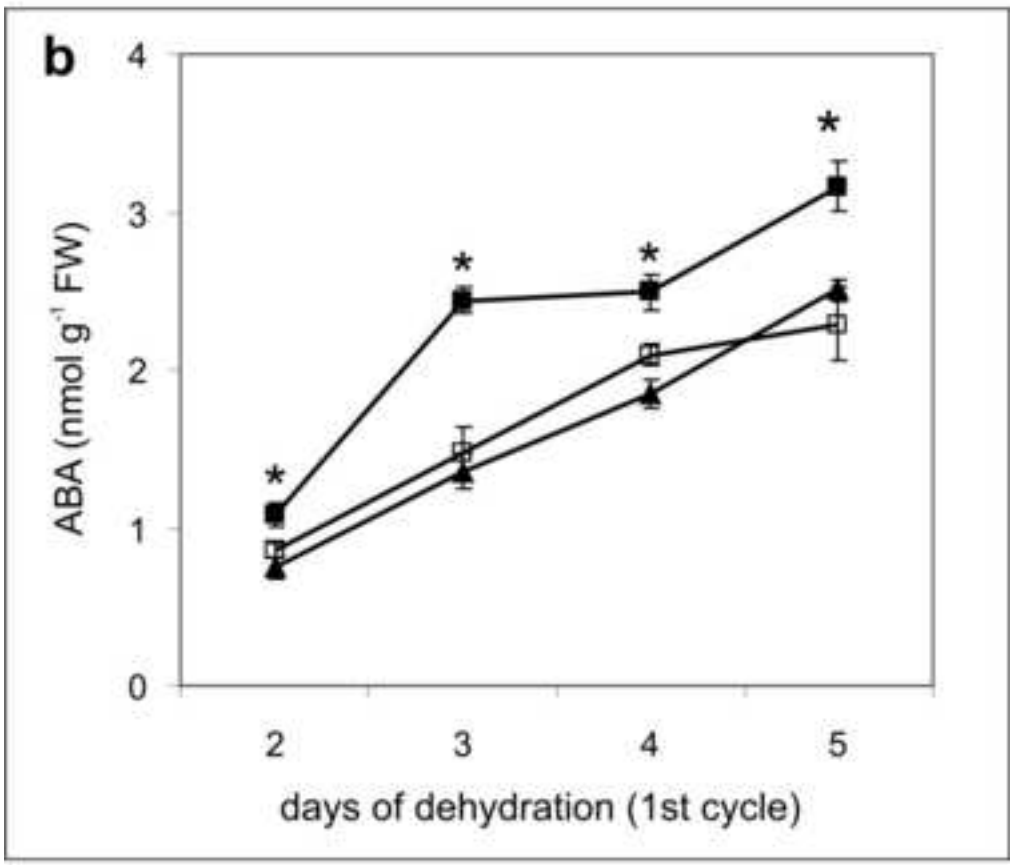

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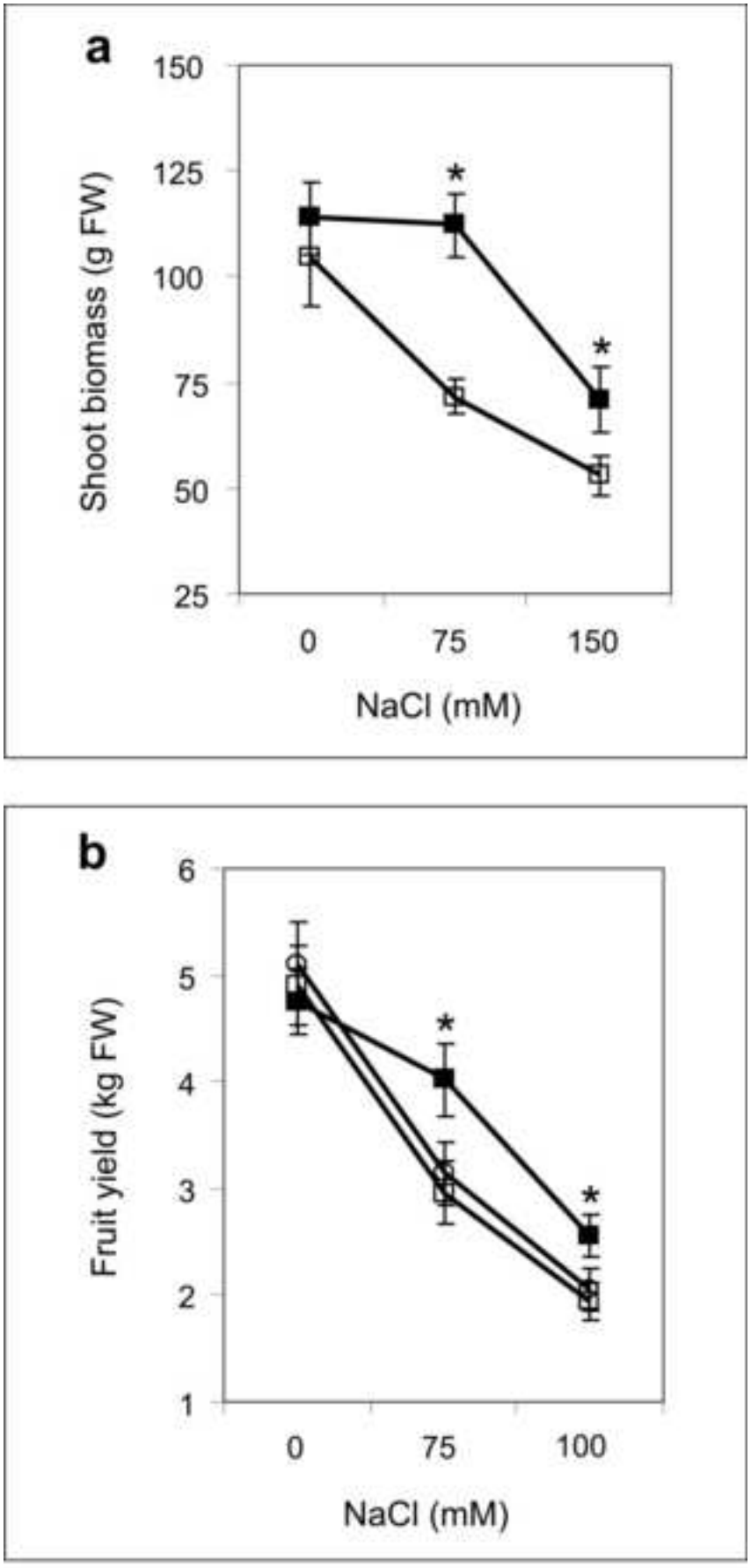

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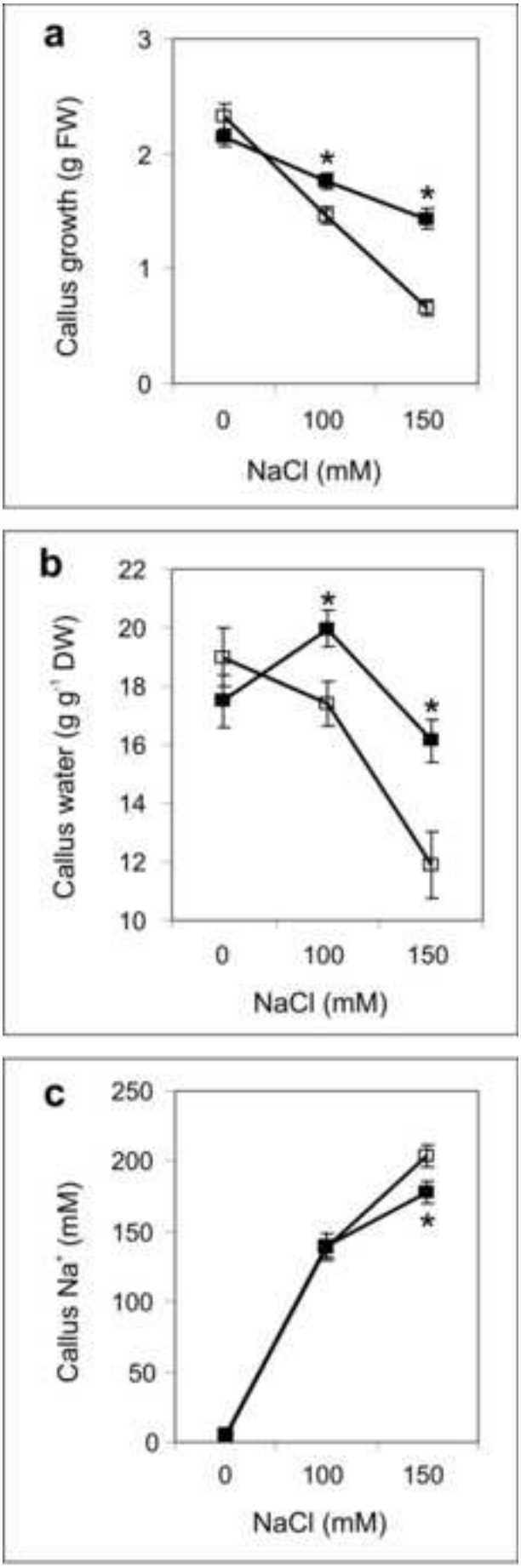

Fig. 5

Table

Table 1 Effect of *TAS14* overexpression on plant physiological response after 30 days of drought treatment

Plant part	Line	Sugars (mM)	K^+ (mM)	Proline (mM)	ABA (nmol g^{-1} FW)	Osmotic potential (MPa)	Water Potential (MPa)	Turgor potential (MPa)
Leaf	WT	71.3 ± 7.7	130.4 ± 2.6	0.93 ± 0.12	1.99 ± 0.25	-1.01 ± 0.023	-0.76 ± 0.031	0.25 ± 0.028
	(-)	87.5 ± 4.8	136.8 ± 4.4	1.03 ± 0.11	2.08 ± 0.36	-0.97 ± 0.041	-0.79 ± 0.029	0.18 ± 0.007
	(+)	113.2 ± 6.7*	127.7 ± 7.3	0.76 ± 0.13	1.60 ± 0.17	-1,11 ± 0.044*	-0.69 ± 0.020*	0.42 ± 0.039*
Root	WT	61.1 ± 6.7	26.7 ± 2.6	0.19 ± 0.02	0.09 ± 0.02	-0.46 ± 0.032		
	(-)	65.6 ± 2.8	33.4 ± 3.7	0.18 ± 0.01	0.08 ± 0.01	-0.40 ± 0.018		
	(+)	70.4 ± 6.7	43.0 ± 2.4*	0.35 ± 0.03*	0.10 ± 0.01	-0.44 ± 0.024		

WT, negative (-) and positive (+) plants were subjected to drought stress by irrigation reduction (30% of the nutrient solution volume added to well-irrigated plants). Values are the mean ± SE of eight plants

* Significant differences between positive plants and their controls (WT and negative plants) at $P < 0.05$

Table

Table 2 Effect of *TAS14* overexpression on plant physiological response after 25 days of salt stress

Plant part	NaCl (mM)	Line	Osmotic potencial (MPa)	Na^+ (mM)	K^+ (mM)	Sugars (mM)	Proline (mM)
Young leaf	75	(-)	-1.19 ± 0.07	37.6 ± 5.3	123.3 ± 11.3	44.2 ± 6.5	3.10 ± 0.41
		(+)	-1.30 ± 0.05*****	45.6 ± 2.6	152.2 ± 9.3*****	58.2 ± 3.4*****	8.01 ± 1.29*****
	150	(-)	-1.40 ± 0.04	92.0 ± 4.8	100.1± 4.5	50.1 ± 4.4	8.40 ± 0.95
		(+)	-1.55 ± 0.07*****	96.0 ± 9.8	125.0 ± 5.6*****	65.0 ± 4.2*****	11.2 ± 1.05*****
Adult leaf	75	(-)	-1.19 ± 0.07	45.0 ± 2.8	121.1 ± 7.9	42.3 ± 3.5	1.60 ± 0.30
		(+)	-1.33 ± 0.03*****	73.5 ± 3.2*****	129.8 ± 6.8	37.4 ± 4.4	2.97 ± 0.70*****
	150	(-)	-1.40 ± 0.02	105.0 ± 8.0	121.0 ± 8.3	39.0 ± 3.6	4.90 ± 0.60
		(+)	-1.89 ± 0.01*****	140.3 ± 12.2*****	128.7 ± 4.0	45.7 ± 5.0	4.60 ± 0.31
Root	75	(-)	-0.90 ± 0.10	131.1 ± 4.2	72.4 ± 4.1	23.4 ± 3.7	1.39 ± 0.16
		(+)	-1.20 ± 0.07*****	176.9 ± 17.3*****	67.9 ± 5.6	40.8 ± 5.3*****	2.08 ± 0.19*****
	150	(-)	-1.33 ± 0.14	224.0 ± 8.4	50.7 ± 9.8	38.2 ± 5.8	1.90 ± 0.04
		(+)	-1.36 ± 0.08	198.5 ± 21.8	56.0 ± 5.0	41.2 ± 5.6	4.80 ± 0.50*****

(-) and (+), negative and positive plants were subjected to 75 and 150 mM NaCl. Values are the mean ± SE of eight plants
* Significant differences between lines at $P < 0.05$

Figure
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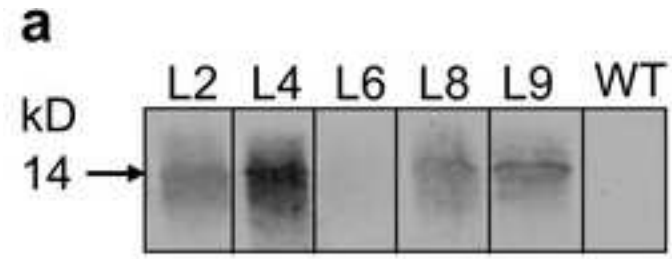

c

	Shoot (g FW)	Fruit (g FW)
WT	750.0 ± 72.3	474.0 ± 51.1
L6	712.8 ± 80.3	466.8 ± 40.3
L4	696.3 ± 79.3	415.5 ± 47.6

Supplementary Fig. 1