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RESEARCH PAPER

Root-synthesized cytokinins improve shoot growth and fruit yield in salinized tomato (*Solanum lycopersicum* L.) plants

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Abstract

Salinity limits crop productivity, in part by decreasing shoot concentrations of the growth-promoting and senescence-delaying hormones cytokinins. Since constitutive cytokinin overproduction may have pleiotropic effects on plant development, two approaches assessed whether specific root-localized transgenic *IPT* (a key enzyme for cytokinin biosynthesis) gene expression could substantially improve tomato plant growth and yield under salinity: transient root *IPT* induction (*HSP70::IPT*) and grafting wild-type (WT) shoots onto a constitutive *IPT*-expressing rootstock (WT/*35S::IPT*). Transient root *IPT* induction increased root, xylem sap, and leaf bioactive cytokinin concentrations 2- to 3-fold without shoot *IPT* gene expression. Although *IPT* induction reduced root biomass (by 15%) in control (non-salinized) plants, in salinized plants (100 mM NaCl for 22 d), increased cytokinin concentrations delayed stomatal closure and leaf senescence and almost doubled shoot growth (compared with WT plants), with concomitant increases in the essential nutrient K^+ (20%) and decreases in the toxic ion Na^+ (by 30%) and abscisic acid (by 20–40%) concentrations in transpiring mature leaves. Similarly, WT/*35S::IPT* plants (scion/rootstock) grown with 75 mM NaCl for 90 d had higher fruit *trans*-zeatin concentrations (1.5- to 2-fold) and yielded 30% more than WT/non-transformed plants. Enhancing root cytokinin synthesis modified both shoot hormonal and ionic status, thus ameliorating salinity-induced decreases in growth and yield.

Key words: ABA, cytokinins, grafting, *IPT*, root zone temperature, root to shoot signalling, salinity, *Solanum lycopersicum*.

Introduction

More than 800 million ha of land worldwide are affected by salinity (Qadir *et al.*, 2007), thus improving crop salt tolerance is a key global agricultural goal. Salt decreases crop yields by reducing growth and inducing leaf senescence due to both an osmotic stress (plant water deficit) and an ionic stress provoked by nutritional imbalances and the accumulation of toxic ions such as sodium (Munns and Tester, 2008). Transgenic overexpression of certain ion transporters can improve salt tolerance by decreasing the uptake of toxic ions and/or altering their compartmentation

within the plant (Zhang and Blumwald, 2001; Shi *et al.*, 2002; Chen *et al.*, 2007). However, since this technology has yet to be exploited in commercial varieties, alternative approaches may also be valuable.

Among the plant hormones acting as 'master regulators' of multiple physiological processes (Davies, 2005), cytokinins (CKs) are especially important in regulating cell division and expansion (Kurakawa *et al.*, 2007) and delaying senescence (Gan and Amasino, 1995; Guo and Gan, 2007). Since both water (Kudoyarova *et al.*, 2007; Havlová *et al.*, 2008) and salt (Albacete *et al.*, 2008; Ghanem *et al.*, 2008) stress decreased plant CK status, attenuating this decrease transgenically [via overexpression of CK biosynthesis genes such as isopentenyltransferase (*IPT*)] might improve crop stress tolerance. However, massively increased (up to 150-fold) CK concentrations of some constitutive [e.g. under the cauliflower mosaic virus (CaMV) *35S* promoter] *IPT* transformants decreased root growth and induced water stress (Smigocki and Owens, 1989; Synková *et al.*, 1999; Pospíšilová, 2003). Tightly regulated transgenic *IPT* expression offers one approach to improving plant stress tolerance. Expression driven by a senescence-specific promoter (*SAG12*; Gan and Amasino, 1995) or maturation-induced promoter (*SARK*; Rivero *et al.*, 2007) delayed the age-dependent decline in photosynthesis (*SAG12::IPT*; Wingler *et al.*, 1998), thus minimizing yield loss of plants grown under heat stress (Xu *et al.*, 2009) and with limiting water supplies (*SARK::IPT*; Rivero *et al.*, 2007, 2009).

Despite intense interest in root-to-shoot signalling of environmental stresses (Davies and Zhang, 1991; Jackson, 1993; Dodd, 2005), whether root CK biosynthesis or delivery of CKs from root to shoot via the xylem (Aloni *et al.*, 2005) can regulate shoot CK concentrations and thence development and senescence, especially when the root system is exposed to environmental stress, remains controversial (Itai and Vaadia, 1971; Jackson, 1993; Faiss *et al.*, 1997; McKenzie *et al.*, 1998; Dodd and Beveridge, 2006). Historically, CKs have been regarded as root synthesized (Letham, 1994; Keiber, 2002; Aloni *et al.*, 2005), although analyses of spatial expression patterns of native *IPT* genes in *Arabidopsis* using their promoter::-reporter constructs suggests that some of these genes are also expressed in aerial organs (Miyawaki *et al.*, 2004; Takei *et al.*, 2004) and influence root CK status via basipetal phloem CK transport (Matsumoto-Kitano *et al.*, 2008). Although young shoot tissues can synthesize CKs (Taylor *et al.*, 1990; Schmülling, 2002; Nordström *et al.*, 2004), several studies have investigated the influence of root-localized CK production on shoot responses.

Root-synthesized CKs may have little physiological impact, since wild-type (WT) tobacco plants grafted on a *35S::IPT* rootstock showed similar lateral shoot growth, senescence, and shoot CK concentrations to WT self-grafts (Faiss *et al.*, 1997). However, McKenzie *et al.* (1998) suggested that a prominent gall at the graft union of those plants blocked xylem CK transport from the roots. In contrast, putatively root-specific *IPT* gene expression under a copper-inducible promoter (Mett *et al.*, 1996) increased shoot CK concentrations, released apical dominance, and delayed leaf senescence in tobacco under optimal growing conditions (McKenzie *et al.*, 1998). Although the reporter gene β-glucuronidase was massively increased in the root after addition of 50 μM $CuSO_4$ to the nutrient solution (Mett *et al.*, 1996), xylem Cu transport to the shoot (Pich and Scholz, 1996) may also have stimulated shoot CK synthesis. Recent reciprocal grafting studies of WT and a quadruple *IPT*-defective *Arabidopsis* mutant (with decreased levels of both iP-type and *t*Z-type CKs) demonstrated that both WT roots and shoots could produce, export, and recover normal CK levels and growth in mutant tissue (Matsumoto-Kitano *et al.*, 2008), thus highlighting (mutant/WT; scion/rootstock) or downgrading (WT/mutant) the physiological significance of root-synthesized CKs. The sometimes conflicting data of studies of root-localized *IPT* expression, within a more general recent context that root hormone supply has little physiological impact on the shoot (Holbrook *et al.*, 2002; Christmann *et al.*, 2007; Dodd *et al.*, 2009), require a re-evaluation of the role of root-synthesized CKs in regulating shoot responses, especially when root systems are exposed to stressful conditions likely to down-regulate root CK production.

However, transgenic IPT expression may produce multiple hormonal phenotypes, potentially obscuring a positive role for root-synthesized CKs. Although *IPT* gene expression regulated by the photosynthetic small subunit promoter (*PSSU::IPT*) both increased shoot CK concentrations and decreased shoot abscisic acid (ABA) concentrations (Synková *et al.*, 1999), leaf ABA concentrations of WT and *SAG12::IPT* (Cowan *et al.*, 2005) and WT and *SARK*::*IPT* transgenic tobacco plants (Rivero *et al.*, 2007) did not differ irrespective of soil moisture. Taking into account the intensive cross-talk between CKs and ABA and the importance of their ratio in regulation of several physiological processes (Dodd, 2003), analysis of ABA levels was performed in *IPT* transformants.

To determine whether root CK biosynthesis is important in mediating the relationship between decreased shoot CK status and salt-induced changes in growth, senescence, and fruit yield (Albacete *et al.*, 2008, 2009, 2010; Ghanem *et al.*, 2008), root *IPT* expression was up-regulated under control of a heat shock-inducible promoter (*HSP70::IPT*; Smigocki, 1991) by transient exposure to elevated root zone temperature (RZT) in short-term (3 weeks) experiments examining vegetative growth, and fruit yield was assessed in long-term (3 months) experiments where WT shoots were grafted onto a rootstock constitutively expressing *IPT* (WT/*35S::IPT*; Smigocki *et al.*, 2000). Augmenting root-to-shoot CK transport improved vegetative growth and ion homeostasis, delayed leaf senescence (induced *HSP70::IPT* plants), and increased fruit yield (WT/*35S::IPT* plants) of salinized tomato, potentially providing a novel strategy to attenuate salt-induced limitations to crop productivity.

Materials and methods

Root IPT induction

Plant material and culture: An *Xba*I/*Xmn*I DNA fragment of 456 bp from the *Drosophila melanogaster HSP70* gene, containing the promoter and 199 nucleotides of untranslated leader sequence, was fused through its 5′-untranslated region to the coding region of the *Agrobacterium tumefaciens IPT* gene from pTiB6S3 (Smigocki, 1991). The resultant *HSP70::IPT* constructs were transferred into *A. tumefaciens* LBA4404 bacteria and used to transform *Solanum lycopersicum* L. cv. UC82-B plants as previously described (Smigocki *et al.*, 2000).

Seeds of WT tomato (*S. lycopersicum* L. cv. UC82-B), transgenic T_3 homozygous *HSP70::IPT* tomato, and internal UC82-B (denoted as 116-9) control (transformed with an empty vector) plants were sown in trays filled with a perlite–vermiculite mix (1:3, v/v) moistened regularly with half-strength Hoagland nutrient solution. Fourteen days after sowing, the substrate was gently washed from the roots and seedlings were placed in PVC plates floating on 52.0 l tanks containing aerated half-strength Hoagland nutrient solution (eight plants per tank). Solutions were refilled every 2 d and renewed every week. The pH was continuously maintained at 5.6–6.0. Plants were grown in a growth chamber under a 16 h photoperiod. Air temperature and relative humidity during the day were 25–28 °C and 70±5%, respectively, and during the night 17–18 °C and 50±5%, respectively. Light intensity at the top of the canopy was ~250 µmol m^{-2} s^{-1} (PPFD). Eighteen days after sowing, seedlings were exposed to 0 (control) or 100 mM NaCl added to the nutrient solution in one step. A total of 240 plants were evaluated in each experiment: 30 plants per genotype, salt, and RZT treatment. Treatments were randomly distributed across available tanks within the growth chamber, and re-randomized for each experiment to avoid positional effects on growth. Although the experiment was repeated three times with similar results (Table 1), the data set presented (Figs 1–7) corresponds to Experiment 1.

Root IPT induction and sampling: To induce root *IPT* gene expression (regulated by a heat-shock promoter with an optimum induction temperature between 37 °C and 42 °C), on days 1, 8, and 22 (after salinization), half the WT and *HSP70::IPT* seedlings from each treatment were temporarily transferred to heated (42 °C) aerated half-strength Hoagland nutrient solutions (by carefully lifting the PVC plates containing the plants from their original solution, and gently placing them into the heated solution). After 2 h treatment (Smigocki, 1991), plants were returned to their normal growing conditions (nutrient solution at room temperature of 20–25 °C). Trays of plants kept at normal RZT were carefully removed and replaced as described above.

RNA was isolated, and plants sampled, 2 h and 48 h, respectively, after each exposure to elevated RZT. Shoots and roots were separated to determine fresh weight (FW). An actively growing leaf, present when salt was applied (leaf 3, numbering from the base of the plant), was tagged for leaf area determination (using a leaf area meter: Model AM 300, ADC BioScientific Ltd, Herts, UK), and ion and hormone analysis. The two youngest

Table 1. Shoot and root fresh weight (FW) of *HSP70::IPT* and WT tomato plants grown in half-strength Hoagland medium in the absence (Control) or presence of 100 mM NaCl (Salinized) for 22 d, and transiently exposed to elevated (RZT) or normal root zone temperature

Results are means of three independent experiments. Data are means ±SD, n=30. Measurements were performed 48 h after the end of the third episode of elevated RZT (22 d of salt treatment). Different letters within each row indicate significant differences between treatments for a given organ according to Student–Newman–Keuls test at P <0.05.

	HSP70::IPT				**WT**			
	Control		**Salt**		**Control**		**Salt**	
	Normal	**RZT**	**Normal**	**RZT**	**Normal**	**RZT**	**Normal**	**RZT**
Shoot FW	238.6±25.7 a,b	217.3±26.3 b	64.1±19.3 d	141.4±26.3 c	245.4±23.5 a,b	231.8±29.7 b	62.9±21.1 d	68.8±10.1 d
Root FW	54.0±19.8 b	37.4±18.6 c	27.9±17.3 d	32.3±9.8 c,d	61.2±19.3 a,b	52.3±19.4 b	27.7±9.7 d	28.6±16.8 d

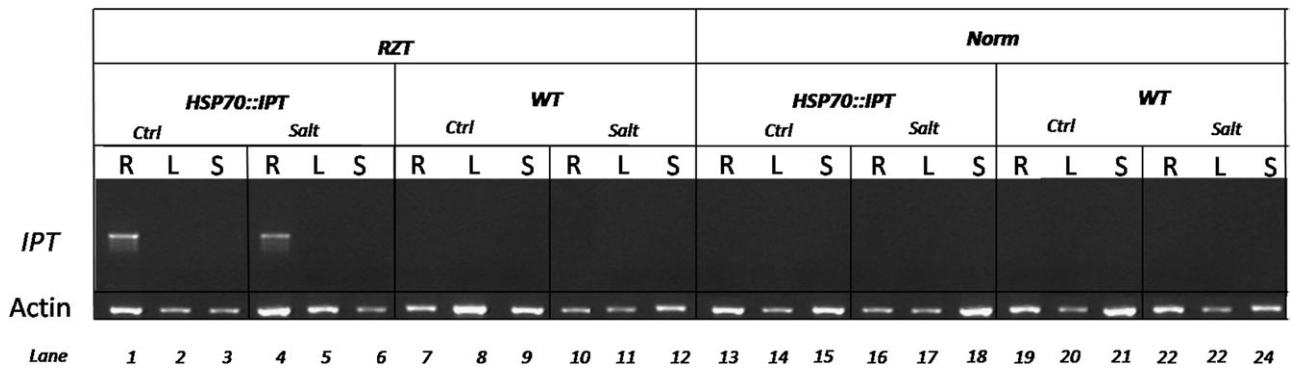

Fig. 1. RT-PCR analysis of *IPT* expression in roots (R), leaves (L) and stems (S) of *HSP70::IPT* and WT tomato plants grown in half-strength Hoagland medium in the absence (Ctrl) or presence of 100 mM NaCl (Salt) and transiently exposed to elevated (RZT) or normal (Norm). RNA samples were isolated 2 h after the end of the elevated root zone temperature treatment. Actin transcripts/cDNAs were used as a PCR control. Lane numbers are indicated at the base of the image.

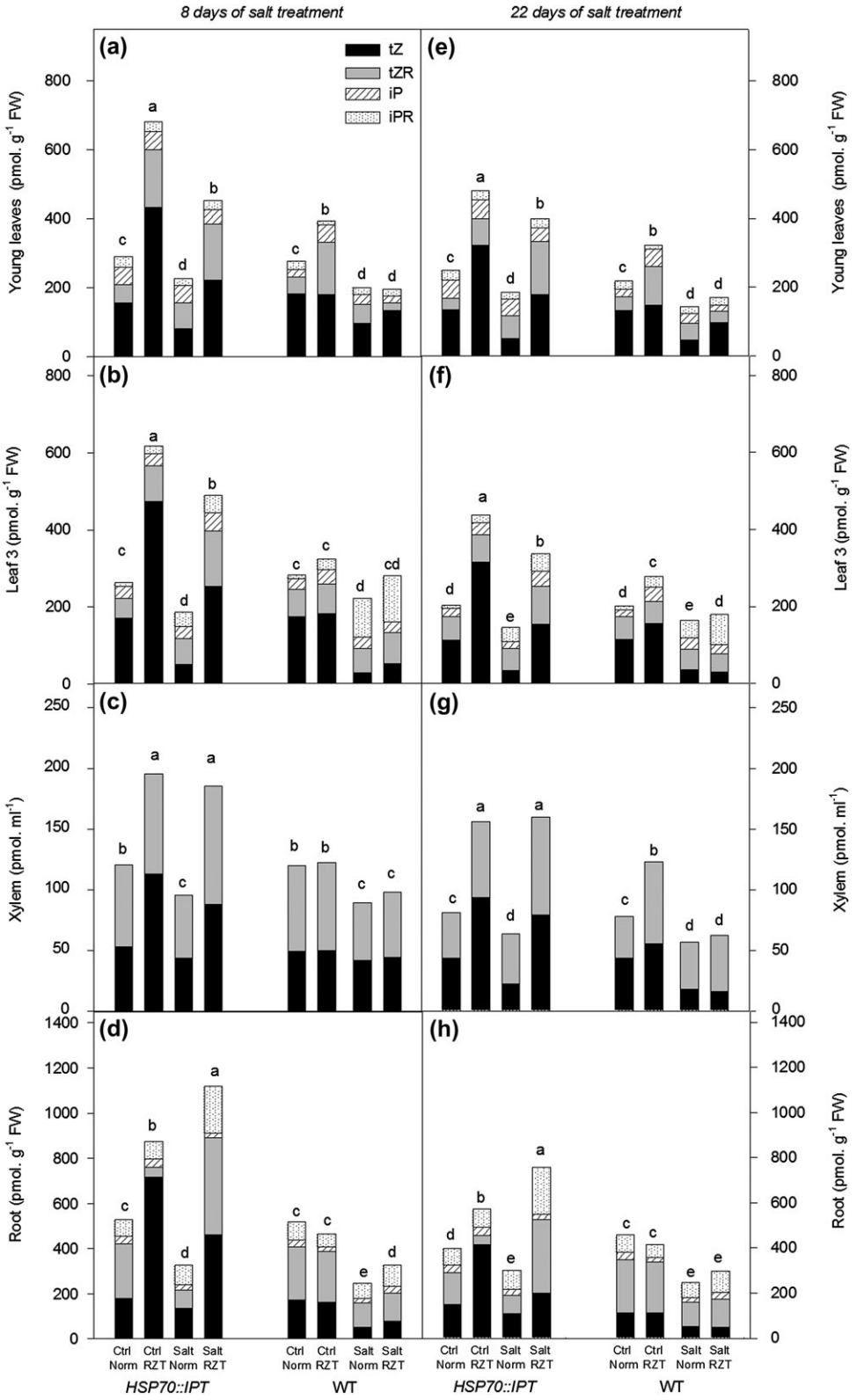

Fig. 2. Bioactive cytokinin (CK) levels in young leaves (a, e), leaf 3 (b, f), xylem sap (c, g), and roots (d, h) of *HSP70::IPT* and WT tomato plants grown in half-strength Hoagland medium in the absence (Ctrl) or presence (Salt) of 100 mM NaCl, and transiently exposed to elevated (root zone temperature) or normal (Norm) RZT. Measurements were performed 48 h after the end of the second (8 d of salt treatment: a–d) and third episodes of elevated RZT (22 days of salt treatment: e–h). Bioactive CKs comprise the following metabolites *trans*-zeatin (*t*Z), isopentenyladenine (iP), and the corresponding ribosides (*t*ZR and iPR). Data are means ±SE, $n=8$. SE was in the range 5–18%. Different letters within each panel indicate significant differences between treatments for a given organ according to Student–Newman–Keuls test at $P<0.05$.

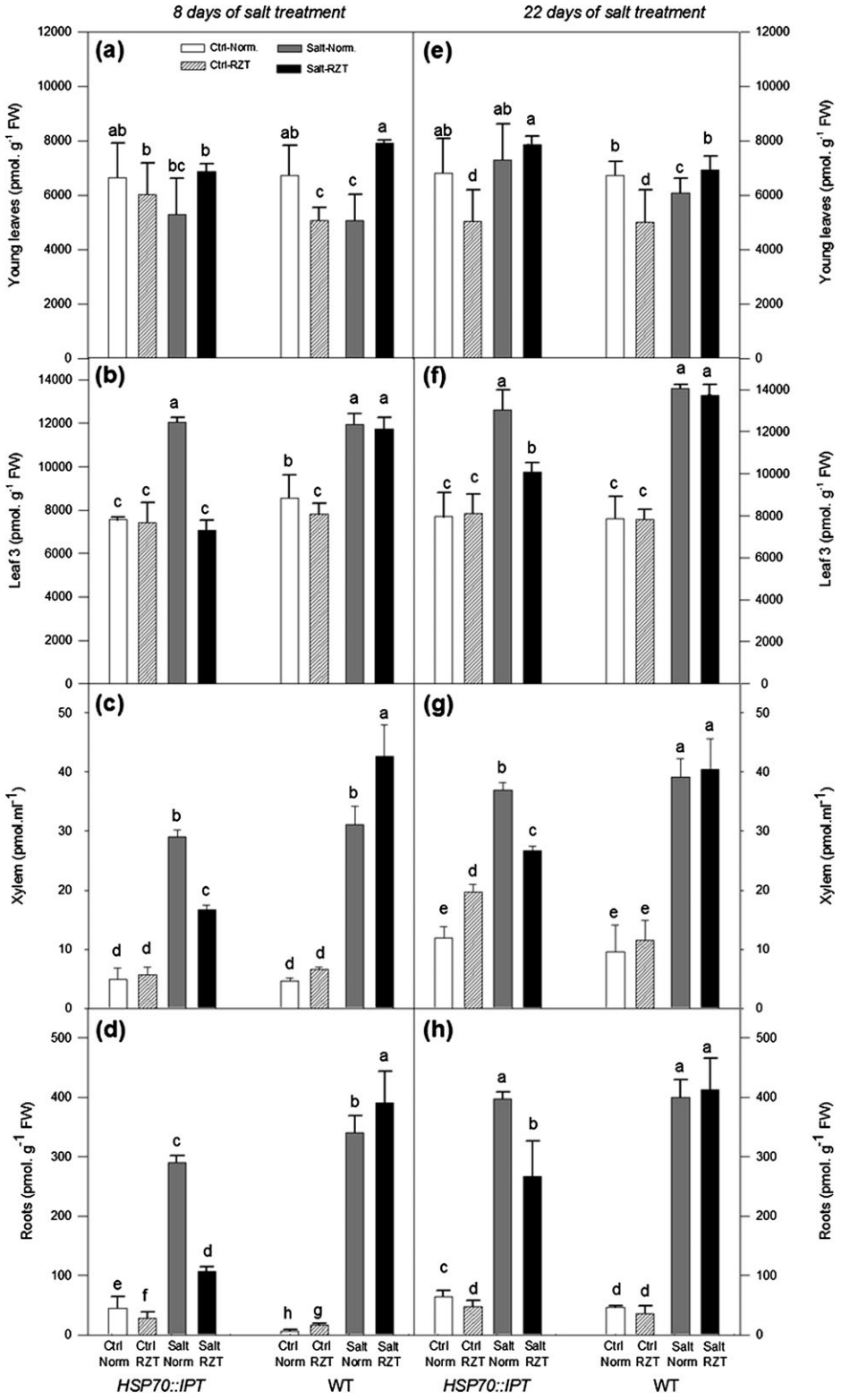

Fig. 3. Abscisic acid (ABA) levels in young leaves (a, e), leaf 3 (b, f), xylem sap (c, g), and roots (d, h) of *HSP70::IPT* and WT tomato plants grown in half-strength Hoagland medium in the absence (Ctrl) or presence (Salt) of 100 mM NaCl, and transiently exposed to elevated (root zone temperature) or normal (Norm) RZT. Measurements were performed 48 h after the end of the second (8 d of salt treatment) and the third episode of elevated RZT (22 d of salt treatment). Data are means ±SE, n=8. Different letters within each panel indicate significant differences between treatments for a given organ according to Student–Newman–Keuls test at P <0.05.

unfolded upper leaves (identified as 'young leaves') were also collected for hormone and ion analysis. Xylem sap was obtained after severing the shoot ~2–3 cm above the root, and applying pressure (–0.5 MPa for control plants and about –0.9 MPa for salinized plants) with nitrogen to the root system with a Scholander pressure chamber (Pérez-Alfocea *et al.*, 2000).

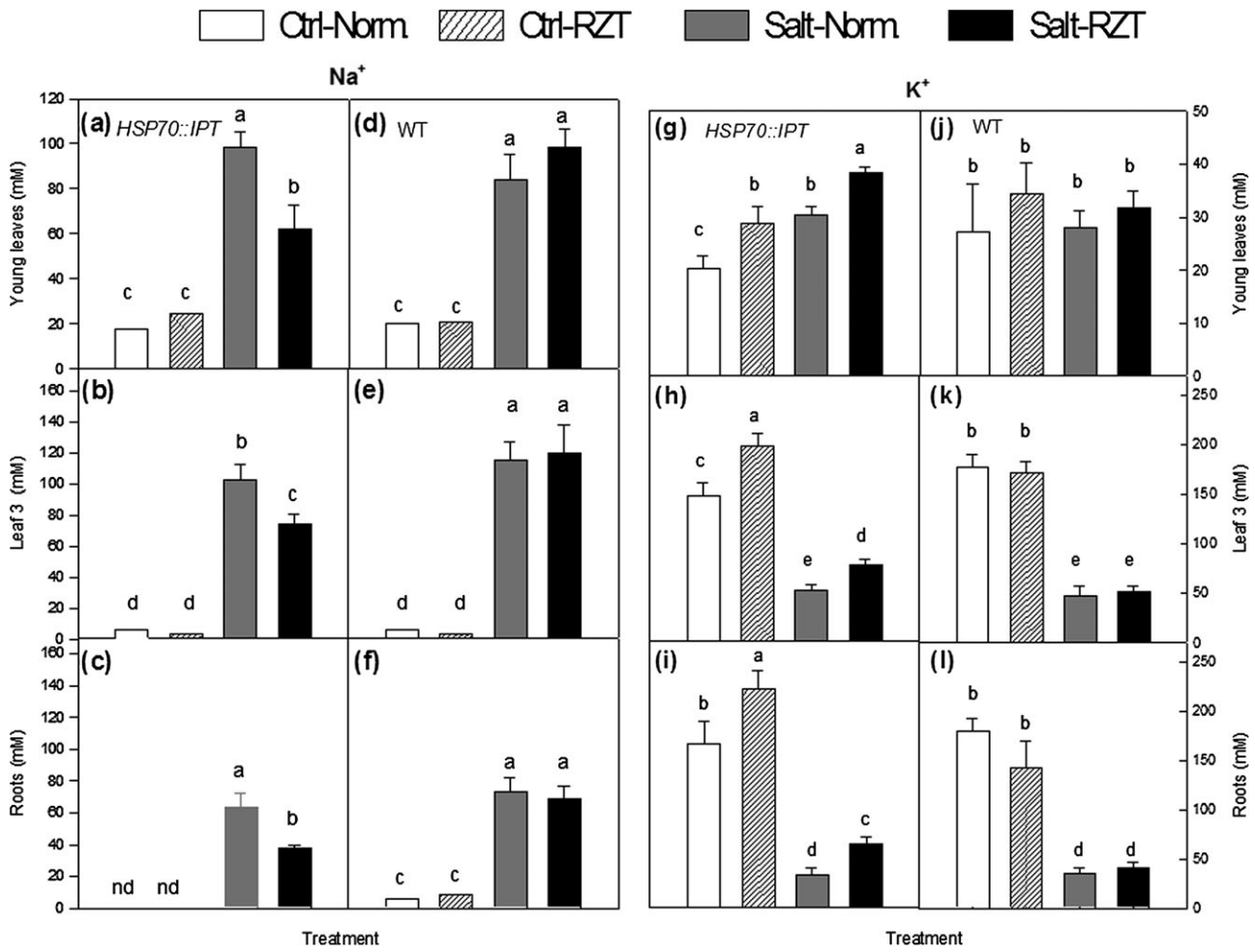

Fig. 4. Sodium (Na^+) (a–f) and potassium (K^+) (g–l) concentrations in young leaves (a, d, g, j), leaf 3 (b, e, h, k), and roots (c, f, i, l) of *HSP70::IPT* and WT tomato plants grown in half-strength Hoagland medium in the absence (Ctrl) or presence of 100 mM NaCl (Salt), and transiently exposed to elevated (root zone temperature) or normal (Norm) RZT. Data are means ±SE, n=8. Measurements were performed 48 h after the end of the third episode of elevated root zone temperature (22 d of salt treatment). Different letters within each panel indicate significant differences between treatments for a given organ according to Student–Newman–Keuls test at P <0.05. nd = not detected.

IPT expression analysis: Total RNA was prepared from leaves and roots using the RNAgents Total RNA isolation system of Promega (Promega Benelux b.v). First-strand cDNA was synthesized using Superscript™ III first strand synthesis for reverse transcription-PCR (RT-PCR; Invitrogen, Life Technologies, The Netherlands) according to the manufacturer's instructions. The amplification procedure for *IPT* and tomato *actin* (used as a constitutive control) genes consisted of 35 cycles; 1 µl of first strand was used as template. Internal oligonucleotides, *IPT forward* (5'-CATCTAATTTTCGGTCCAACTTGCA-3'), *IPT reverse* (5'-CGATATCCATCGATCTT-3'), *actin forward (*5'-ATTCCCTGACTGTTTGCTAGT-3'), and *actin reverse* (5'-TCCAACACAATACCGGTGGT-3'), were designed, and standard RT-PCR performed. After an initial denaturation step at 94 °C for 4 min, each cycle consisted of 30 s at 94 °C, 30 s at 55 °C of annealing temperature, 1 min extension at 72 °C, followed by a final extension of 7 min at 72 °C. Amplifications were conducted using the GoTaq DNA polymerase (Promega Benelux b.v). PCR products were analysed by 1.5% (w/v) Tris/acetic acid/EDTA/agarose electrophoresis. The PCR products of each gene were sequenced using an automatic sequencer (Genetic Analyser 3100, Applied Biosystems, Belgium) and the 'BigDye Terminator v1.1 cycle sequencing kit'. The resulting nucleotide sequences were aligned and compared using ClustalX.

Hormone extraction and analysis: Phytohormones (CKs and ABA) were extracted as previously reported in Dobrev and Kaminek (2002) and adapted in Novák *et al.* (2003) and Ghanem et al. (2008). Plant material (1 g FW of leaf, root, or fruit) was homogenized in liquid nitrogen and placed in 5 ml of a cold (–20 °C) extraction mixture of methanol/water/formic acid (15:4:1, v/v/v, pH 2.5). After overnight extraction at –20 °C, solids were separated by centrifugation (20 000 *g*, 15 min) and re-extracted for 30 min in an additional 5 ml of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and plant pigments and evaporated to dryness. The residue was dissolved in 5 ml of 1 M formic acid and loaded on an Oasis MCX mixed mode (cation-exchange and reverse phase) column (150 mg, Waters, USA) pre-conditioned with 5 ml of methanol followed by 5 ml of 1 M formic acid. To separate different CK forms (nucleotides, bases, ribosides, and glucosides) from ABA, the column was washed and eluted stepwise with different appropriate solutions as indicated (Dobrev and Kaminek, 2002). After each solvent passed through the columns, they were purged briefly with air. Solvents were evaporated at 40 °C under vacuum. Samples then dissolved in a water/acetonitrile/formic acid (94.9:5:0.1, v/v/v) mixture for high-performance liquid chromatography/mass spectrometry (HPLC/MS) analysis. Xylem sap samples were filtered

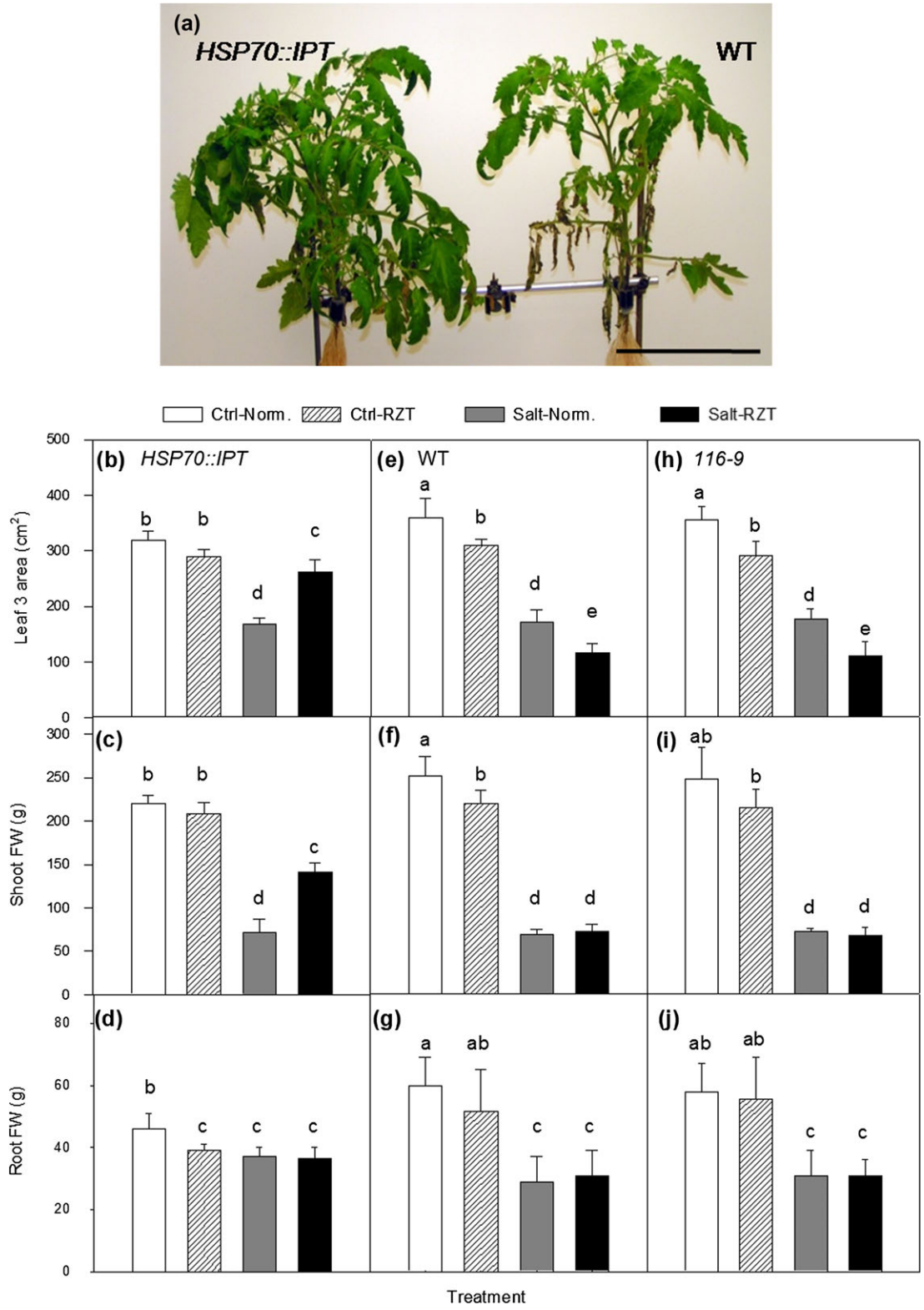

Fig. 5. (a) Appearance of WT (right) and *HSP70::IPT* (left) tomato plants grown in the presence of 100 mM NaCl for 22 d and exposed to elevated RZT. Scale bar is 30 cm. (b–j) Leaf 3 area (cm^2) (b, e, h) and shoot (c, f, i) and root (d, g, j) fresh weight (FW) of *HSP70::IPT*, 116-9 line (internal control), and WT tomato plants grown in half-strength Hoagland medium in the absence (Ctrl) or presence of 100 mM NaCl (Salt) for 22 d, and transiently exposed to elevated (RZT) or normal (Norm) RZT. Data are means ±SE, n=10. Measurements were performed 48 h after the end of the third episode of elevated RZT (22 d of salt treatment). Different letters within each panel indicate significant differences between treatments for a given organ according to Student–Newman–Keuls test at P <0.05.

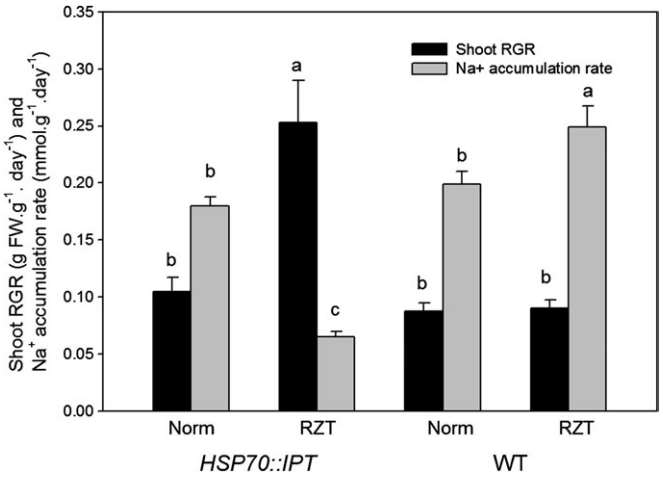

Fig. 6. Shoot relative growth rate (RGR) and sodium (Na^+) accumulation rate (mmol per g of shoot FW acquired and per day, average of both analysed leaf 3 and young leaves) during 22 d growth of *HSP70::IPT* and WT tomato plants in half-strength Hoagland medium in the presence of 100 mM NaCl, and transiently exposed to elevated (root zone temperature) or normal (Norm) RZT. Data are means ±SE of nine plants. Different letters within each panel indicate significant differences between treatments for according to Student–Newman-Keuls test at P <0.05.

through 13 mm diameter Millex filters with 0.22 μm pore size and nylon membrane (Millipore, Bedford, MA, USA), and injected directly onto the LC-MS/MS system.

LC-MS/MS analysis of leaf, root and xylem sap was performed as previously described (Dobrev *et al.*, 2002; Novák *et al.*, 2008) using an Ion Trap Mass Spectrometer Finnigan MAT LCQ-MS^n (Thermo Fisher, Waltham, MA, USA) equipped with an electrospray interface. Detection and quantification were carried out using a Finnigan LCQ MS ion trap LC-MS operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with deuterated CKs and ABA ($[^2H_6]$*cis-trans*-abscisic acid, Olchemin Ltd, Olomouc, Czech Republic) as internal standards. For analyses of endogenous CKs, 50 pmol of each of the following 15 deuterium-labelled standards were added: $[^2H_5]$Z, $[^2H_5]$ZR, $[^2H_5]$Z9G, $[^2H_5]$ZOG, $[^2H_5]$ZROG, $[^2H_6]$iP, $[^2H_6]$iPR, $[^2H_6]$iP9G, $[^2H_3]$DHZ, $[^2H_3]$DHZR, $[^2H_3]$DHZ9G, $[^2H_7]$DHZOG, $[^2H_5]$ZMP, $[^2H_3]$DHZMP, and $[^2H_6]$iPR5'MP (Apex Organics, Honiton, UK). Recovery percentages ranged between 92% and 95%. *cis*-Zeatin derivatives were determined using the retention times and MS spectra of unlabelled standards and the response ratio of their *trans*-zeatin counterparts. Of 47 CK derivatives of interest, the concentrations of 21 were above the detection limit: *t*Z, *t*ZR, *t*ZOG, *t*ZROG, *t*Z9G, *t*ZR5'MP, *c*Z, *c*ZR, *c*ZOG, *c*ZROG, *c*Z9G, *c*ZR5'MP, DHZ, DHZR, DHZOG, DHZROG, DHZ9G, iP, iPR, iP9G, and iPR5'MP (Supplementary Fig. S1 available at *JXB* online). The detection limit was calculated for each compound as 3.3 σ/S, where α is the standard deviation of the response and S is the slope of the calibration curve. Each sample was injected at least twice. Fruit *trans*-zeatin concentrations were determined according to the LC-MS/MS method previously described

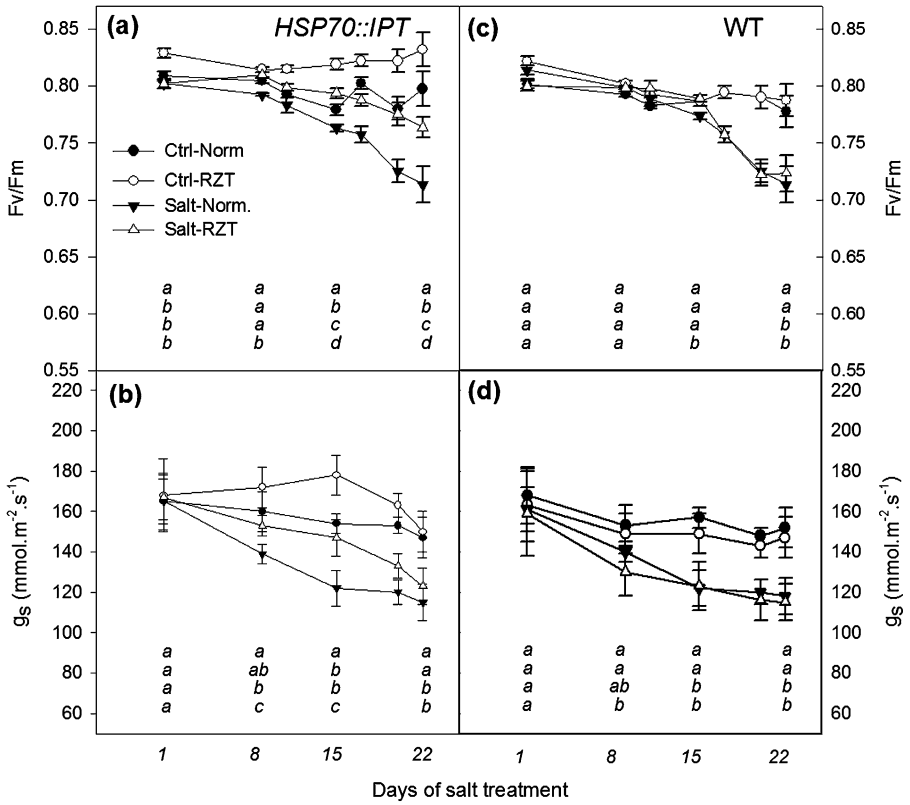

Fig. 7. Maximum photochemical efficiency (F_v/F_m) (a, c) and stomatal conductance (g_s) (b, d) of leaf 3 of *HSP70::IPT* (a, b) and WT (c, d) tomato plants grown in half-strength Hoagland medium in the absence (circles) or presence (triangles) of 100 mM NaCl for 22 d, and either exposed to elevated RZT (open symbols) or cultivated under normal nutrient solution temperature (filled symbols). Different letters at the base of each panel indicate significant differences between the treatments, in order of top to base on each panel, according to Student–Newman–Keuls test at P <0.05, n=5.

(Albacete *et al.*, 2008; Ghanem *et al.*, 2008). Treatment differences in tissue hormone status were consistent irrespective of whether concentrations were calculated on an FW or dry weight (DW) basis (data not shown).

Ion (Na^+ and K^+) quantification: Leaf and root tissues were oven-dried at 70 °C for 48 h and 50 mg DW was digested in 35% (v/v) HNO_3 at 80 °C. Ions were dissolved in 0.1 M HCl and concentrations determined (in triplicate for each sample) using an inductively coupled argon plasma emission spectrophotometer (YJ48; Jobin Yvon, Edison, NJ, USA) calibrated with certified standard solutions.

Chlorophyll fluorescence: Modulated chlorophyll fluorescence was measured in dark-adapted (30 min) leaves using a pulse-modulated chlorophyll fluorometer FMS-2 (Hansatech Instruments Ltd, Norfolk, UK) as reported previously (Ghanem *et al.*, 2008). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as $(F_m–F_0)/F_m$.

Grafting experiments

To test whether root-localized CK production could improve fruit yield of salinized plants grown under commercial greenhouse conditions, seedlings from the semi-determinate cultivar P-73 were grafted as previously described (Albacete *et al.*, 2009) onto a determinate commercial cultivar UC-82B (P-73/UC-82B, denoted as WT/WT) and on a transformant overexpressing the *IPT* gene as rootstocks under the control of the constitutive CaMV *35S* promoter (P-73/*35S::IPT*; denoted as WT/*35S::IPT*) (Smigocki *et al.*, 2000). The particular *35S::IPT* transformant selected had a moderate 30% higher leaf *trans*-zeatin concentration (compared with azygous plants) after 50 d of salinization without apparent phenotypic alterations in the whole plant (data not shown). Four plants per combination and salinity level were transferred to a polyethylene greenhouse after the grafts had established, using perlite as substrate. Plants were distributed in a planting pattern of 2 m between rows and 0.5 m between plants within rows, and cultivated as one stem by eliminating all axillary buds weekly. A standard fertilization mixture for tomato was applied by a drip irrigation system. Salt treatment started 10 d after transfer and continued for 3 months. Electrical conductivities of the fertilization solutions were 1.2 dS m^{-1} (control) and 8.7 dS m^{-1} (75 mM NaCl). Fruit yield, number, and weight were determined, and actively growing (20 d and 35 d after anthesis) and ripe fruits were collected for CK analysis as described above.

Statistics

Data from transient root *IPT* induction experiments were subjected to an analysis of variance (ANOVA II) using the SAS software (SAS System for Windows, version 8.02) with mean discrimination achieved using the Student–Newman–Keuls test at the 5% level. Since WT and internal control (denoted as line 116-9 and transformed with an empty vector) plants showed similar growth responses in all experiments (Fig. 5), only data from WT plants are presented. Data of grafting experiments were subjected to two-way ANOVA (main effects of graft combination and salinity) with a *t*-test for small sample size based on the *t*-distribution using the SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA) used to discriminate means within a salinity treatment.

Results

Elevated RZT induced IPT *transgene expression only in root tissues*

Elevated RZT (exposure to 42 °C nutrient solution for 2 h to induce the promoter region controlling *IPT* gene expression) caused *IPT* gene expression in roots (Fig. 1, lanes 1 and 4) but not leaves (lanes 2 and 5) or stems (lanes 3 and 6) of *HSP70::IPT* plants in both control (0 mM NaCl) and salt-stressed (100 mM NaCl) conditions. No *IPT* expression was detected in roots or leaves of WT plants exposed to elevated RZT (Fig. 1, lanes 7–12), or control WT or *HSP70::IPT* plants maintained at normal RZT throughout the experiment (Fig. 1, lanes 13–24). Thus transient exposure of *HSP70::IPT* plants to elevated RZT successfully established root-localized *IPT* gene induction.

Salinity decreased CK concentrations, except in HSP70::IPT *plants exposed to elevated RZT*

Individual CK metabolites were assigned to groups based on their structure and function as previously described (Havlova *et al.*, 2008): bioactive CKs (*t*Z, *t*ZR, iP, and iPR), CK deactivation forms (*N*-glucosides: *t*Z9G and iP9G), CK storage forms (*O*-glucosides: *t*ZOG, *t*ZROG, *c*ZOG, and *c*ZROG), and CK phosphates (CK biosynthesis intermediates: *t*ZR5'MP, *c*ZR5'MP, and iPR5'MP). As *cis*-zeatin (*c*Z) and its riboside (*c*ZR) are recognized by some CK receptors, but lack physiological activity in most CK bioassays (Havlova *et al.*, 2008), *cis*-zeatin derivatives *c*Z, *c*ZR, and *c*Z9G were considered separately, while *c*ZMP was included in other phosphates and *c*ZOG and *c*ZROG were included in CK storage forms. Total CK concentrations, classified as above, are available (Supplementary Fig. S1 at *JXB* online). Treatment differences in spatial patterns of bioactive CK metabolites are presented (Fig. 2).

Under both control and salt treatments, no genotypic differences in total bioactive CK concentrations were found at either 8 d or 22 d under normal RZT in young leaves (Fig. 2a, e), the more mature leaf 3 (Fig. 2b, f), xylem sap (Fig. 2c, g), or roots (Fig. 2d, h).

Elevated RZT increased bioactive CK concentrations (by 60–130%) in all compartments of *HSP70::IPT* plants on both measurement occasions, independently of salinization. In contrast, in WT plants, elevated RZT increased bioactive CK concentrations (by 10–40%) essentially in young leaves (days 8 and 22; Fig. 2a, e), and leaf 3 and xylem sap of control plants (day 22; Fig 2f, g). In salinized WT plants, elevated RZT only increased bioactive CKs in leaf 3 (days 8 and 22; Fig. 2b, f) and roots (day 8, Fig. 2d).

Generally, salinization significantly decreased (by 20–50%) bioactive CK concentrations in all measured plant compartments, to a similar degree in WT and *HSP70::IPT* plants grown under normal RZT, and in WT plants exposed to elevated RZT, especially for *t*Z and *t*ZR (Fig. 2). However, this salinity-induced decrease was counteracted in *HSP70::IPT* plants exposed to elevated RZT; compared with salinized WT plants exposed to elevated RZT, induced *HSP70::IPT* plants showed higher bioactive CKs concentrations not only in roots (3-fold; Fig. 2d, h), but also in leaves and xylem sap (2-fold; Fig. 2a–c, e–g), mainly due to greatly increased *t*Z and *t*ZR. Although salinity decreased CK concentrations (by 20–35%) in leaves of *HSP70::IPT* plants exposed to elevated RZT (compared with control

HSP70::IPT plants exposed to elevated RZT), this effect was not detected in xylem sap (Fig 2c, g) or was the opposite in roots (Fig. 2d, h). Taken together, these data indicate that the CK accumulation in WT plants exposed to elevated RZT (mostly restricted to the leaves under control conditions) was much less than in *HSP70::IPT* plants.

Salinity increased ABA concentrations, but to a lesser extent in HSP70::IPT *plants exposed to elevated RZT*

Under normal RZT and control conditions, ABA concentrations of *HSP70::IPT* and WT plants were similar in young leaves, leaf 3, and xylem sap on both measurement occasions (Fig. 3a–c, e–g). Roots of *HSP70::IPT* plants had higher (1.5- to 5-fold) ABA concentrations under these conditions (cf. columns 1 and 5 of Fig. 3d, h).

Under control conditions, the effects of elevated RZT varied according to genotype and plant compartment. Elevated RZT decreased ABA concentrations (by 15–30% averaged over both genotypes and measurement occasions) in young leaves (Fig. 3a, e), had no effect in mature leaves (Fig. 3b, f), increased ABA concentrations 1.8-fold in xylem sap of *HSP70::IPT* plants only on day 22 (Fig. 3g), and consistently decreased (by 30–40%) root ABA concentrations of *HSP70::IPT*, but not WT, plants (Fig. 3d, h).

Although effects of salinization were minimal in young leaves, ABA concentrations increased significantly in mature leaves (1.5-fold), xylem sap (4- to 5-fold), and roots (5- to 25-fold) of *HSP70::IPT* and WT plants grown under normal RZT, and WT plants grown with elevated RZT (Fig. 3b–d, f–h). However, exposure of *HSP70::IPT* plants to elevated RZT attenuated this salinity-induced increase in ABA in the mature leaf, xylem sap, and roots, hence these plants had 2- to 3-fold lower ABA concentrations in those organs than salinized WT plants. Taken together, these data indicate that root *IPT* gene induction attenuated salinity-induced ABA accumulation.

Salinity-induced perturbation of ionic status was ameliorated in HSP70::IPT *plants exposed to elevated RZT*

At the end of the experiment, under normal RZT, control WT and *HSP70::IPT* plants had similar Na^+ concentrations in young (Fig. 4a, d) and mature (Fig. 4b, e) leaves and similar K^+ concentrations in roots (Fig. 4i, l). However, WT plants had higher Na^+ concentrations in roots (cf. Fig. 4c, f) and higher K^+ concentrations in leaves (cf. Fig. 4g, h, j, k) than *HSP70::IPT* plants.

Transiently elevating the RZT of control plants had no effect on Na^+ concentrations in either genotype (cf. first two columns in each panel of Fig. 4a–f), but significantly increased K^+ concentrations in all analysed organs of *HSP70::IPT* plants (Fig. 4g–i) but not WT plants (Fig. 4j–l).

Salinity significantly increased Na^+ concentrations throughout the plant (Fig. 4a–f), but exposure of *HSP70::IPT* plants to elevated RZT attenuated the salinity-induced increases in Na^+ in all plant compartments by 20 mM (roots) to 40 mM (young leaves) (Fig. 4a–c). Furthermore, salinity significantly decreased K^+ concentrations (>50%) in mature leaves and roots (Fig. 4h, k, i, l) and either increased (*HSP70::IPT* plants; Fig. 4g) or had no effect (WT plants; Fig. 4j) on K^+ concentrations in young leaves. However, exposure of *HSP70::IPT* plants) to elevated RZT ameliorated (by 20%) the decrease in K^+ concentrations in leaf 3 (Fig. 4h) and roots (Fig. 4i), and actually increased K^+ concentrations of young leaves (Fig. 4g), but had no significant effects in WT plants (Fig 4j–l). Taken together, these data indicate that the effects of salinity on plant ion status were modified by *IPT* gene induction.

Elevated RZT decreased growth under control conditions, but enhanced shoot growth of salinized HSP70::IPT *plants*

Since WT and 116-9 (transformed with empty vector) plants showed similar growth irrespective of treatments (Fig. 5e–j), only WT data are described. Irrespective of RZT treatment, WT and *HSP70::IPT* plants were phenotypically normal (Fig. 5a), although *HSP70::IPT* plants tended to have a 10% lower biomass (P=0.38) than WT plants prior to imposition of salinity and RZT treatments (18 d after germination). Under control conditions at normal RZT, WT plants had a greater leaf 3 area (Fig. 5b, e), and a higher shoot (1.1-fold; cf. Fig. 5c, f) and root FW (1.3-fold; cf. Fig. 5d, g) than *HSP70::IPT* plants. Elevated RZT abolished these genotypic differences in the shoot but not in the roots, decreased leaf area (Fig. 5e) and shoot FW (Fig. 5f) of WT plants by 20%, but had no effect on leaf area (Fig. 5b) and shoot FW (Fig. 5c) of *HSP70::IPT* plants. Elevated RZT inhibited root growth of WT and *HSP70::IPT* plants by ~15%. Similar results were obtained when data from three independent experiments were pooled (Table 1).

Under normal RZT, salinization decreased leaf 3 area, and shoot and root FW of both WT and *HSP70::IPT* plants. In WT plants, elevated RZT magnified the salinity-induced growth reduction of leaf 3 area, but had no additional impact on shoot and root FW. In *HSP70::IPT* plants, elevated RZT did not affect the salinity-induced growth reduction in root biomass, but doubled leaf 3 area and shoot FW. Thus shoot FW of salinized *HSP70::IPT* plants exposed to elevated RZT was only 30% less than that of control plants, while that of salinized WT plants was 60% less (Fig. 5c, f). On average, the magnitude of the increased shoot relative growth rate of salinized *HSP70::IPT* plants exposed to elevated RZT was equivalent to the magnitude of the decrease in leaf Na^+ concentration, compared with the other treatments (Fig. 6). Taken together, these data indicate that root *IPT* gene induction prevented or attenuated negative impacts of elevated RZT or salinity in the shoot.

Elevated RZT increased stomatal conductance and delayed salt-induced senescence in HSP70::IPT *plants*

Under control conditions, elevated RZT increased the maximum quantum efficiency of PSII (F_v/F_m) in

HSP70::IPT plants compared with those plants maintained at normal RZT (Fig. 7a). In contrast, RZT treatment did not change F_v/F_m in WT plants, irrespective of salt treatment (Fig. 7b). Salinization for 17 d sharply decreased F_v/F_m values of WT plants independently of RZT (Fig. 7b), while this salinity-induced decrease in F_v/F_m was delayed by 1 week in *HSP70::IPT* plants exposed to elevated RZT (Fig. 7a).

Elevated RZT increased stomatal conductance (g_s) of control *HSP70::IPT* plants by 20%, but had no effect on WT plants (Fig. 7c, d). From day 9 until the end of the experiment, salinity decreased g_s of WT plants by 16% independently of RZT (Fig. 7d). Salinized *HSP70::IPT* plants at normal RZT also showed this decreased g_s, but exposure to elevated RZT maintained g_s comparable with control plants during the first 2 weeks of salinization. However, this positive impact disappeared by the end of the experiment.

CK-overproducing rootstocks increased both fruit trans-*zeatin concentration and yield*

Under control conditions, the rootstock had no significant impact on any measured fruit yield-related parameters (Table 2). However, after 3 months growing under moderate salinity, WT/*35S::IPT* (scion/rootstock) plants had a 30% higher fruit yield than WT/WT plants, mainly due to 25% more fruit and a significantly higher fruit weight (Table 2).

CK analyses of actively growing (20–35 d after anthesis) and ripe fruits revealed that fruits of WT/*35S::IPT* plants had 2-fold higher *t*Z concentrations than fruits from WT/WT plants under both control and salinized conditions (Table 2). Salinity decreased fruit *t*Z concentration by 5-fold compared with control plants of both graft combinations (Table 2).

Discussion

Salinity-induced leaf growth inhibition and premature senescence were correlated with decreased root, xylem sap, and leaf bioactive CK concentrations (Albacete *et al.*, 2008; Ghanem *et al.*, 2008; cf. columns 1, 3 and 5, 7 of Fig. 2). Root-synthesized CKs may regulate these shoot responses, since rootstocks conferring better growth and fruit yield under salinity had increased shoot xylem CK concentrations with minimal impact on xylem Na^+ concentrations (Albacete *et al.*, 2009). However, it is not clear whether root-localized *IPT* expression alters plant growth and development and/or delays leaf senescence of plants exposed to environmental stress, since previous studies purportedly addressing this issue (i) showed conflicting results (cf. Faiss *et al.*, 1997; McKenzie *et al.*, 1998); (ii) did not intentionally impose environmental stress (Matsumoto-Kitano *et al.*, 2008); or (iii) localized *IPT* expression in the leaves (Gan and Amasino 1995; Synkova *et al.*, 1999; Rivero *et al.*, 2007; Xu *et al.*, 2009). It was hypothesized that transient root *IPT* induction (*HSP70::IPT*) and grafting WT shoots onto a constitutive *IPT*-expressing rootstock with moderately higher CK production (WT/*35S::IPT*) would augment root CK biosynthesis and xylem transport, thus ameliorating deleterious effects of salinity on shoot growth and fruit yield.

It was first necessary to establish whether *IPT* gene expression was localized to the roots after exposure to elevated RZT. Since the root zone was insulated from the aerial environment by floating the hydroponically grown plants on PVC plates, and the duration of elevated RZT was relatively short (2 h), leaf and stem temperature measurements did not detect any shoot warming (data not shown). Although some heat transfer through xylem sap may have occurred, RT-PCR did not detect any *IPT* transgene expression in stems or leaves (Fig. 1). Despite this

Table 2. Fruit yield, number, mean fresh weight of individual fruits (FW), and *trans*-zeatin concentrations at specified growth stages [days after anthesis (DAA)] in grafted plants cultivated in the absence (control) or presence of 75 mM NaCl for 3 months

Fruit yield-related parameters (n=4)

Genotype[a]	Control			75 mM NaCl		
	Yield (g)	No. of fruit	Fruit FW (g)	Yield (g)	No. of fruit	Fruit FW (g)
WT/WT	3122±170 a	35.0±1.6 a	89.7±3.9 a	877±91 b	20.2±2.2 b	43.4±0.5 b
WT/*35S::IPT*	3665±169 a	35.3±1.7 a	104.9±6.4 a	1133±107 a	25.0±2.5 a	45.4±1.0 a

***Trans*-zeatin (pmol g^{-1} FW, n=6)**

	Control			75 mM NaCl		
	20 DAA	35 DAA	Ripe	20 DAA	35 DAA	Ripe
WT/WT	227.1±24.2 b	373.1±17.3 b	95.3±16.4 b	62.5±7.8 b	68.0±5.5 b	103.1±3.6 b
WT/*35S::IPT*	568.8 ±16.4 a	746.6 ±30.6 a	161.5± 7.8 a	116.8±4.6 a	125.9±6.4 a	144.1±5.0 a

[a] Genotypes were the cultivar P-73 either grafted onto rootstocks of the commercial cultivar UC-82B (WT/WT) or the same genotype overexpressing the *IPT* gene under control of the constitutive CaMV *35S* promoter (WT/*35S::IPT*). Values marked with different letters within the same column are significantly different according to a two-tailed *t*-test for small sample size based on the *t*-distribution. Means±SD.

apparent tight spatial and temporal control of *IPT* expression, leaf 3 area, and shoot and root FW of *HSP70::IPT* plants grown with normal RZT were 11% less than those of corresponding WT plants (cf. column 1 of Fig. 5b, e, c, f, and d, g), in agreement with the 10% lower biomass of the transgenic population before starting the treatments. In contrast, considerable 'leakiness' of heat shock-inducible promoters in tobacco under control conditions, particularly *in vitro* (Smart *et al.*, 1991; Smigocki, 1991), caused pronounced phenotypic alterations (i.e. shorter, greener, bigger leaves, and delayed senescence) linked to increased CK concentrations *in planta*. This was not the case here (Fig. 5a); thus *HSP70::IPT* and WT plants had similar CK concentrations (cf. columns 1, 5 and 3, 7 in Fig. 2a–c, e–g) under normal RZT, and elevated RZT was essential to induce root *IPT* gene expression (Fig. 1) to increase CK concentrations (Fig. 2) of *HSP70::IPT* plants.

In all plant compartments measured, elevated RZT caused higher CK concentrations in *HSP70::IPT* plants than corresponding WT plants (cf. columns 2, 6 and 4, 8 in Fig. 2). However, exposure of WT plants to elevated RZT usually increased the bioactive CK concentration in leaves (cf. columns 5, 6 of Fig. 2a, e, b, f) but not roots (Fig. 2d, h), perhaps due to increased xylem CK delivery, since xylem CK concentration was sometimes increased (Fig. 2g) while stomatal conductance was apparently unaffected by elevated RZT (Fig. 7d). Further detailed measurements of the temporal responses of xylem CK concentration, stomatal behaviour, and native *IPT* root gene expression in WT plants following exposure to elevated RZT are necessary to substantiate this hypothesis.

Although elevated RZT increased shoot CK concentrations of control WT plants, it decreased leaf 3 area, and shoot and (slightly) root biomass of control WT plants (cf. columns 1, 2 of Fig. 5e, f, g), and also leaf 3 area of salinized WT plants (cf. columns 3, 4 of Fig. 5e). This shoot growth inhibition was not mediated by increased leaf ABA concentration (cf. columns 5, 6 of Fig. 3a, b, e, f); instead ABA concentrations decreased (up to 30%) in young leaves (Fig. 3a, b) consistent with a role for ABA in maintaining tomato shoot growth (Sharp *et al.*, 2000; Dodd *et al.*, 2009). Alternative explanations for shoot growth inhibition by elevated RZT include decreased shoot water status (He *et al.*, 2001) and/or increased production of the growth-inhibitory plant hormone ethylene (Qin *et al.*, 2007). Thus it was desirable to minimize plant exposure to elevated RZT. However, elevated RZT did not inhibit leaf and shoot growth (Fig. 5b, c) of control *HSP70::IPT* plants, probably due to increased CK concentrations (Fig. 2a, b, e, f) stimulating cell division and expansion, and thus compensating negative impacts of elevated RZT. Instead, inhibitory effects of elevated RZT on *HSP70::IPT* plants were restricted to the roots of non-salinized plants (cf. columns 1, 2 of Fig. 5d) which had 15% less biomass, consistent with previously reported root growth inhibition by exogenous (Bertell and Eliasson, 1992) and endogenous CKs (Werner *et al.*, 2001). In contrast, shoot-localized *IPT* expression stimulated root biomass (*SARK::IPT*; Rivero *et al.*, 2007), length, and number (*SAG12::IPT*; Xu *et al.*, 2009) by up to 3-fold under drought and heat stress, respectively. This is unlikely to be a direct promotion of root growth by additional root CKs, but rather an indirect effect of greater photoassimilate availability in the roots due to delayed canopy senescence.

The stimulation of CK synthesis of *HSP70::IPT* plants by elevated RZT resulted in concentrations similar to those reported in WT tomato xylem sap (Kudorayova *et al.*, 2007) and *IPT*-transformed tomato shoots (Groot *et al.*, 1995), but higher (~10-fold) than reported in WT tomato leaves (Walker and Dumbroff, 1981; Rahayu *et al.*, 2005; Kudorayova *et al.*, 2007). Absolute CK concentrations vary according to plant developmental stage, tissue sampled, and plant nutrition. The magnitude of CK accumulation in *HSP70::IPT* plants depended on salinity treatment (cf. columns 2, 4 in Fig. 2): it was greater in leaves under control than saline conditions (Fig. 2a, b, e, f), similar in both control and saline conditions in xylem sap (Fig. 2c, g), and greater in roots under saline than control conditions due principally to an accumulation of CK ribosides (Fig. 2d, h). Paradoxically, the higher root CK concentrations of salinized *HSP70::IPT* plants after elevated RZT did not cause additional root growth inhibition (Fig. 5d), perhaps due to the reduced levels of the free forms *t*Z and iP (Fig. 2d, h). In spite of an apparent doubling of shoot CK concentration (cf. columns 1, 2 of Fig 2a, b, e, f) in non-salinized *HSP70::IPT* plants, shoot growth was independent of RZT treatment (Fig. 5b, c) either since absolute CK concentrations were not growth limiting or due to negative impacts of elevated RZT. However, additional shoot CKs increased leaf expansion 1.5-fold (Fig. 5b), shoot growth 1.9-fold (Fig. 5c), and maximum efficiency of PSII (F_v/F_m) (Fig. 7a, b) in salinized plants, while only F_v/F_m increased in control plants (Fig. 7a). Indeed, the impacts of root-localized *IPT* expression on shoot CK concentrations under stress were of a similar magnitude to the improved growth rate. Thus the average 2- to 2.5-fold increase in shoot biomass and relative growth rate (Figs 5c, 6) was associated with a 2- to 2.6-fold increase in leaf CK concentration (cf. columns 3, 4 of Fig. 2a, b, e, f), while the 4.5-fold increase in biomass of droughted tobacco was associated with up to a 5-fold increase in leaf CK concentration (*SARK::IPT*; Rivero *et al.*, 2007). However, depending on the promoter used and the growing conditions, it generally seems that the physiological impacts of overcoming decreased CK status (via transgenic *IPT* expression) were most pronounced in plants experiencing abiotic stress.

The attenuated growth inhibition of salinized *HSP70::IPT* plants exposed to elevated RZT may be via the relative maintenance of cytokinin-mediated cell division and cell wall extensibility (Rayle *et al.*, 1982) and/or improved carbon status due to both delayed stomatal closure and leaf senescence (Fig. 7a–d). However, other hormonal or ionic changes may also be involved. Salinized *HSP70::IPT* plants exposed to elevated RZT also had lower ABA concentrations in mature leaves, xylem, and roots (cf. columns 3, 4 of Fig. 3b–d, f–h), which may

directly delay stomatal closure (Fig. 7c, d) and/or decrease antagonism of CK-dependent stomatal opening (Dodd, 2003), allowing more photosynthesis. A lower ABA concentrations in photosynthetically active leaves may also indirectly delay senescence, photoinhibition, and photooxidation during the osmotic phase of salinity (Ghanem *et al.*, 2008). However, decreased ABA concentration, perhaps mediated by decreased foliar Na^+ concentrations (Montero *et al.*, 1998), is unlikely to explain increased growth directly, since ABA-deficient mutants grew less than WT plants when salinized (Mäkelä *et al.*, 2003; Mulholland *et al.*, 2003). Furthermore, leaf ABA concentrations showed limited (up to 1.5-fold) variation with salinity treatment in young (actively growing) *HSP70::IPT* leaves (Fig. 3a, e), in contrast to the larger variations (up to 3-fold) in leaf CK concentrations, which seem directly responsible for the improved shoot growth of induced *HSP70::IPT* plants under salinity.

However, increasing shoot levels of bioactive CKs also significantly increased leaf and root K^+ (control and saline conditions; cf. Fig. 4g–l) and decreased Na^+ concentrations (only under salinity, Fig. 4a–f) of *HSP70::IPT* plants compared with WT plants. To the best of our knowledge, this is the first demonstration that modulating CK biosynthesis alters K^+ and Na^+ concentrations, contributing to improved crop salt tolerance. Electrophysiological studies with excised barley roots demonstrated that exogenous (kinetin) application directly increased root cell plasmalemma K^+ uptake (Shabala *et al.*, 2009), suggesting a potential mechanism for the positive zeatin–K^+ correlation seen in rootstock-mediated improvement of tomato salt tolerance (Albacete *et al.*, 2009). The decreased Na^+ concentrations of salinized, induced *HSP70::IPT* plants (30–40 mM less than WT plants and *HSP70::IPT* plants grown at normal RZT) may minimize or delay the toxic Na^+ effect (Ghanem *et al.*, 2008; Munns and Tester, 2008), perhaps due to dilution of Na^+ ions by additional growth. This idea is supported by the correspondence of shoot growth improvement and decrease in leaf Na^+ concentration (Fig. 6). Although xylem Na^+ concentrations were not assayed, a higher delivery of Na^+ from root to shoot due to enhanced transpiration (Fig. 7) may explain the decreased root Na^+ concentrations (Fig. 4c) despite no additional root growth (Fig. 5d). This may also suggest direct (gene expression) or indirect (membrane potential, energy availability) effects on selective ion transport systems. Indeed, decreased Na^+ concentration may occur via selective K^+/Na^+ uptake, as reported in several isolated tissues (Sastry *et al.*, 1973), and/or increasing the exclusion efficiency of the Na^+/H^+ antiporters by providing more energetic substrates from the shoot (Malagoli *et al.*, 2008). Although rootstock-influenced leaf xylem CK and Na^+ concentrations were not correlated across a range of rootstocks (Albacete *et al.*, 2009), further exploration of the role of CKs in controlling root cell ion transporter expression or activity seems warranted.

Another technique for localizing root *IPT* expression, that avoided dynamic changes in RZT, was used in longer term experiments. Grafting WT plants onto a constitutively expressing *IPT* rootstock increased fruit yield (by 30%) compared with salinized WT/WT plants, probably due to increased shoot development (as suggested by the 25% increase in fruit number) and the higher *tZ* concentrations (1.5- to 2-fold) in the actively growing fruits (Table 2) promoting cell division and expansion, thus slightly increasing (by 5%) fruit weight. In contrast, transient elevated fruit CK concentrations (up to 9-fold) very early in tomato fruit development did not increase final fruit size or total fruit yield per plant in the absence of stress (*AGPase::IPT*; Luo *et al.*, 2005). However, *IPT* expression in senescing leaves increased individual tomato fruit weight by 20% (*SAG12::IPT*; Swartzberg *et al.*, 2006), probably due to an increased sink assimilate import because of delayed source leaf senescence. Shoot growth maintenance (induced *HSP70::IPT* plants) and increased fruit production (WT/*35S::IPT* plants) under salinity may result not only from an increased source capacity to produce and/or export more assimilates due to both increased leaf area and delayed senescence, but also from an increased import and/or utilization of assimilates in sink organs achieved by an increased cell population attracting photoassimilates and/or an increased capacity to utilize imported sucrose metabolically (Roitsch and González, 2004). Thus topical (i.e. to the surface) application of exogenous CK (kinetin 10^{-5} M) increased sink activity (radiolabelled sucrose transport) of developing salinized fruits (Albacete, 2009). This idea is also supported by functional approaches that decreased CK concentration and shoot growth of tobacco by overexpressing the CK-degrading enzyme cytokinin oxidase; those plants had lower shoot sink activities due to both decreased cell number in leaf meristems (Werner *et al.*, 2001) and sucrolytic activities (Werner *et al.*, 2008). Similarly, growth inhibition of CK receptor- (Nishimura *et al.*, 2004) and CK-activating enzyme- (Kurakawa *et al.*, 2007) defective mutants was associated with lower meristematic activity, confirming a key role for a threshold CK concentration or sensitivity in regulating plant growth, which seems critical under abiotic stress.

Conclusion

Although induced root *IPT* gene expression could restrict root growth, increased root-to-shoot CK transport improved salt tolerance by increasing vegetative and fruit growth and also delaying leaf senescence and maintaining stomatal conductance and PSII efficiency, thereby avoiding or delaying the accumulation of toxic ions (Ghanem *et al.*, 2008; Munns and Tester, 2008; Albacete *et al.*, 2009). Expressing *IPT* in the roots also decreased the (fruit) yield penalty caused by salinity, and may allow a more efficient use of saline water (3–6 dS m^{-1} is typical of aquifers used to abstract water for semi-hydroponic tomato culture in southern Spain). While additional tools to regulate root cytokinin production spatially and temporally, alone, or in combination with other hormones, may provide additional crop improvement possibilities, it will be important to

determine whether this approach is valuable in ameliorating salinity-induced growth inhibition of other species grown in other cropping systems.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Total cytokinin (CK) levels in young leaves, leaf 3, and roots of *HSP70::IPT* and WT tomato plants grown in half-strength Hoagland medium in the absence (Ctrl) or presence (Salt) of 100 mM NaCl, and transiently exposed to elevated (RZT) or normal (Norm) root zone temperature.

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