Overproduction of ABA in rootstocks alleviates salinity stress in tomato shoots

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Abstract

To determine whether root-supplied ABA alleviates saline stress, tomato (Solanum lycopersicum L. cv. Sugar Drop) was grafted onto two independent lines overexpressing the SlNCED1 (9-cis-epoxycarotenoid dioxygenase) gene (NCED OE) and wild type rootstocks. After 200 days of salinity irrigation (EC = 3.5 dS m-1), plants with NCED OE rootstocks had 30% higher fruit yield, but root biomass and lateral root development was reduced. Although NCED OE rootstocks upregulated ABA-signalling (AREB, ATHB12), ethylene-related (ACCs, ERFs), aquaporin (PIPs) and stress-related (TAS14, KIN, LEA) genes, downregulation of PYL ABA receptors and signalling components (WRKYs), ethylene synthesis (ACOs) and auxin responsive factors occurred. Elevated SlNCED1 expression enhanced ABA levels in reproductive tissue while ABA catabolites accumulated in leaf and xylem sap suggesting homeostatic mechanisms. NCED OE also reduced xylem cytokinin transport to the shoot and stimulated foliar 2-isopentenyl adenine (iP) accumulation and phloem transport. Moreover, increased xylem gibberellin GA3 levels in growing fruit trusses was associated with enhanced reproductive growth. Improved photosynthesis without changes in stomatal conductance was consistent with hormone-mediated alteration of leaf growth and mesophyll structure, which combined with lower assimilate requirement in the roots and systemic changes in hormone balances could explain enhanced vigour, reproductive growth and yield under saline stress.

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Abstract

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Keywords

Abscisic acid, 9-*cis* -epoxycarotenoid dioxygenase, plant hormones, root gene expression, salt stress, rootstocks, tomato (*Solanum lycopersicum*).

Introduction

Limited water availability is a shared component of drought and salinity stresses that constrains crop growth and yield. Additionally, salinity stress limits plant growth and agricultural productivity through nutritional imbalance and ion toxicity. Roots sense their environment, triggering transcriptomic and biochemical responses that allow the plant to adapt to such conditions through local and systemic responses, with hormones playing a key role in such adaptive responses (Achard *et al.* 2006). Root-targeted alteration of hormone metabolism and signalling has been proposed as a biotechnological strategy to overcome the effects of saline soils, and to enable this we must understand the specific adaptive roles of plant hormones (Ghanem *et al.* 2011b; Albacete, Martínez-Andújar & Pérez-Alfocea 2014).

Crops dynamically regulate their root system architecture (RSA) in response to environmental stresses to fulfil their mineral and water requirements. In dry and saline soils, plants reduce lateral root initiation and elongation while promoting root hair density and the growth of the primary root to reach deeper water and nutrient sources (Xiong, Wang, Mao & Koczan 2006; Ma *et al.* 2010; Brown *et al.* 2012; Xu *et al.* 2013). Depending on the level of salt tolerance of the plant species or genotype, low-moderate salinity (2-8 dS m^{-1}) can promote root growth while high salt levels (8-16 dS m^{-1}) restrict root development (Julkowska & Testerink 2015).

Among the different plant hormones, tissue-specific ABA levels (and responses) change dynamically according to developmental and environmental stimuli. Although ABA is generally considered to inhibit growth of well-watered plants, low ABA concentrations (< 1 μM) can stimulate root growth of Arabidopsis (Ephritikhine,

Fellner, Vannini, Lapous & Barbier-Brygoo 1999; Fujii, Verslues & Zhu 2007). Moreover, wild-type (WT) ABA levels are necessary to sustain root growth in maize seedlings grown under low water potential (Sharp & LeNoble 2002), and for leaf expansion and shoot development in tomato (Sharp, LeNoble, Else, Thorne & Gherardi 2000) and Arabidopsis (LeNoble, Spollen & Sharp 2004) under well-watered conditions. ABA may stimulate growth by restricting the biosynthesis of ethylene, a growth inhibitor (reviewed in Sharp et al., 2004). Within the roots, ABA alters gene expression that induces changes in RSA (Sharp *et al.* 2004), increases root hydraulic conductivity (Thompson *et al.* 2007a), modifies nutrient and ionic transport and changes primary metabolism leading to osmotic adjustment (Sharp & LeNoble 2002; Martínez-Andújar*et al.* 2020b).

ABA is seemingly exported to the shoot as a root-to-shoot signal, since plants growing in dry or saline soil can show stomatal closure before shoot water status (the trigger for ABA accumulation) begins to decline (Gowing, Jones & Davies 1993; Dodd 2005). Stress-induced increases in xylem sap ABA concentration are independent of shoot water status and often inversely correlated with stomatal conductance (g_s) (Wilkinson & Davies 2002). However, experiments with reciprocal grafts of ABA-deficient and WT plants showed that stomatal closure of WT scions in response to dry (Holbrook 2002) or saline (Li, de Ollas & Dodd 2018) soil was rootstock independent. Instead, roots in drying soil alkalise xylem sap causing a redistribution of existing pools of ABA within the leaf that affects stomatal closure (Wilkinson, Corlett, Oger & Davies 1998), and other non-ABA chemical signals such as sulphate (Malcheska *et al.* 2017) or jasmonic acid (De Ollas, Arbona, Gómez-Cadenas & Dodd 2018) may also be involved. ABA detected in the root system may either be synthesized locally or translocated from the shoot via the phloem (McAdam, Brodribb & Ross 2016), and ABA can recirculate between roots and shoots, with roots either acting as a sink for ABA or as a net exporter of ABA to the shoot, depending on plant nutrient and water status (Peuke 2016).

Genetically increasing endogenous ABA levels is a promising strategy to improve resistance to abiotic stresses such as drought and salinity. The enzyme 9-*cis* -epoxycarotenoid dioxygenase (NCED) is rate-limiting for ABA biosynthesis, and over-expression of *NCED* genes increased ABA content of tissues, as first shown in tobacco and tomato by overexpressing the tomato gene *SlNCED* (Thompson *et al.*2000, 2007a b). This work provided transgenic tomato lines with different levels of expression of *SlNCED1* and ABA contents (SP12 and SP5) and offers the opportunity to study the effects of high ABA on root-to-shoot communication. In previous reciprocal grafting experiments between WT, SP12 and SP5, ABA in xylem sap collected from detopped roots was mainly determined by the root genotype, as might be expected in the absence of the shoot. Also, root cultures (again independent of the shoot) of SP12 and SP5 had higher ABA content that WT, thus overexpression of *SlNCED1* was sufficient to increase ABA biosynthesis in the root alone (Thompson *et al.* 2007b), despite the much lower level of NCED substrate available in roots compared to leaves (Taylor, Sonneveld, Bugg & Thompson 2005). In contrast, stomatal conductance in well-watered reciprocal grafting experiments was significantly affected only by the shoot genotype (Thompson *et al.* 2007b). Overexpression of NCED has now been explored in many systems, and its limiting effect on stomatal conductance confers improved water use efficiency (WUE) (Thompson *et al.* 2007a) and resistance to terminal drought (withdrawal of irrigation in pot experiments). The latter effect, e.g. in tobacco (Qin & Zeevaart 2002), grapevine (He *et al.* 2018), and petunia (Estrada-Melo, Ma, Reid & Jiang 2015) is dominated by the lower transpiration rate and slower soil moisture depletion. NCED overexpression also increased growth relative to WT under osmotic stress (NaCl, mannitol) in tobacco (Zhang, Yang, Lu, Cai & Guo 2008) and improved transpiration and reduced chloride accumulation in Arabidopsis grown in "a 150 mM chloride dominant solution" (Zhang, Yang, You, Fan & Ran 2015). However, the effect of rootstocks overexpressing NCED on plant growth and yield responses to saline soil has not been investigated.

ABA interacts with other hormones to mediate local and systemic stress responses (Sah, Reddy & Li 2016): it antagonizes the growth inhibitory effects of ethylene production in tomato shoots (Sharp *et al.*2000), Arabidopsis shoots (LeNoble *et al.* 2004), and maize roots (Spollen, Lenoble, Samuels, Bernstein & Sharp 2000), and also during grain-filling in wheat (Yang, Zhang, Liu, Wang & Liu 2006). Moreover, root-supplied ABA from WT rootstocks was sufficient to revert xylem ACC concentrations, foliar ethylene production and leaf area of ABA-deficient scions (Dodd, Theobald, Richer & Davies 2009). However, night-time maize leaf

expansion of water-stressed plants did not appear to be regulated by either ABA or ethylene (Voisin *et al.* 2006), but probably by more complex hormone interactions.

Many hormones (ABA, ethylene, JA and brassinosteroids) modify the development of RSA in saline stress conditions (Achard *et al.*2006; Osmont, Sibout & Hardtke 2007; Zolla, Heimer & Barak 2010; Duan*et al.* 2013; Geng *et al.* 2013). The integration of auxin and cytokinin antagonistic mechanisms might be mediated by gibberellins, because auxin induces degradation of DELLA proteins and enhances cell cycle activity, whereas gibberellins limit the growth inhibition mediated through cytokinin (reviewed in Petricka et al., 2012). Although salinity leads to root, xylem and leaf ABA accumulation in tomato (Albacete, Martínez-Andújar, Pascual, Acosta & Pérez-Alfocea 2008b; Li*et al.* 2018), it is not clear whether it directly controls plant responses, since other hormonal factors (such as ethylene precursor ACC and the ratio ACC/ABA) co-varied with the productivity (biomass), photosynthetic parameters and WUE (Cantero-Navarro *et al.* 2016). These two root-derived hormones were positively (ABA) or negatively (ACC) correlated with productivity in a salinized population of plants in which a common scion was grafted onto rootstocks representing a recombinant inbred line population from the cross *S. lycopersicum*× *S. cheesmaniae* (Albacete *et al.* 2009).

Grafting is a common commercial practice in many woody and herbaceous horticultural species (Albacete *et al.* 2014), and easily applied in the field. Tomato is one of the most important economic crops in the world and it is commonly propagated by grafting high productivity scions onto vigorous rootstocks to alleviate soilborne diseases and abiotic stress effects (Bletsos & Olympios 2008; Martínez-Andújar, Albacete & Pérez-Alfocea 2020a). Cultivated tomato is moderately tolerant to salinity with a threshold of tolerance of 2.5 dS m^{-1}but there is a subsequent yield loss of 10% for each unit of salinity increase (François & Maas 1994), which means that 30-40% yield losses due to salinity are quite common in many horticultural areas such as the tomato-producing region of Southeast Spain. Root-specific traits such as RSA, sensing of edaphic stress and root-to-shoot communication can be exploited to improve resource (water and nutrients) capture and plant development under resource-limited conditions. Root system engineering and rootstock breeding provides new opportunities to maintain sustainable crop production under changing environmental conditions. We hypothesises that grafting a commercial tomato cultivar scion onto ABA over-producing tomato rootstocks would enhance growth and yield under saline conditions, potentially through multiple local and systemic mechanisms.

Material and methods

Plant material, growth and grafting conditions

Two independent tomato transgenic lines, SP5 and SP12, in the genetic background of the wild-type (WT) cultivar Ailsa Craig (AC) (Thompson*et al.* 2007b) were used in this study as rootstocks of the commercial cherry variety Sugar Drop (SD, Unigenia Semillas, Murcia, Spain). SP5 and SP12 transgenic rootstocks constitutively overexpress the *SlNCED1* gene (Thompson *et al.* 2000), under the control of the Gelvin superpromoter (SP) and contain elevated ABA levels compared to WT, with SP5 accumulating more ABA than SP12 (Thompson*et al.* 2007a b). Since germination rates differed between genotypes, different sowing dates were used to synchronise development of the three genotypes: SP12 and SP5 seeds were sown one and two weeks before the WT, respectively, as described previously (Martinez-Andujar*et al.* 2020b). Seeds of the scion SD were sown 5 days earlier than AC seeds (12 days earlier than SP12 and 19 days earlier than SP5) to ensure equal stem diameters at grafting. For all genotypes, seeds were sown in commercial vermiculite, watered with deionized water and kept at 26-28oC and 80-90% relative humidity in the dark until germination. Grafting was performed using the splicing method at the two to three true leaf stages (3–4 weeks after sowing) where the scion was attached at the first node of the rootstock (Savvas *et al.* 2011). Grafting with the two transformants and the WT AC resulted in the following three graft combinations: SD/SP5, SD/SP12 and SD/AC.

One month later, when the grafted plants were well established, they were cultivated under commercial-like conventional plastic greenhouse conditions using a sand substrate during an autumn-winter season, in Almeria area (Spain). Fertilizers and water were supplied by a drip fertigation. From 10 days after

transplanting, a low salinity treatment with an electroconductivity (EC) of 3.5 dS m^{-1} was applied for a period of 200 days. Six plants per graft combination were randomly cultivated and distributed in blocks. After 130 days of salt treatment (DST), the second fully expanded mature leaf over the fourth truss (with actively growing fruits) of 6 plants per graft combination was assayed for various physiological parameters (described below), then detached to weigh and determine leaf area using an LI-3100AC area meter (LI-Cor, Lincoln, NE, USA). Plant stem diameter was also measured at the second node level using an electronic LCD digital vernier caliper (0-150 mm).

At the end of the experiment (200 DST), the shoot and root were detached and weighed to determine biomass. Young fully expanded leaves and young roots were immediately frozen in liquid nitrogen and stored at -80degC for hormonal and gene expression analysis.

Yield and exudate measurements

Phloem exudate was collected using the method described by Perez-Alfocea et al. (2000). The distal stem with the shoot apex and the two youngest expanded leaves were excised and the basal 2-3 cm immediately immersed in a 150 mL glass containing 30 mL of 20 mM EDTA (pH 6, adjusted with LiOH to avoid interactions with cation measurements). Each container with the plant material was placed in a plastic bag and hermetically sealed. The exudate was obtained by incubating the plant material for 20 h in the dark at room temperature.

Total yield was calculated using all the fruits collected from each plant during the harvest period. Fully ripe fruits were harvested weekly for two months. The truss length and fruit weight were also recorded in the 3rd truss. Fruit at green and mature stages were harvested for hormonal analysis. Leaf, root and truss xylem sap was obtained by applying a pneumatic pressure (between -0.6 and -0.7 MPa) to excised organs. Sap was collected with a pipette, immediately frozen in liquid nitrogen and stored at -80degC for hormonal analysis.

Photosynthesis and gas exchange measurements

Throughout the experiment (at 130, 163 and 180 DST), photosynthesis (A_N), stomatal conductance (g_s) and substomatal CO_2(Ci) were measured in the youngest fully expanded leaves (one leaf per plant) using a CIRAS-2 (PP Systems, Massachusetts, USA) between 09.00 h and 12.00 h (lights were turned on at 08.00 h). CO_2 was set at ambient levels (400 ppm) and radiation matched the chamber conditions (1500 µmol $m^{-2}s^{-1}$ PPFD). Intrinsic water-use efficiency (WUE_i) was calculated as the ratio between the values ofA_N and g_S .

Plant hormone extraction and analysis

The main classes of plant hormones, cytokinins [*trans-* zeatin (t-Z), zeatin riboside (ZR) and isopentenyladenine (iP)], gibberellin A3 (GA_3), indole acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), as well as the ABA catabolites, dihydrophaseic acid (DPA) and phaseic acid (PA) were extracted and analysed as described previously in Albacete et al. (2008) with some modifications. Fresh plant material (0.1 g FW of leaf or root) was homogenized in liquid nitrogen and incubated in 1 mL of cold (-20°C) extraction mixture of methanol/water (80/20, v/v) for 30 min at 4ºC. Solids were separated by centrifugation (20,000 g, 15 min at 4ºC) and re-extracted for another 30 min at 4ºC with 1 mL of extraction solution. Pooled supernatants were passed through Sep-Pak Plus C18 cartridges (previously conditioned with 3 mL of extraction buffer) to remove interfering lipids and some plant pigments. The supernatant was collected and evaporated under vacuum at 40ºC. The residue was dissolved in 1 mL methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA) and placed into opaque microcentrifuge tubes.

Ten µL of filtered extract (xylem, leaf or root) were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra wereobtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA,

USA). To quantify the plant hormones, calibration curves were constructed for each analysed component (0, 1, 10, 50, and 100 μg L^{-1}). ABA catabolites were identified by extracting the exact mass of the target catabolite from the full scan chromatogram obtained in the negative mode, adjusting a mass tolerance of [?] 1 ppm. The concentrations were semi-quantitatively determined from the extracted area of the derivative peak by using the calibration curve of ABA.

RNA isolation for real-time quantitative PCR and microarray hybridisation

Total RNA from frozen tomato roots (150 mg) was extracted using TRI-Reagent (Sigma-Aldrich, St Louis, MO, USA). Contaminating genomic DNA was removed by 20 min incubation at 37ºC with 4 units of DNase I (Thermo Fisher Scientific, Waltham, MA, USA). After DNase I inactivation at 70ºC for 15 min, RNA was ethanol-precipitated and resuspended in 30 mL of diethylpyrocarbonate (DEPC)-treated water.

First-strand cDNA synthesis and Real-time quantitative PCR

The expression of a set of ABA, stress, hormone and root-development related genes previously selected (Ferrández-Ayela *et al.* 2016; Martínez-Andújar *et al.* 2020b) was analysed in roots by real-time quantitative PCR (RT-qPCR). First strand cDNA was synthesised with one μg of purified RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA). The resulting cDNA was diluted by adding 40 μL of sterile distilled water.

Primers were designed to amplify 79 to 143 bp of the cDNA sequences as described previously (Ferrández-Ayela et al., 2016). To avoid amplifying genomic DNA, forward and reverse primers were designed to hybridize across consecutive exons, except in the case of *SlNCED1 gene* . RT-qPCR reactions were prepared with 5 μL of the SsoAdvanced SYBR Green Supermix (Bio-Rad, USA), 1 μM of specific primer pairs, 0.8 μL of cDNA and DNase-free water (up to 10 μL of total volume reaction). PCR amplifications were carried out in 96-well optical reaction plates on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Three biological and two technical replicates were performed per genotype and treatment. The thermal cycling program started with a step of 30 s at 95°C, followed by 40 cycles (5 s at 95degC, 10 s at 55degC and 20 s at 72degC), and a melt curve (from 65degC to 95degC, with increments of 1degC every 5 s). Dissociation kinetic analyses and agarose gel loading and sequencing of the PCR product was used to confirm its specificity.

Primer pair validation and relative quantification of gene expression levels were performed using the comparative Ct method (Schmittgen & Livak 2008). Data were represented as the relative gene expression normalized to the Ct value for the tomato housekeeping gene*SlACTIN2* (Solyc04g011500) as previously described (Ferrandez-Ayela et al., 2016). In each gene, mean fold-change values relative to the expression levels of WT were used for graphic representation. ΔCt values were analyzed using SPSS 21.0.0 (SPSS Inc., USA) by applying the Mann-Whitney U test for determining statistical differences between samples (P-value [?] 0.05).

Microarray hybridisation and data analysis

Four biological replicates per genotype were used for RNA extraction using the method described above. RNA (200 ng) was used for cDNA synthesis and Cy3-labelling using the Low Input Quick Amp Labelling Kit for One-Colour Microarray-Based Gene Expression Agilent analysis (Agilent, Santa Clara, CA, USA). Linearly amplified and labelled cDNA (1.65 μg) was hybridised for 17 h at 65ºC on 4 X 180 k format 60-mer oligonucleotide probes designed against the *S. lycopersicum* cv. Heinz 1706 build SL2.40 (annotation 2.3) genome (Agilent design ID = 069672; see Gene Expression Omnibus (GEO) record GPL21602). Each array contained ˜5 probes for 34,619 transcripts. Arrays were imaged using an MS200 microarray scanner using only the 480 nm laser using the autogain feature of the NimbleScan software (Roche NimbleGen, Madison, WI, USA). Image (tiff) files were imported into the Agilent Feature Extraction software for quality control assessment, grid alignment and expression value extraction at the probe and transcript level with the RMA algorithm (Irizarry *et al.* 2003) used to carry out background subtraction, quantile normalisation and summarisation via median polish, and output log2 normalised gene expression levels (GEO record GSE79307)(Ferrández-Ayela *et al.* 2016). Linear Models for Microarray Data (package LIMMA in R) was

then used to fit linear models to pairs of samples, identifying genes that contrasted the most between the experimental pairs (Smyth 2004). Transcripts were deemed to be differentially expressed if they showed a Benjamini-Hochberg adjusted$P$ *[?]* 0.05 when comparing rootstocks genotypes.

Leaf anatomy and scanning electron microscopy (SEM)

For mesophyll structure imaging, leaf sections samples were prefixed in 3% glutaraldehyde solution in 0.1 M cacodylate buffer (during 3 hours at 4degC), rinsed in 0.1 M cacodylate buffer and 0.1 M sucrose, then kept overnight. The next day, samples were fixed in 1% tetroxide (during 2 hours) and rinsed again in 0.1 M cacodylate buffer and 0.1 M sucrose and kept overnight. The fixed material was dehydrated with an acetone series (30%, 50%, 70%, 90% and 100%) for 10 minutes at each concentration. Samples were dried in the critical point dryer (LEICAEM CPD 030) and coated with gold, before being examined under SEM (JEOL-6100 model). Stomatal density and epidermal cell size were determined in the adaxial and abaxial surface of mature fully expanded leaves using SEM micrographs at 330x magnification.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) to test the main effects of genotype. Genotypic means were compared using Tukey's test at 0.05 of confidence level. All analyses were performed using SPSS for Windows (Version 22.0, SPSS Inc., Chicago, IL, USA).

Results

Plant growth, gas exchange and yield

To determine whether rootstock ABA overproduction can alleviate salt stress, two independent tomato transgenic lines, SP5 and SP12, in the genetic background of the wild-type (WT) cultivar Ailsa Craig (AC), as previously reported (Thompson *et al.* 2000), were used in this study as rootstocks of the commercial cherry variety Sugar Drop. At the end of the growing cycle (up to 200 days of irrigation with saline water), plants grafted onto NCED OE rootstocks had almost twice the leaf area, leaf and shoot biomass (shoot fresh weight; SFW), and stem diameter of plants grafted onto WT rootstocks (Figure 1a; Table 1). However, the root biomass of SP12 and SP5 rootstocks was 30% and 60% smaller than WT rootstocks, respectively (Table 1). Visually, these NCED OE grafts had less a complex root system architecture (the spatial configuration of a root system in the soil), than the WT (Figure 1c). Moreover, plants grafted onto NCED OE rootstocks had up to 20-30% increases in length and weight of the 3rd fruiting truss, fruit number, fruit weight and total fruit yield (Table 1; Figure 1b). Thus, NCED OE rootstocks promoted shoot (and fruit) growth at the expense of root system growth.

Plants grafted onto NCED OE rootstocks had higher photosynthesis rate (A_N) on certain measurement occasions, with similar g_s (Figure 2a, b) and transpiration (data not shown) to plants grafted on WT rootstocks. Accordingly, NCED OE rootstocks increased WUEi (Figure 2b). Electron microscopy revealed that leaves of scions grafted on SP12 rootstocks had altered leaf and mesophyll structure, with a more disorganized palisade and spongy cell layers (Figure 2c), and smoother and more elongated epidermis and trichome cells in the adaxial surface (Figure 2e; Table 2) than those grafted on WT rootstocks. Those differences could explain the lower sub-stomatal CO_2 concentration (Ci) in the leaves grafted onto the NCED OE lines (Figure 2d). The SP12 rootstock also seems to lead to fewer epicuticular wax crystals on both adaxial and abaxial leaf surfaces, without affecting stomatal density and aperture (Figure 2e; Table 2), supporting the lack of effect on g_s (Figure 2b) and transpiration. Thus, NCED OE rootstocks affected leaf structure and function.

Hormones

Since hormones mediate many physiological changes (Ghanem *et al.*2008; Albacete *et al.* 2008a), we profiled several root and shoot tissues and xylem and phloem exudates of grafted plants and measured their hormone levels (Figures 3, 4; Table S1).

Generally, NCED OE grafts had relatively few significant effects on ABA concentration in tissues and transport pathways compared to the WT rootstock (Figure 3a). Interestingly, the NCED OE rootstocks significantly increased ABA concentrations in the xylem sap of a flowering truss 180 days after transplanting, but those differences decreased during green fruit stage and disappeared at maturity stage. Moreover, mature fruit (juice) ABA concentration of plants grafted onto SP12 rootstocks was more than 2-fold higher than in plants grafted on WT rootstocks. Leaf phloem exudate ABA concentrations decreased in plants grafted on NCED OE rootstocks (Figure 3a). SP12 rootstocks had higher root and root xylem sap concentrations of the ABA catabolites phaseic acid (PA) and dihydrophaseic acid (DPA) respectively, with leaves of plants grafted on SP12 having higher DPA concentrations (Figure 3b). Thus, rootstock NCED OE had significant effects on ABA (and metabolites) concentrations only in some shoot tissues.

Plants grafted onto NCED OE rootstocks had lower total CKs (t -Z and iP type) in the xylem sap of roots and flowering truss, as well as in leaf tissue and green fruits mainly due to lower t -Z levels (Figure 4; Table S1). The different graft combinations had similar$t$ -Z and iP concentrations in leaf xylem sap and root tissues. However, iP type CK concentrations on leaf tissue (130 DST) and leaf phloem exudate were 5-14-fold higher in plants grafted on NCED OE rootstocks than on WT rootstocks, with iP the only hormone increasing in leaf phloem exudate (Figure 4; Table S1). Thus, rootstock NCED OE significantly affected CK concentrations in root xylem sap and shoot tissues.

Rootstock genotype also significantly affected auxin (IAA) and ethylene precursor (ACC) measurements. Leaf phloem exudate and root tissue ACC concentrations were 3-25 times lower in plants grafted on NCED OE rootstocks, while they had a higher ACC concentration in xylem sap of a mature fruit truss (Figure 4; Table S1). Leaf phloem exudate and xylem of mature fruit truss had up to 6-fold lower IAA concentrations when grafted on the SP5 rootstock (Figure 4; Table S1), otherwise there were no significant rootstock impacts on IAA levels. Similar to ABA, xylem sap of trusses at flowering and green-fruited stages had 7.5 to 4-fold more GA_3 when grafted on NCED OE rootstocks, with these differences disappearing at fruit maturity (Figure 4). However, leaf xylem GA_3 concentration of plants grafted on NCED OE rootstocks was 65-80% lower than when grafted on WT rootstock. Furthermore, root xylem JA concentration of plants grafted on SP5 was lower, even though plants grafted on NCED OE rootstocks had leaf JA concentrations that were more than twice that of plants grafted on WT rootstocks at 80 DST (Table S1); however, these differences disappeared at 130 DST (Figure 4). No significant rootstock differences in JA concentrations occurred in other tissues at the time points analyzed (Figure 4). The NCED OE rootstocks had few significant impacts on SA, except for 3-10 fold lower concentrations in leaf xylem and phloem exudates and a similar increase in ripe fruits (Figure 4, Table S1). Thus, NCED OE rootstocks also occasionally affected tissue and transport fluid concentrations of other acidic hormones.

Gene expression

To determine the molecular basis of the physiological changes, the same graft combinations were grown for 200 days and samples of roots taken for whole gene transcriptome profiling using microarrays. We also used RT-qPCR to confirm the expression of selected genes. More than 1300 transcripts were differentially expressed in NCED OE rootstocks, compared to WT. From this set, more than 850 were down-regulated, while almost 500 were up-regulated. A common set of 365 and 237 genes were down- and up-regulated in SP rootstocks, compared to WT grafts (Figure 5a, b; Table S2-S5). To interpret the gene expression data in a physiological context, we focused on analyzing DEG related to hormone pathways. We started by focusing on ABA-related genes because of the known role of *NCED.* Both PCR and transcriptomic data showed that*SlNCED1* gene expression was higher in SP5 than SP12 (Figure 6a; Table S2 and S3), confirming previous results (Thompson *et al.*2007a; Martinez-Andujar *et al.* 2020b).

Other ABA-metabolic genes were mostly not affected, corroborating their lack of differential regulation in roots of whole plants under control conditions (Martinez-Andujar *et al.* 2020b). *AREB1* (Solyc04g078840) and *ATHB12* (Solyc01g096320) were induced in SP12 and SP5 rootstocks respectively, while other ABA signalling-related genes *WRKY* s (e.g. *WRKY80/WRKY6* , Solyc03g095770) and ABA-receptor *PYL* s (e.g., *PYL6* , solyc05g052420) were down-regulated in the NCED OE grafts, indicating a reduced response/or

sensitivity to ABA compared to the WT (Figure 6a). Thus, increased *SlNCED1* gene expression altered some ABA signalling components, but there was no widespread effect on ABA signalling responses.

Regarding stress-related genes (Figure 6b; Table S2 and S3), the *TAS14* (Solyc02g084850), *KIN2* (Solyc03g095510) ,*LEA* (Solyc03g116390), *MYB49* (Solyc10g008700) and *MYB62* (Solyc03g119370) were upregulated in SP12 rootstocks, while most of those and other *MYB* genes were not affected or down-regulated in SP5 rootstocks (Figure 6a). Most aquaporin *PIP* genes analyzed were down-regulated in NCED OE rootstocks (Figure 6c), while *PIP1.7* (Solyc03g096290) in SP5 and *NIP6.1* (Solyc03g117050) in SP12 were upregulated (Figure 6c). Thus, increased *SlNCED1* gene expression either directly or indirectly generally decreased genes associated with response to stress and water transport.

Rootstock NCED overexpression seems to interact with other hormone-related genes in the roots. These rootstocks downregulated *IPT7* (Solyc01g080150), and a beta-glucosidase gene (Solyc03g119080) involved in biosynthesis of bioactive CKs (Figure 7a). The ACC synthase genes (*ACC2* , Solyc01g095080; *ACS1a* , Solyc08g081540) and most ethylene response factors (*ERF* s) genes were upregulated in both SP lines (Figure 7d). NCED OE rootstocks showed considerable changes in the ACC oxidase gene family, with 2 and 1 ACC oxidase genes upregulated in SP12 and SP5, respectively, while 13 ACC oxidase genes were downregulated in SP5 and 6 in SP12 (Figure 7d; Table S2 and S3). Thus, these results are consistent with NCED OE rootstocks having enhanced ACC synthesis, but with less conversion through to ethylene due to the majority of ACC oxidase genes being down-regulated, and more active ethylene signalling pathways. The rootstocks should have diminished CK biosynthesis.

SP12 rootstocks showed increased expression of genes involved in IAA conjugation (*IAAsGH3,* Solyc02g064830) but decreased expression of genes involved in IAA flux (*PIN9,* Solyc10g078370), along with the downregulation of most auxin responsive proteins (Figure 7e; Table S2 and S3). A gene involved in GA-deactivation (*GA2ox3* , Solyc01g079200 -qRT-PCR data) was downregulated in both NCED OE rootstocks. However, while a GA biosynthesis gene was upregulated only in SP12 (*GA20ox-2* , Solyc01g108870), four GA2oxidases (involved in GA deactivation) were induced only in SP5 (Figure 7b; Table S2 and S3). Thus, *SlNCED1* gene expression decreased auxin activity in the roots, while SP5 rootstocks showed greater changes in GA-related gene expression than SP12 rootstocks.

Transcriptomic data revealed that many JA-related genes in SP lines (*LOX, JA1* , *MJE* , *JAZ*) were downregulated, particularly in SP5 (Figure 7c; Table S2 and S3). RT-qPCR analysis revealed that *JA2* was also downregulated in SP5, but up-regulated in SP12, confirming the data obtained in the roots of whole NCED OE plants (Martinez-Andujar *et al.* 2020b).

Overall, the SP5 and SP12 lines showed some differential gene expression besides *NCED1* and *NCED2* genes, indicating differential ABA sensitivity and GA activity (reduced in SP5), and ethylene signalling and auxin activity (reduced in SP12). Those differences could potentially explain the reduced root growth of SP5 rootstocks compared to plants grafted on SP12 and WT rootstocks.

Discussion

Roots sense a complex soil environment and change their architecture and function to optimize resources and restore plant functional equilibrium. Rootstock-specific *SlNCED1* overexpression altered root ABA biosynthesis, shoot phenotypes and enhanced stress-tolerance, likely via multiple mechanisms including altered root-to-shoot signalling (Dodd, 2005; Perez-Alfocea *et al* . 2010). NCED OE rootstocks increased vegetative and reproductive growth, with enhanced xylem ABA concentrations in flower trusses and ABA catabolites (PA and DPA) in root, xylem sap and leaves (Figure 3) and diminished root system development (Figure 1; Table 1), although changes in root xylem ABA were more evident in younger vegetative stages (Figure S1). Thus root ABA biosynthesis and catabolism is not only enhanced, but ABA is exported to the shoots, although did not accumulate in most tissues analyzed. There are multiple changes in other hormone groups in many different tissues (Figure 4; Table S1), suggesting that *SlNCED1* plays a complex role in regulating growth. Thus, it is necessary to understand how NCED OE in the roots alters shoot phenotype through both local and systemic responses affecting root gene expression and root-shoot communication in the plant.

NCED OE rootstocks have reduced gene expression for ABA receptors and signalling components

Rootstock *SlNCED1* overexpression (Figure 6a) was consistent with transgene expression level in own-rooted plants (Thompson *et al.*2007b; Martinez-Andujar *et al.* 2020b), implying that shoot-to-root signalling has little effect on constitutive (root-specific in grafted plants) *SlNCED* expression. Although bulk root ABA status did not increase in the adult plants (Figure 3a), previously ABA in root exudates from approximately 7 week old detopped plants (Thompson *et al* . 2007a), in root cultures (Thompson*et al* . 2007b) and in bulk root tissue and xylem sap of younger ungrafted plants (Martinez-Andujar *et al* . 2020b) was elevated; in addition, in grafted plants the bulk root ABA was determined by the root genotype and was elevated in SP5 and SP12 (Thompson *et al.*2007b). Therefore, the lack of bulk root ABA accumulation in this study is consistent with increased export (Figure S1) and catabolism of ABA (Figure 3b).

Many genes were down- or up-regulated in NCED OE rootstocks compared to the WT grafts (Figure 5). Amongst those genes, 7 *PYL* ABA receptors and 3 *WRKY* factors were downregulated in NCED OE roots, suggesting decreased sensitivity to ABA, as in own-rooted plants grown in optimal conditions (Martinez-Andujar *et al.* 2020b). Several ABA *PYR/PYL* receptors are highly expressed in tomato roots compared to other tissues (Gonzalez-Guzman et al., 2014), allowing root system adaptation to low water potential including via modulation of osmoregulation and architectural changes (Sharp *et al.* 2004; Des Marais *et al.* 2012; Duan *et al.* 2013). For example,*PYL8* plays an essential role in regulating root ABA sensitivity in *Arabidopsis* (Antoni *et al.* 2013), promoting lateral root growth by enhancing *MYB77* -dependent transcription of auxin-responsive genes (Zhao *et al.* 2014). Loss-of-function of several *pyr/pyl* loci impaired ABA signalling, causing a robust ABA-insensitive root phenotype (Park *et al.* 2009; Gonzalez-Guzman *et al.*2014). Thus, downregulation of *PYL* s in NCED OE rootstocks may account for their limited root system development and sensitivity to saline stress.

Despite the proposed decreased ABA sensitivity, genes involved in ABA biosynthesis (*FLC/AAO,* Solyc07g066480) and signalling (*AREB* , Solyc04g078840*; ATHB12,* Solyc01g096320) were only slightly induced or not affected in either SP rootstock compared to WT. Furthermore, transcriptomic and RT-qPCR data of stress-related genes (*TAS14* , Solyc02g084850; *KIN2* , Solyc03g095510; *LEA,*Solyc03g116390 and some *MYB* s) indicate SP12 was more responsive than WT rootstocks to low saline stress, with an attenuated response in SP5. These different transcriptomic responses between SP rootstocks mimicked the responses of own-rooted plants grown under optimal conditions (Martinez-Andujar *et al.* 2020b), implying that each SP rootstock senses a different stress intensity that optimizes physiological responses according to both basal and NCED OE ABA levels.

Grafted plants with NCED OE rootstocks may have improved assimilate supply to the shoot

Reduced root growth of NCED OE rootstocks may be beneficial (Lambers, Atkin & Millenaar 2002; Lynch 2018), as excessive lateral root production increases sink competition for internal and external resources (primarily carbohydrates, but also water and nutrients) needed for root growth and exudate production. Decreased assimilate allocation to the root may also explain the greater vegetative growth and fruit yield of shoots grafted to NCED OE rootstocks (Table 1). Furthermore, scions grafted on SP12 rootstocks maintained photosynthetic activity under low salinity (Figure 2 a, b) without changingg_s , thereby increasing intrinsic WUE; this concurs with the previous observation in reciprocal grafting experiments under non-stressed conditions where g_s was only reduced when an NCED OE scion was present, and this had modest effects on A_N; NCED OE rootstocks had no effect ong_s (Thompson *et al.* 2007b).

Elevated ABA tissue concentrations, with or without environmental stresses, can promote developmental changes in stomata and leaf anatomy that mimic the effects of water deficit (Quarrie & Jones 1977; Franks & Farquhar 2001; Galmes *et al.* 2011). Enhanced cuticular wax deposition and changes in its composition can protect photosynthesis and water status (Ziv, Zhao, Gao & Xia 2018). In this study, grafting scions onto NCED OE rootstocks under salinity stress increased the elongation of leaf epidermal cells and reduced the number of cuticular wax crystals on leaf adaxial and abaxial surfaces (Figure 2e; Table 2). In agreement, autotetraploid Rangpur lime rootstocks with high ABA levels were compared with the diploid equivalent with

lower ABA levels: this showed a reduced expression for the wax synthesis *WAX2* gene in scions grafted to the high ABA autotetraploid rootstocks (Allario*et al.* 2013). These results contradict an earlier study in tomato using ABA-deficient mutants and exogenous application of ABA where there was a positive relationship between ABA level and wax deposition (Martin, Romero, Fich, Domozych & Rose 2017). The reduction in wax deposition due to NCED OE rootstocks could possibly be explained by a direct down regulation of wax synthesis pathways, or as a secondary effect where ABA alleviates salinity stress, allows greater leaf expansion and consequently a dilution of wax deposition or a mitigation of stress-induced wax synthesis.

Rootstocks can improve photosynthesis by affecting leaf structure to enhance mesophyll conductance to CO_2(g_m) (Fullana-Pericas, Conesa, Perez-Alfocea & Galmes 2020). Indeed, g_m was negatively correlated with sub-stomatal and/or ambient CO_2concentration under long-term stress (Flexas *et al.* 2012, 2013). Here, grafting onto NCED OE rootstocks disorganized laminar mesophyll structure (Figure 2c), which could explain the decreased *Ci*(Figure 2d) through enhanced CO_2 diffusion to the cells (Flexas *et al.* 2012, 2013).

Overall, NCED OE rootstocks may have improved tomato plant performance under low salinity via at least two mechanisms that improved assimilate supply for scion growth: i) altered ABA metabolism and signalling restricted root growth, consistent with reduced root sink strength, making more assimilate available for other sinks; ii) increasedA_N and decreased sub-stomatal CO_2associated with changes in leaf mesophyll structure would have increased assimilate supply.

NCED OE rootstocks alter cytokinin status in the scion and affect root-shoot signalling

Plants grown on NCED OE rootstocks had lower xylem sap concentrations of bioactive CKs in the leaves and in fruit trusses (Figure 4; Table S1), supporting the operation of an antagonistic interaction with ABA (Gawronska, Deji, Sakakibara & Sugiyama 2003; Ghanem *et al.*2011a; Peleg & Blumwald 2011), consistent with the finding that NCED OE rootstocks have downregulated expression of CK-metabolic genes (Figure 7a). Despite this inhibited root-to-shoot CK signalling, shoot-to-root CK signalling was activated with phloem iP concentrations increasing, possibly a putative signal to restore root CK status (Hirose, Takei, ... & 2008 2008; Matsumoto-Kitano *et al.* 2008). Moreover, changes in foliar iP accumulation in scions grafted on NCED OE rootstocks correlated with leaf area and A_N(r = 0.85 and 0.73; P [?] 0.01) and could also explain the changes in the leaf mesophyll structure, since this hormone preferentially accumulates in the leaf mesophyll and vascular bundles (Veselov *et al.* 2018). Indeed, both ABA and iP have been proposed as signalling components of the reticulate leaf phenotype in*Arabidopsis* , where there is altered mesophyll structure and reduced CO_2 fixation capacity (Lundquist, Rosar, Brautigam & Weber 2014). These results suggest that the iP/ABA-mediated mesophyll alteration is favoring CO_2 assimilation in this case, probably by facilitating its diffusion to the carboxylation sites into the cells (Flexas *et al.* 2012, 2013). Moreover, iP-type CKs have been related with xylem development and plant growth vigor and yield in tomato (Qi *et al.* 2020). Thus, ABA-CK interactions in rootstock-mediated improvement of the scion physiology require further investigation, especially since root-to-shoot CK-mediated plant vigor under salinity (Albacete *et al.* 2008a, 2009, 2014; Ghanem *et al.* 2011a) was associated with decreased ABA levels.

Ethylene-related responses in NCED OE grafted plants

ABA signalling enables plants to maintain shoot and root growth in both well-watered and droughted tomato (Sharp et al., 2000, 2004; Dodd et al. 2009) and *Arabidopsis* (LeNoble *et al.* 2004) plants by suppressing ethylene production (Sharp *et al.* 2000; Spollen*et al.* 2000; LeNoble *et al.* 2004). Surprisingly, NCED OE rootstocks upregulated genes for biosynthesis of the ethylene precursor ACC (*ACC2,* Solyc01g095080; ACS1a, Solyc08g081540) and ethylene signalling (several *ERFs*), while most genes responsible for the final step in ethylene biosynthetic genes (e.g. *ACCO,*Solyc07g049550; ACCO-like protein, Solyc12g006380) were down-regulated, especially in SP5 (Figure 7d). Yet, root and leaf phloem ACC concentrations were significantly reduced, as in own-rooted NCED OE plants (Martinez-Andujar *et al.* 2020b). Since diminished lower (lateral) root development in the NCED OE rootstocks is consistent with the phenotype of the ethylene overproducing mutant *epinastic*under control (Negi, Sukumar, Liu, Cohen & Muday 2010) and saline (Ortiz 2017) conditions, higher up-regulation of *ERF* s in SP5 rootstocks may be involved (Figure 7d). Whether

these local changes in ethylene response (and production) are involved in systemic signalling is less clear, as mature reproductive tissues of scions grafted on NCED OE rootstocks had increased ACC levels (Figure 4; Table S1). Overall, the differences existing between SP12 and SP5 lines suggest that complex ABA-ethylene interactions regulate root growth by altering ABA sensitivity and signalling, while long-distance ACC signalling cannot be ruled out.

NCED OE rootstocks mostly down-regulate auxin signalling

An antagonistic interaction between ABA and auxin modulates the lateral root developmental program in Arabidopsis (De Smet *et al.* 2003),*Medicago truncatula* (Gifford, Dean, Gutierrez, Coruzzi & Birnbaum 2008; Ariel, Diet, Crespi & Chan 2010) and peanuts (Guo, Chen, Wang, Xiao & Chen 2012). ABA induces root tip expression of the auxin transporter genes *AUX1* and *PIN2* , activating proton secretion, thereby promoting primary root elongation and root hair development under moderate water deficit (Xu *et al.* 2013). NCED OE rootstocks downregulated most auxin-responsive and auxin-induced genes (*ARFs* , *MYBs* , *SAURs*) and the auxin transporter*PIN9 (* Solyc10g078370), while upregulating the auxin deactivation gene *IAASGH3* (Solyc02g064830) in SP12 (qRT-PCR data) (Figure 7e), without changing root IAA concentration (Figure 4). These changes modulate stress-dependent ABA-auxin interactions, thereby decreasing lateral and main root development (Shkolnik-Inbar & Bar-Zvi 2010; Duan*et al.* 2013; Hong, Seah & Xu 2013; Song & Liu 2015; Ma *et al.* 2018) as observed in the whole plants under control conditions (Martinez-Andujar *et al.* 2020b).

Altered gibberellin metabolism in NCED OE rootstocks

In own-rooted plants, GA deactivation genes were induced in SP12 roots under control conditions, but moderate salinity alleviated this compared to WT plants (Martinez-Andujar *et al.* 2020b). Similarly, the*GA2ox-3* (Solyc01g079200) gene (GA deactivation) was downregulated in SP5 and SP12 rootstocks. The different interactions between the SP lines could be explained by the different effects on *ERF* s, since 6/9 genes were up-regulated in SP5 and only 3/9 in SP12. *ERFs*induce GA2oxidases to inactivate GAs, while ABA-dependent stabilization of DELLA proteins that inhibit GA signalling (Julkowska & Testerink 2015), would be alleviated in NCED OE grafts, probably due to the reduced ABA sensitivity. Interestingly, while this differential regulation may explain local root growth response, a systemic GA signal could also transferred to the scion since NCED OE rootstocks significantly increased xylem sap GA_3 concentrations in the fruit trusses, consistent with the elongated truss phenotype observed (Figure 1).

Jasmonic acid metabolism and signalling are repressed in NCED OE rootstocks

As in own-rooted plants (Martinez-Andujar *et al.* 2020b), NCED OE rootstocks downregulated the JA biosynthetic (*JA1* , Solyc05g007180*; LOX,* Solyc03g096460), JA conjugation (*MEJ* s, Solyc03g044820 and Solyc03g070380) and signalling (*JAZ, Solyc06g068930*) genes, to a greater extent in SP5 than SP12. Interestingly, the ABA-activated NAC transcription factor*JA2* , which promotes stomatal closure by inducing expression of the ABA biosynthetic gene *NCED1* and acts as a regulatory loop to monitor endogenous ABA status (Munoz-Espinoza, Lopez-Climent, Casaretto & Gomez-Cadenas 2015), was induced in SP12, but inhibited in SP5 rootstocks (RT-qPCR data, Figure 7c). Despite this negative JA-ABA interaction, transient foliar JA accumulation (1.3 fold higher at 80 DST, Table S1) was concurrent with reduced ABA accumulation in scions grafted on NCED OE rootstocks.

Conclusion

Grafting WT scions onto constitutively ABA-overproducing rootstocks produced local (root) and systemic (scion) responses mediated by root-shoot communication. Evidence that *SlNCED1* overexpression in rootstocks caused a change in ABA root-to-shoot signalling included increased ABA concentrations in scion reproductive tissues and increased ABA catabolites in leaves, but lower ABA in leaf phloem. ABA overproduction altered stress-mediated responses by: decreasing root expression of *PYL* ABA receptors; reduced auxin signalling (lower auxin concentration in leaf phloem and decreased root expression of auxin responsive factors); enhanced root expression of most ethylene signalling gene (*ERFs*); and decreased lateral root

development. Moreover, rootstock NCED overexpression down-regulated root expression of CK biosynthesis genes and reduced *t* -Z in root xylem sap and leaf, suggesting reduced CK transport from root to shoot. However, iP increased in the leaf and leaf phloem, potentially as part of feedback loop to restore CK homeostasis. The modified leaf growth and anatomy and associated increase in photosynthesis induced by NCED overexpression in rootstocks could be explained by the known actions of the iP and JA accumulating in the leaf and leaf phloem. Enhanced GA_3in truss xylem sap was consistent with the observed increases in truss length, weight and overall yield. Considering whole plant source-sink relationships, the stimulation of leaf photosynthesis and reduction in root assimilate requirements could explain the more productive scion phenotypes (vegetative vigour, truss length, fruit number and yield) when grafted on NCED OE rootstocks. Overall, NCED OE rootstocks may be of great value in generating plants with higher yields under abiotic stresses (Figure 8).

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Data availability statement

All raw and processed microarray data are openly available in the Gene Expression Omnibus (GSE79307) at*https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79307*.

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Figure legends

Figure 1 . Images of a mature leaf (**a**), the 2nd fruit trusses (**b**) and the root (**c**) from representative plants of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1} (equivalent to 35 mM NaCl) for 100 days under greenhouse conditions.

Figure 2 . Variation of net photosynthesis rate (A_N) after 130, 163 and 180 DST of tomato cv. Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1}(equivalent to 35 mM NaCl) (**a**). Net photosynthesis (A_N), stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i) of tomato cv. Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1} for 180 days under greenhouse conditions. Different letters indicate significant differences between graft combination (n =3, P [?] 0.05) (**b**). Scanning electron micrograph (SEM) of transverse sectioning of tomato leaf (300x) showing the differences in epidermis and mesophyll layers between cv. Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE line SP12 (SD/SP12) grown under 3.5 dS m^{-1} for 180 days under greenhouse conditions (**c**). Substomatal CO_2(Ci) of cv. Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1} for 180 days under greenhouse conditions (**d**). SEM visualization (330x) of adaxial (left) and abaxial (right) leaf surfaces of cv Sugar Drop grafted onto WT AC (SD/AC) and the NCED OE line SP12 (SD/SP12) grown under 3.5 dS m^{-1} for 180 days under greenhouse conditions (**e**).

Figure 3 . Abscisic acid (ABA) concentrations in mature fruit juice (180 DST), mature, green and flower truss xylem sap (180 DST), leaf (130 DST), leaf phloem (180 DST), leaf xylem sap (130 DST), root xylem sap (200 DST) and root (200 DST) of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under a 3.5 dS m^{-1} (equivalent to 35 mM NaCl) under greenhouse conditions. Different letters indicate significant differences between genotypes (n =3, P [?]0.05) (**a**). Dihydrophaseic acid (DPA) and phaseic acid (PA) concentrations in leaf (130 DST), root xylem sap (200 DST) and root (200 DST) of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE line SP12 (SD/SP12) grown under 3.5 dS m^{-1} under greenhouse conditions (**b**). * indicate statistically significant difference between graft combinations (n=3, P [?] 0.05).

Figure 4 . HeatMap of the variation of *trans* -zeatin (t -Z), isopentenyl adenine (iP), 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA), gibberellin A3 (GA_3), jasmonic acid (JA) and salicylic acid (SA) concentrations in mature fruit juice (180 DST), mature truss xylem sap (180 DST), green fruit juice (180 DST) green fruit xylem sap (180 DST), flower truss xylem sap (180 DST), leaf (130 DST), leaf phloem (180 DST), leaf xylem sap (130 DST), root xylem sap (200 DST) and root (200 DST) of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1} (equivalent to 35 mM NaCl) under greenhouse conditions. -1 and -2 indicate significant decrease at P [?] 0.05 and P [?] 0.01, respectively; 0 indicates not significant effects and +1 and +2 indicate significant increase at P [?] 0.05 and$P$ [?] 0.01, respectively. ND, not detected.

Figure 5 . Venn diagram showing the intersection of the differentially expressed genes identified in roots (**a**) and upregulated and downregulated genes in roots of SD/SP5 against SD/AC, SD/SP12 against SD/AC and SD/SP5 + SD/SP12 against SD/AC grown under 3.5 dS m^{-1} (equivalent to 35 mM NaCl) for 200 days under greenhouse conditions (**b**).

Figure 6 . ABA (**a**) stress (**b**) and aquaporin (**c**) related genes differentially expressed in root tissues comparing plants of SD/SP12 and SD/SP5 against SD/AC in response to 3.5 dS m^{-1} (equivalent to 35

mM NaCl) for 200 days under greenhouse conditions. Real time PCR quantification (RT-qPCR) of some ABA-related selected genes is also given.

Figure 7 . Cytokinin (CK)(**a**), gibberellin (GA) (**b**), jasmonic acid (JA)(**c**), ethylene (**d**) and auxin (**e**) related genes differentially expressed in root tissues comparing plants of SD/SP12 and SD/SP5 against SD/AC in response to 3.5 dS m^{-1}(equivalent to 35 mM NaCl) for 200 days under greenhouse conditions. Real time PCR quantification (RT-qPCR) of some ABA-related selected genes is also given.

Figure 8 . Proposed model to explain the performance of ABA overproducing rootstocks under salinity conditions. The improved growth and yield phenotype of the plants grafted onto NCED OE rootstocks can be explained through local (root) and systemic (scion) responses mediated by root-to-shoot communication. (**a**) At local level in the root, ABA overproduction seems to interfere with stress mediated response by decreasing root expression of ABA receptors (PYLs) and signalling components (*WRKYs*), thus altering sensitivity to ABA. The reduced ABA sensitivity in the roots appear to diminish auxin activity (ARFs, auxin transport from the shoot) and increase ethylene-related processes (*ERFs, ACCs*) leading to reduced RSA (mainly lateral roots). Inhibited *IPT* gene supports diminished CK synthesis in the rootstock and *t* -Z transport to the shoot. (**b**) At the systemic level in the scion, although root-to-shoot ABA signal has not been detected, a higher transport to the shoot cannot be ruled out (increased NCED expression and ABA catabolites). The increased foliar iP accumulation and phloem transport (in response to reduced *t* -Z transport from the roots) along with transitory increase of ABA and JA in leaf tissue seems to modify leaf growth and mesophyll structure leading to improved photosynthesis (A_N) activity. Moreover, the induced xylem GA_3 in growing fruits seems to enhance reproductive growth. Improved photosynthesis and reduced root growth lead to optimized source-sink relations in benefit of scion development and yield. Arrow and bar heads indicate positive and negative regulation, respectively.

Supplementary Figure legends

Figure S1 . Abscisic acid (ABA) concentrations in root xylem sap of tomato cv Ailsa Craig self-grafted (AC/AC) and grafted onto the NCED OE line SP12 (AC/SP12) (38- days old), cultivated hydroponically under control and salt conditions (100 mM NaCl) for 21 days. * indicates a significant difference between AC/AC and AC/SP12 within each treatment according to the Tukey test (P [?] 0.05).

Tables

Table 1 . Shoot fresh weight (SFW), mature leaf fresh weight (LFW), leaf area, stem diameter (SD), root fresh weight (RFW), RFW/SFW ratio, truss length (TL), 3rd truss fresh weight (TFW) and fruit yield of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5), grown under 3.5 dS m^{-1} (equivalent to 35 mM NaCl) after 130 (Leaf FW, Leaf area, SD and TL) and 200 (SFW, RFW and Total yield) DST (mean +- SE). Different letters indicate significant differences among graft combinations ($n = 6$, P [?] 0.05). P -values from ANOVA testing of the effect of the genotype on all parameters are shown.

	SD/AC	SD/SP12	SD/SP5	P (ANOVA)
SFW (g)	483.33±21.55 **c**	1105.00±65.00 **a**	848.00±88.23 **b**	<0.001**
Leaf FW (g)	9.22±0.85 **b**	25.88±1.77 **a**	28.31±2.73 **a**	<0.001**
Leaf area (cm^2)	150.49±13.21 **b**	418.66±32.53 **a**	421.03±31.64 **a**	<0.001**
SD (mm)	4.49±0.18 **b**	5.80±0.17 **a**	6.09±0.34 **a**	0.001**
RFW (g)	35.25±1.44 **a**	25.22±2.06 **b**	14.33±0.84 **c**	<0.001**
RFW/SFW	0.074±0.004 **a**	0.023±0.003 **b**	0.018±0.003 **b**	<0.001**
3rdTL (cm)	26.00±2.90 **b**	34.88±1.71 **a**	34.17±2.37 **a**	0.049*
3rdTFW (g)	237.42±13.81 **b**	342.27±33.29 **a**	343.19±29.62 **a**	0.014*
Yield (kg/plant)	1.14±0.12 **b**	1.83±0.33 **a**	1.57±0.12 **a**	0.050*

Table 2 . Stomatal density in adaxial and abaxial leaf surfaces and cell size in adaxial epidermis of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE line SP12 (SD/SP12), grown under 3.5 dS m^{-1} (equivalent to 35 mM NaCl) after 200 days of treatment (mean ± SE). *P* -values from ANOVA testing of the effect of the genotype on all parameters are shown.

		SD/AC	SD/SP12	*P* (ANOVA)
Stomatal density (n^o/mm^2)	Abaxial	125.67±8.67	120.67±5.81	0.657
	Adaxial	2.68±1.25	3.30±1.27	0.754
Cell size (adaxial epidermis)	Width (µm)	42.71±2.15	40.42±2.21	0.475
	Length (µm)	62.78±2.25	109.79±5.64	$<0.001^{**}$
	Area (μm^2)	2670.17±115.69	4402.10±115.69	$<0.001^{**}$

Supplementary Table legends

Tabla S1 . *Trans* -zeatin (*t* -Z), zeatin riboside (ZR), isopentenyl adenine (iP) 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA) gibberellin A3 (GA_3), jasmonic acid (JA) and salicylic acid (SA) concentrations in mature fruit juice (180 DST), mature truss xylem sap (180 DST), green fruit juice (180 DST) green fruit xylem sap (180 DST), flower truss xylem sap (180 DST), leaf (80 and 130 DST), leaf phloem (180 DST), leaf xylem sap (130 DST), root xylem sap (200 DST) and root (200 DST) of tomato cv Sugar Drop grafted onto the WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1}(equivalent to 35 mM NaCl) under greenhouse conditions (mean ± SE). Different letters indicate significant differences among graft combinations ($n = 3$, P [?] 0.05). * and ** indicate significant differences between SD/SP12 or SD/SP5 and SD/AC at P[?] 0.05 and P [?] 0.01, respectively. ND, not detected.

Table S2 . Differentially expressed genes (DEG), comparing SD/SP5 against SD/AC. The Log FC values are given with their mean relative expression level, the adjusted P values and B values.

Table S3 . Differentially expressed genes (DEG), comparing SD/SP12 against SD/AC. Log FC values are given with their mean relative expression level, the adjusted P values and B values.

Table S4 . Differentially expressed genes (DEG), comparing SD/SP5 and SD/SP12 against SD/AC. Log FC values are given with their mean relative expression level, the adjusted P values and B values.

Table S5 . Differentially expressed genes (DEG), comparing SD/SP12 against SD/SP5. Log FC values are given with their mean relative expression level, the adjusted P values and B values.