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 (NCED OE) overexpressing the *SlNCED1* gene (9-*cis*-epoxycarotenoid dioxygenase) and wild type rootstocks. After 200 days of saline irrigation (EC = 3.5 dS m^{-1}), plants with NCED OE rootstocks had 30% higher fruit yield, but decreased root biomass and lateral root development. Although NCED OE rootstocks upregulated ABA-signalling (*AREB,* ATHB12), ethylene-related (*ACCs, ERFs*), aquaporin (*PIP*s) and stress-related (*TAS14, KIN, LEA*) genes, downregulation of *PYL* ABA receptors and signalling components (*WRKYs*), ethylene synthesis (*ACO*s) 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 GA_3 levels in growing fruit trusses were associated with enhanced reproductive growth. Improved photosynthesis without changes in stomatal conductance was consistent with reduced stress sensitivity and hormone-mediated alteration of leaf growth and mesophyll structure. Combined with increases in leaf nutrients and flavonoids, systemic changes in hormone balance could explain enhanced vigour, reproductive growth and yield under saline stress.

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 (Brown *et al.* 2012; Xu *et al.* 2013; Koevoets, Venema, Elzenga & Testerink 2016; Li *et al.* 2021) 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). Phenotypic comparisons between wild-type (WT) and ABA-deficient mutants demonstrates that WT ABA levels are necessary to sustain primary 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).

Plants growing in dry or saline soil can show stomatal closure before shoot water status (the trigger for leaf ABA accumulation) begins to decline (Gowing, Jones & Davies 1993; Dodd 2005), coincident with root ABA accumulation and export to the shoot as a root-to-shoot signal (Zhang and Davies 1989; 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 de-topped 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). Lower transpiration rate and slower soil moisture depletion of these NCED-overexpressing lines maintains turgor of tobacco (Qin & Zeevaart 2002), grapevine (He *et al.* 2018), and petunia (Estrada-Melo, Ma, Reid & Jiang 2015) in drying soil. 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 1-aminocyclopropane-1-carboxylic acid (ACC)

concentrations and foliar ethylene production of ABA-deficient scions, while enhancing their leaf area (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 (Duan *et al.* 2013; Geng *et al.* 2013; Qin, He & Huang 2019; Vissenberg, Claeijs, Balcerowicz & Schoenaers 2020; Waidmann, Sarkel & Kleine-Vehn 2020) Gibberellins might mediate the integration of auxin and cytokinin antagonistic mechanisms, because auxin induces degradation of DELLA proteins and enhances cell cycle activity, whereas gibberellins limit cytokinin-mediated growth inhibition (reviewed in Petricka et al., 2012). Although salinity causes 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 the 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 commonly applied to many woody and herbaceous horticultural species in commercial practice (Albacete *et al.* 2014). Tomato is one of the most important economic crops in the world and 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 hypothesise 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 culture

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 (Martínez-Andújar *et al.* 2020b). Seeds of the scion SD were sown 5 days earlier than AC seeds (7 days earlier than SP12 and 14 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 three graft combinations: SD/SP5, SD/SP12 and SD/AC (Figure S1).

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 Almería area (Spain). Fertilizers and water were supplied by a drip fertigation. From 10 days after transplanting, a low salinity treatment with an electrical conductivity (EC) of 3.5 dS m^{-1} was applied for a period of 200 days (Figure S1). Six plants per graft combination were randomly cultivated and distributed in blocks.

Plant phenotyping

Throughout the experiment (after 130, 163 and 180 days of salt treatment, 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 of A_N and g_S.

After 130 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 above), 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 -80°C for hormonal and gene expression 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 -80°C for hormonal analysis. Phloem exudate was collected using the method described by Pérez-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 also harvested for hormonal analysis.

Nutritional, hormonal and flavonoid analysis

For ionome composition, leaves were dried for 48 h at 80◦C, milled to a powder and 200 mg dry tissue was digested with a HNO_3:HClO (2:1, v/v) solution. Samples were analyzed by using inductively coupled plasma spectrometry (ICP-OES, Thermo ICAP 6000 Series). Total C and N contents were determined in 200 mg of dry leaf material by the combustion method using an elemental analyser (LECO TRUSPEC, The Netherlands).

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)) and flavonoids (luteolin, taxifolin, genistein, quercetin and cyanidin) 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 (dihydrophaseic acid (DPA) and phaseic acid (PA)) and flavonoids (luteolin, taxifolin, genistein, quercetin and cyanidin) 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 peaks using the calibration curve of ABA (catabolites) or the total area (flavonoids).

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 95°C, 10 s at 55°C and 20 s at 72°C), and a melt curve (from 65°C to 95°C, with increments of 1°C 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 (Ferrández-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 $P \leq 0.05$ when comparing rootstocks genotypes.

The molecular pathways where differentially expressed genes were involved in the biosynthesis of plant hormones (Figure S2) and hormone signal transduction (Figure S3) were marked in the relevant KEGG pathways (Kanehisa & Goto 2000).

Leaf anatomy and scanning electron microscopy (SEM)

For mesophyll structure imaging, the third fully expanded mature leaf samples were prefixed in 3% glutaraldehyde solution in 0.1 M cacodylate buffer (during 3 hours at 4°C), 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.

Assay of root xylem ABA under salinity stress in grafted plants

In grafted plants with either WT (AC) or SP12 roostocks, the effect of salinity on ABA accumulation was investigated: 60-day old self-grafted WT plants (AC/AC) and WT scion grafted onto the rootstock of NCED OE line SP12 (AC/SP12) were cultivated for 21 days in 0.5 L pots using vermiculite as substrate and irrigated with ½ strength Hoagland nutrient solution alone (control) and supplied with 35, 70 and 100 mM NaCl (salinity). At the end of the experiment, root xylem sap ABA concentration was analyzed as described previously.

ABA sensitivity

Surface-sterilized (washed in 5% NaOCl) WT and SP12 seeds were germinated in Petri dishes containing 1/5 Hoagland nutrient solution supplemented with 10 g L^{-1} agar and 1% sucrose. Seedlings were transferred to culture medium supplied with 0, 1.5, 3 and 5 µM (+)-*cis, trans*-ABA (Sigma-Aldrich, USA) when the two cotyledons were developed (6 days for WT and 9 days for SP12). After 30 days of ABA treatment, main total root length was measured using WinRHIZO software (Pro 2016, Regent, Canada).

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, leaf nutrients 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 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, b). However, the root biomass of SP12 and SP5 rootstocks was 30% and 60% smaller than WT rootstocks, respectively (Figure 1b). 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 1a). 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 (Figure 1b). Thus, NCED OE rootstocks promoted shoot (and fruit) growth but reduced the root system growth.

Plants grafted onto NCED OE rootstocks had higher photosynthesis rate (A_N) on certain measurement occasions (Figure 2a), with similar g_s (Figure 2b) 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 1) 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 1), supporting the lack of effect on g_s (Figure 2b) and transpiration. Foliar C, N, P, K, Na, B and Zn concentrations did not differ between graft combinations, but plants grafted onto NCED OE rootstocks had increased S, Mg, Ca and Mn concentrations, but decreased Fe concentrations (Table 2). Thus, NCED OE rootstocks affected leaf structure, nutritional status and function.

Hormone accumulation

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

Generally, NCED OE grafts produced few significant effects on ABA concentrations in tissues and transport pathways compared to the WT rootstock (Figures 3a, 4). 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 PA and 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 few 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 roots sampled for whole gene transcriptome profiling using microarrays, with 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). While ethylene, flavonoid and carbon metabolism related genes were among the most up-regulated in NCED OE rootstocks, several proteases and peroxidases were particularly abundant among the down-regulated genes (Table 3).

To highlight any classes of genes that are over-represented in the differentially expressed genes, GO terms were searched for higher difference in the frequency between the differentially expressed transcripts and all the transcripts included in the microarray (Figure 5c). When comparing SP rootstocks to WT, differentially expressed genes were enriched in several classes, including serine type endopeptidases, defense response genes, oxygen binding, snoRNA binding, chlorophyll binding and glucuronosyltransferase activity (Figure 5c).

To interpret the gene expression data in a physiological context, we analysed DEGs related to hormone metabolism (Figure S2) and signalling (Figure S3) pathways, initially 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; Martínez-Andújar *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 (Martínez-Andújar *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). Additional experiments determined the sensitivity of root responses to salinity and ABA. Root xylem sap ABA accumulation of SP12 rootstocks grafted to WT scions increased under control conditions compared to the WT rootstocks (22.8 *vs* 5.8 ng ml^{-1}, $P<0.01$, respectively), but it was stable as salt concentrations increased from 35 to 100 mM NaCl (Figure 6b). However, stress-induced root xylem ABA accumulation in the rootstocks of WT self-grafted plants diminished as salt concentrations increased. Whereas root length of WT plants almost halved as exogenous ABA concentrations increased from 1.5 to 5 µM, SP12 root length increased with exogenous ABA concentration (Figure 6c). Thus, increased *SlNCED1* gene expression altered some ABA perception and signalling components, and reduced sensitivity to stress.

Regarding stress-related genes (Figure 7a; 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 7a). Most aquaporin *PIP* genes analyzed were down-regulated in NCED OE rootstocks (Figure 7b), while SP12 rootstocks upregulated *PIP1.7* (Solyc03g096290) in SP5 and *NIP6.1* (Solyc03g117050) (Figure 7b). Both NCED OE rootstocks upregulated two genes involved in flavonoid synthesis, a flavanone 3-hydroxylase-like protein (Solyc03g080190) and a flavonoid oxidoreductase (cytochrome P450, Solyc03g111290) (Table 3, Figure 7c). To investigate whether other upregulated genes in the root affect leaf metabolites, flavonoids were analyzed in root xylem sap and leaves of grafted plants (Figure 7d). Luteolin and cyanidin concentrations increased in xylem sap and leaves of plants grafted on the SP12 rootstock, with no significant differences in taxiolin, genistein and quercetin concentrations. Thus, increased *SlNCED1* gene expression either directly or indirectly generally decreased genes associated with response to stress and water transport, but increased flavonoid biosynthetic genes.

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 8a). While SP12 upregulated a GA biosynthesis gene (*GA20ox-2*, Solyc01g108870), SP5 upregulated four GA2oxidases that are involved in GA deactivation (Figure 8b; Table S2 and S3). Furthermore, both NCED OE rootstocks downregulated a gene involved in GA-deactivation (*GA2ox3*, Solyc01g079200 - qRT-PCR data). Transcriptomic data revealed

that many JA-related genes in SP lines (*LOX, JA1*, *MEJA*, *JAZ*) were downregulated, particularly in SP5 (Figure 8c; 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 (Martínez-Andújar *et al.* 2020b).

Both NCED rootstocks upregulated the ACC synthase genes (*ACC2*, Solyc01g095080; *ACS1a*, Solyc08g081540) and most ethylene response factors (*ERF*s) (Figure 9a; Table 3). SP12 and SP5 rootstocks upregulated 2 and 1 ACC oxidase genes respectively, but downregulated 6 and 13 ACC oxidase genes respectively (Figure 9a; Table S2 and S3). SP12 rootstocks 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 9b; Table S2 and S3).

Overall, these results are consistent with NCED OE rootstocks having enhanced ACC synthesis and ethylene signaling pathways, but with less conversion to ethylene as the majority of ACC oxidase genes were down-regulated. Moreover, *SlNCED1* gene overexpression decreased root auxin activity, while SP5 rootstocks showed greater changes in GA-related gene expression than SP12 rootstocks. The NCED OE rootstocks should have diminished CK biosynthesis.

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; Pérez-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 roots, root xylem sap and leaves (Figure 3) and diminished root system development (Figures 1, 6c). However, changes in root xylem ABA were more evident in young vegetative plants and diminished with salt stress, compared to the WT (Figure 6b; Martínez-Andújar *et al.* 2020b). Although root ABA biosynthesis and catabolism is enhanced and ABA is exported to the shoots, it did not accumulate in most tissues analyzed. Alternatively, multiple changes in other hormone groups in many different tissues (Figure 4; Table S1) suggest 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.

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; Martínez-Andújar *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 fruiting plants (Figure 3a), previously ABA in root exudates from approximately 7 week old de-topped plants (Thompson *et al.* 2007a), in root cultures (Thompson *et al.* 2007b) and in bulk root tissue and xylem sap of younger ungrafted plants (Martínez-Andújar *et al.* 2020b) was elevated. Moreover, bulk root ABA concentration of grafted plants was determined by the root genotype and increased in SP5 and SP12 (Thompson *et al.* 2007b), as in the root xylem sap prior to stress (Figure 6b). Therefore, the lack of bulk root ABA accumulation in this study is consistent with increased root export (Figures 3a, 6b) and catabolism of ABA (Figure 3b).

NCED OE rootstocks showed differential gene expression compared to the WT grafts (Figure 5). NCED OE roots downregulated 7 *PYL* ABA receptors and 3 *WRKY* factors, consistent with decreased sensitivity to ABA (Figure 6c), as in own-rooted plants grown in optimal conditions (Martínez-Andújar *et al.* 2020b). Several ABA *PYR/PYL* receptors are highly expressed in tomato roots compared to other tissues (González-Guzmán 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; Fernandez *et al.* 2020) . Loss or gain-of-function of several *pyr/pyl* loci reduced (Park *et al.* 2009; González-Guzmán *et al.* 2014) or enhanced (Fernández et al., 2020; García-Maquilón *et al*., 2021) root ABA sensitivity and signalling, respectively, altering the root phenotype. Moreover, NCED OE rootstocks downregulated most auxin-responsive and auxin-induced genes (*ARFs*, *MYBs*, *SAURs*) and the auxin transporter *PIN9*, while upregulating the auxin deactivation gene *IAASGH3* in SP12 (Figure 9b), without changing root IAA concentration (Figure 4). Therefore, antagonistic ABA-auxin interactions can account for 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 in the whole plants under control conditions (Martínez-Andújar *et al.* 2020b). Furthermore, genes involved in ABA biosynthesis (*FLC/AAO*), signalling (*AREB, ATHB12*) and stress responses (*MYBs, PIPs*) were slightly induced, not affected or attenuated in SP rootstocks (Figures 6a; 7a, b). Thus, downregulation of *PYL*s in NCED OE rootstocks may account for their reduced sensitivity to ABA and saline stress and limited root system development, favoring resource allocation to the vegetative and reproductive structures of the scion.

Enhanced photosynthesis of grafted plants with NCED OE rootstocks

Interestingly, NCED OE rootstocks enhanced leaf nutritional (S, Mg, Ca, Mn) status without affecting leaf Na concentration (Table 2), thus uncoupling root function from (diminished) root growth. Moreover, scions grafted on SP12 rootstocks maintained photosynthesis under low salinity (Figure 2 a, b) without changing g_s, thereby increasing intrinsic WUE (Figure 2b). Similarly, reciprocal grafting experiments under non-stressed

conditions indicated that only NCED OE scions decreased g_s with only modest effects on A_N, while NCED OE rootstocks had no effect on g_s (Thompson *et al.* 2007b).

Irrespective of environmental stresses, elevated ABA tissue concentrations can promote developmental changes in stomata and leaf anatomy that mimic the effects of water deficit (Quarrie & Jones 1977; Franks & Farquhar 2001; Galmés *et al.* 2011). Enhanced cuticular wax deposition and changes in its composition can protect photosynthesis (Ziv, Zhao, Gao & Xia 2018). In this study, grafting scions onto NCED OE rootstocks increased elongation of leaf epidermal cells and reduced the number of cuticular wax crystals on leaf adaxial and abaxial surfaces (Figure 2e; Table 1). Similarly, scions grafted onto autotetraploid Rangpur lime rootstocks with high ABA levels had higher expression of the wax synthesis *WAX2* gene than scions grafted onto the diploid equivalent with lower ABA levels (Allario *et al.* 2013). In contrast, there was a positive relationship between ABA level and wax deposition in ABA-deficient tomato mutants and following exogenous ABA application (Martin, Romero, Fich, Domozych & Rose 2017). NCED OE rootstocks may diminish wax deposition by directly downregulating wax synthesis pathways, or indirectly by alleviating salinity stress, thereby allowing greater leaf expansion and consequently diluting wax deposition or attenuating stress-induced wax synthesis. Furthermore, rootstocks can improve photosynthesis by affecting leaf structure to enhance mesophyll conductance to CO_2 (g_m) (Fullana-Pericàs, Conesa, Pérez-Alfocea & Galmés 2020), with g_m negatively correlated to sub-stomatal and/or ambient CO_2 concentration under long-term stress (Flexas *et al.* 2012, 2013). Here, grafting onto NCED OE rootstocks disorganized laminar mesophyll structure (Figure 2c), possibly explaining decreased *Ci* (Figure 2d) by enhancing CO_2 diffusion to the cells (Flexas *et al.* 2012, 2013).

Other rootstock-derived metabolites may also protect root and leaf function. Two genes involved in flavonoid synthesis, a flavanone 3-hydroxylase-like protein and a flavonoid oxidoreductase, were among the most upregulated genes in NCED OE rootstocks (Table 3; Figure 7c). Flavonoid accumulation leads to chilling and salt stress tolerance in tomato and *Arabidopsis* by reducing ROS accumulation and sensitivity to ABA (Mahajan & Yadav 2014; Meng, Zhang, Deng, Wang & Kong 2015; Li, Liu & Yao 2017), which is supported by the down-regulation of several peroxidase genes in the NCED OE rootstocks (Table 3). Furthermore, rootstock-derived flavonoids were xylem-transported to the leaves (Albacete *et al.* 2015).

Overall, NCED OE rootstocks enhanced tomato productivity under low salinity via at least three mechanisms that improved assimilate supply for scion growth: i) altered ABA metabolism and signalling restricted root growth, making more assimilate available for other sinks; ii) enhanced leaf nutrition and protection; iii) increased A_N and decreased sub-stomatal CO_2 associated with changes in leaf mesophyll structure.

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

Plants grown on NCED OE rootstocks had lower xylem sap concentrations of bioactive CKs in leaves and fruit trusses (Figure 4; Table S1) and downregulated root expression

of CK-metabolic genes (Figure 8a), supporting an antagonistic interaction with ABA (Gawronska, Deji, Sakakibara & Sugiyama 2003; Ghanem *et al.* 2011a; Peleg & Blumwald 2011). Despite attenuated root-to-shoot CK signalling, activation of shoot-to-root CK signalling (enhanced phloem iP concentrations) might act as a putative signal to restore root CK status (Hirose, Takei, … & 2008 2008; Matsumoto-Kitano *et al.* 2008). Moreover, leaf area and A_N were positively correlated with foliar iP accumulation (r = 0.85 and 0.73; $P \leq 0.01$) across the different graft combinations, possibly explaining altered leaf mesophyll structure, since this hormone preferentially accumulates in the leaf mesophyll and vascular bundles (Veselov *et al.* 2018). By facilitating CO_2 diffusion to carboxylation sites (Flexas *et al.* 2012, 2013), iP/ABA-mediated mesophyll alteration favored CO_2 assimilation. Indeed, both ABA and iP have been proposed as signalling components of the reticulate leaf phenotype in *Arabidopsis*, which has altered mesophyll structure and reduced CO_2 fixation capacity (Lundquist *et al.* 2014). Interestingly, a phosphoglycerate mutase gene (Solyc04g072800), whose function is reduced in reticulate mutants (Lundquist *et al.* 2014), was 2 and 1.4-fold upregulated in SP12 and SP5 rootstocks, compared to the WT (Table 3). This enzyme is key in ATP production and reducing power from glycolysis (Zhao & Assmann 2011) and could contribute to active transport and root assimilatory processes such as nutrient uptake and Na^+ exclusion (Malagoli, Britto, Schulze & Kronzucker 2008; Munns, Passioura, Colmer & Byrt 2020) and nitrate or sulphate reduction (Wang et al. 2004), thereby enhancing leaf nutrient status. Moreover, iP-type CKs were related with greater xylem development and plant growth, vigor and yield in tomato (Qi *et al.* 2020). 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, ABA-CK interactions in rootstock-mediated improvement of the scion physiology require further investigation,

Ethylene and gibberellin related responses in NCED OE grafted plants

ABA signalling maintains 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 (Figure 9a). Root and leaf phloem ACC concentrations were significantly reduced, as in own-rooted NCED OE plants (Martínez-Andújar *et al.* 2020b). Since diminished (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 may be involved (Figure 9a). *ERFs* induce GA2oxidases to inactivate GAs and root growth by stabilizing DELLA proteins (Julkowska & Testerink 2015; Hetherington, Kakkar, Topping & Lindsey 2021). Whether these local changes in ethylene and GA responses are involved in systemic signalling is less clear, as reproductive tissues of scions grafted on NCED OE rootstocks had increased

ACC and GA_3 levels (Figure 4; Table S1). These enhanced GA_3 levels are consistent with the elongated truss phenotype (Figure 1). Overall, ABA-ethylene-GA interactions seem involved in regulating root growth, while long-distance ACC and GA signalling cannot be ruled out.

NCED OE rootstocks also upregulated other stress-adaptive processes (Table 3) involved in membrane protection (Glycerol-3-phosphate acyltransferase, Solyc07g056320) through lipid metabolism (Ziv *et al.* 2018; Zhao *et al.* 2020) and epigenetic regulation (Bromodomain containing 2, Solyc09g015660) through RNA binding and chromatin remodeling (Chaturvedi & Rao 2016; Liu *et al.* 2017). Finally, regulation of pathogenesis–related proteins and subtilin-like proteases genes seems highly sensitive to elevated natural (Zhang, Cao, Li, Chen & Xu 2019) or transgenic (this study) constitutive ABA production, which deserves further investigation.

Conclusion

Grafting WT scions onto constitutively ABA-overproducing rootstocks produced local (root) and systemic (scion) responses mediated by root-shoot communication. Evidence that rootstock *SlNCED1* overexpression changed root-to-shoot ABA signalling included increased ABA concentrations in scion reproductive tissues and increased ABA catabolites in leaves, but lower leaf phloem ABA concentrations. ABA overproduction altered stress-mediated responses associated with: 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. Increased root glycolytic activity may mediate increased nutrient uptake and flavonoid synthesis and transport for stress protection in the scion. Rootstock NCED overexpression modified leaf growth and anatomy and enhanced photosynthesis, possibly due to iP, JA and ABA accumulation in the leaf and leaf phloem. Enhanced GA_3 in truss xylem sap was consistent with increased truss length, weight and overall yield. Considering whole plant source-sink relationships, the stimulation of leaf photosynthesis and reduction in root assimilate requirements for biomass 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 10).

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703 **Conflict of interest**

704 The authors declare that they have no conflict of interest.

705 **Author contributions**

706 F.P.-A. planned and designed the research, A.M.-P. and C.M.-A. performed all the stress
707 experiments, A.F.-A. and J.M.P.-P. executed the qPCR analysis, A.J.T., F.M., L.E.-L.,
708 Z.K. and M.G. carried out the transcriptomic analysis, C.M.-A., A.M-P., P.A.M.-M. and
709 A.A. performed the physiological analysis, A.A. carried out the hormone profiling
710 experiments, C.M.-A. performed the data analysis, C.M.-A. and F.P.-A. wrote the original
711 draft preparation, C.M-A., I.C.D., A.J.T. and F.P-A. reviewed and edited the final
712 manuscript.
713

714 **Data availability statement**

715 All raw and processed microarray data are openly available in the Gene Expression
716 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, the 2nd fruit trusses and the root 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 in greenhouse conditions (**a**). Shoot fresh weight (SFW), mature leaf fresh weight (LFW), leaf area, stem diameter (SD), root fresh weight (RFW), RFW/SFW ratio, 3rd truss length (TL), 3rd truss fresh weight (TFW) and fruit yield after 130 (LFW, 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, $P \leq 0.05$). *P*-values from ANOVA testing of the effect of the genotype on all parameters are shown (**b**).

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) Different letters indicate significant differences between graft combination (n=3, $P \leq 0.05$) (**a**). Net photosynthesis (A_N), stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i) after 180 DST. Different letters indicate significant differences between graft combination (n=3, $P \leq 0.05$) (**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 after 180 DST (**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) after 180 DST (**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) after180 DST (**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 3.5 dS m^{-1} (equivalent to 35 mM NaCl) in 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). * indicates statistically significant difference between graft combinations (n=3, P ≤ 0.05) (**b**).

Figure 4. HeatMap of the variation of abscisic acid (ABA), trans-zeatin (t-Z), isopentenyl adenine (iP), 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA), gibberellin A3 (GA3), 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) in greenhouse conditions. -1 and -2 indicate significant decrease at $P \leq 0.05$ and $P \leq 0.01$, respectively; 0 indicates not significant effects and +1 and +2 indicate significant increase at $P \leq 0.05$ and $P \leq 0.01$, 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 in greenhouse conditions (**b**). GO terms related to the differentially expressed transcripts in SD/SP5 + SD/SP12 against SD/AC whose proportion is different from all the tomato transcripts contained in the microarray (**c**).

Figure 6. ABA 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 in greenhouse conditions. Real time PCR quantification (RT-qPCR) of some ABA-related selected genes is also given (**a**). Root xylem sap ABA concentration (as a percentage with respect to control conditions -no salt, data in the embedded table- for each genotype) as a function of salt concentration in the medium (35, 70 and 100 mM NaCl) of tomato cv Ailsa Craig self-grafted (AC/AC, open circles) and grafted onto the NCED OE line SP12 (AC/SP12, closed circles) during 27 days. Each point represents the mean value of four replicates. Different letters indicate significant differences between treatments within each graft combination ($P \leq 0.05$). * and ** indicate significant difference between graft combinations at $P \leq 0.05$ and $P \leq 0.01$, respectively (**b**). The relationship between main root total length (RL) and ABA concentration in the culture medium (0, 1.5, 3 and 5 µM ABA) in tomato cv Ailsa Craig (AC, open circles) and the transgenic line SP12 (SP12, closed circles) grown *in vitro* during 30 days. Each point represents the mean value of four replicates along with its standard error. Different letters indicate significant differences between treatments within each graft combination ($P \leq 0.05$). * and ** indicate significant difference between graft combinations at $P \leq 0.05$ and $P \leq 0.01$, respectively (**c**).

Figure 7. Stress (**a**) aquaporin (**b**) and flavonoids (**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 in greenhouse conditions. Real time PCR quantification (RT-qPCR) of some selected genes is also given. Luteolin, taxifolin, genistein, quercetin and cyanidin peak area in root xylem sap and leaves 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} for 180 days in greenhouse conditions. * and ** indicate significant difference between SD/AC and SD/SP12 at $P \leq 0.05$ and $P \leq 0.01$, respectively (**d**).

Figure 8. Cytokinin (CK) (**a**), gibberellin (GA) (**b**) and jasmonic acid (JA)(**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 9. Ethylene (**a**) and auxin (**b**) 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 10. Proposed model to explain how ABA overproducing rootstocks improve growth and yield under saline conditions, by affecting local (root) and systemic (scion) responses mediated by root-to-shoot communication. (**a**) In the roots, ABA overproduction seems to interfere with stress mediated response by decreasing root expression of ABA receptors and signalling components, thus altering sensitivity to ABA. Decreased ABA sensitivity in the roots appears to diminish auxin activity (ARFs, auxin transport from the shoot) and increases ethylene-related processes (*ERFs, ACCs*) leading to reduced RSA (mainly lateral roots). Lower *IPT* gene expression diminishes rootstock CK synthesis and *t*-Z transport to the shoot. (**b**) In the scion, increased ABA catabolites in fruiting plants and ABA accumulation in young plants indicates that a root-to-shoot ABA signal cannot be ruled out. Increased foliar iP accumulation and phloem transport (in response to reduced *t*-Z transport from the roots) along with transient foliar ABA and JA accumulation seems to modify leaf growth and mesophyll structure leading to improved photosynthesis (A_N) activity. Increased transport of nutrients and flavonoids to the leaves also protects leaf function. Moreover, increased xylem GA_3 in growing fruits seems to enhance reproductive growth. Improved photosynthesis and reduced root growth optimise source-sink relations to benefit scion development and yield. Arrow and bar heads indicate positive and negative regulation, respectively.

Supplementary Figure legends

Figure S1. Schematic diagram of the experimental design.

Figure S2. KEGG pathways of hormone biosynthesis. Wide arrows mark the affected pathways in the overexpression lines. Thick red arrows show increased gene expression, thick blue arrows mark decreased expression in the affected part of the pathway.

Figure S3. KEGG pathways of hormone signal transductions. Wide arrows mark the affected pathways in the overexpression lines. Thick red arrows show increased gene expression, thick blue arrows mark decreased expressions in the involved part of the pathway.

Tables

Table 1. Stomatal density in abaxial and adaxial 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

Table 2. Total carbon (C) total nitrogen (N), phosphorus (P), potassium (K), sulphur (S), magnesium (Mg), calcium (Ca), sodium (Na), iron (Fe), manganese (Mn), boron (B) and zinc (Zn) concentrations in the leaf of cv Sugar Drop grafted onto WT AC (SD/AC) and the NCED OE lines SP12 (SD/SP12) and SP5 (SD/SP5) grown under 3.5 dS m^{-1} for 130 days in greenhouse conditions (mean ± SE). Different letters indicate significant differences among graft combinations (n = 6, $P \leq 0.05$). P-values from ANOVA testing of the effect of the genotype on all parameters are shown.

Nutrient (mg g^{-1} DW)	SD/AC	SD/SP12	SD/SP5	*P* (ANOVA)
C	21.40±0.79	23.98±1.20	22.82±1.64	0.249
N	373.50±4.71	362.47±4.77	364.72±5.64	0.295
P	1.32±0.12	2.03±0.36	1.54±0.20	0.105
K	36.63±5.73	31.94±3.97	29.65±2.0	0.531
S	8.44±0.90 b	12.20±0.59 a	12.35±1.46 a	<0.05
Mg	5.50±0.09 b	8.48±0.89 ab	9.96±1.85 a	<0.05
Ca	24.17±1.15 b	42.81±2.72 a	48.21±6.865 a	<0.01
Na	5.98±0.80	4.92±0.25	5.34±0.85	0.618
Fe	1.20±0.12 a	0.47±0.04 b	0.40±0.02 b	<0.01
Mn	0.07±0.01 b	0.13±0.01 a	0.10±0.01 ab	<0.05
B	0.07±0.00	0.06±0.01	0.07±0.00	0.958
Zn	0.04±0.01	0.03±0.01	0.03±0.01	0.837

1199 **Table 3**. Differentially expressed genes comparing SD/SP12 and SD/SP5 combined against SD/AC. The 25 most
1200 upregulated genes (largest logFC values) and the 25 most downregulated genes (smallest, most negative logFC values)
1201 are given with their mean relative expression (AveExpr) level and the adjusted *P* value (Adj.P.val).

ID	LogFC	AveExpr	Adj.P.Val	Description
Upregulated genes				
Solyc07g056570.1.1	6.74	11.71	$2.15E^{-4}$	**9-cis-epoxycarotenoid dioxygenase**
Solyc01g095080.2.1	1.74	11.11	$1.70E^{-3}$	**1-aminocyclopropane-1-carboxylate synthase**
Solyc04g072800.2.1	1.70	9.90	$2.50E^{-4}$	**2 3-bisphosphoglycerate-dependent phosphoglycerate mutase**
Solyc09g015660.2.1	1.51	11.61	$8.26E^{-7}$	**Bromodomain containing 2**
Solyc03g080190.2.1	1.50	12.54	$7.13E^{-5}$	**Flavanone 3-hydroxylase-like protein**
Solyc02g093040.2.1	1.49	8.77	$1.15E^{-3}$	**Cathepsin B-like cysteine proteinase**
Solyc03g111290.1.1	1.46	10.23	$1.58E^{-4}$	**Cytochrome P450**
Solyc01g011450.1.1	1.45	7.47	$4.01E^{-6}$	**Unknown Protein**
Solyc05g007950.2.1	1.44	10.74	$8.07E^{-4}$	**Ribonuclease T2**
Solyc12g017460.1.1	1.41	10.84	$1.93E^{-5}$	**GDSL esterase/lipase At1g28590**
Solyc06g052020.2.1	1.40	11.47	$1.65E^{-6}$	**Unknown Protein**
Solyc04g012050.2.1	1.35	11.64	$8.85E^{-5}$	**Ethylene responsive transcription factor 2a**
Solyc09g098160.2.1	1.27	12.00	$4.01E^{-4}$	**Pirin-like protein**
Solyc07g056320.2.1	1.26	9.55	$7.11E^{-4}$	**ER glycerol-phosphate acyltransferase**
Solyc03g111720.2.1	1.25	12.79	$1.26E^{-3}$	**Peptide methionine sulfoxide reductase msrA**
Solyc08g013760.1.1	1.24	7.47	$1.10E^{-3}$	**F-box family protein (AHRD V1 ***- B9GFH4_POPTR)**
Solyc10g055200.1.1	1.22	9.92	$6.40E^{-4}$	**Disease resistance response**
Solyc06g065870.2.1	1.17	9.17	$1.33E^{-3}$	**Unknown Protein**
Solyc07g054470.1.1	1.14	9.09	$2.58\ E^{-4}$	**Unknown Protein**
Solyc03g096670.2.1	1.14	10.93	$4.87\ E^{-4}$	**Integrin-linked kinase-associated serine/threonine phosphatase 2C**
Solyc08g044490.1.1	1.14	6.27	$6.90E^{-5}$	**Kinesin heavy chain-like protein**
Solyc01g006170.2.1	1.13	9.45	$1.60E^{-4}$	**rRNA processing protein ebna1-binding protein-related**
Solyc01g110960.2.1	1.13	8.81	$2.23E^{-6}$	**Glutamic acid-rich protein**
Solyc03g111300.1.1	1.10	10.15	$4.68E^{-4}$	**Cytochrome P450**
Solyc03g007170.2.1	1.10	10.82	$7.59E^{-5}$	**FK506-binding protein 4, Peptidyl-prolyl cis-trans isomerase**
Downregulated genes				
Solyc04g063350.2.1	-1.93	8.89	$2.90E^{-4}$	**3-methyl-2-oxobutanoate dehydrogenase (2-methylpropanoyl-transferring)**
Solyc06g059870.1.1	-1.96	9.63	$4.00E^{-4}$	**Unknown Protein**
Solyc03g020080.2.1	-1.97	11.93	$1.59E^{-4}$	**Proteinase inhibitor II**
Solyc02g068170.1.1	-2.04	8.85	$5.34E^{-6}$	**Unknown Protein**
Solyc10g036660.1.1	-2.06	8.85	$5.34E^{-6}$	**Unknown Protein**
Solyc09g072700.2.1	-2.07	6.93	$1.65E^{-6}$	**Peroxidase 57**
Solyc07g044900.1.1	-2.08	5.63	$1.37E^{-6}$	**Unknown Protein**
Solyc01g006300.2.1	-2.17	8.36	$5.77E^{-6}$	**Peroxidase**
Solyc08g079930.1.1	-2.31	8.63	$5.61E^{8}$	**Subtilisin-like protease**
Solyc12g087940.1.1	-2.32	9.27	7.13^{E-5}	**Aspartic proteinase nepenthesin-1**
Solyc08g079850.1.1	-2.35	7.64	$5.62E^{-7}$	**Subtilisin-like protease**
Solyc04g076190.1.1	-2.37	8.62	$1.08E^{-5}$	**Aspartic proteinase nepenthesin-1**
Solyc08g079890.1.1	-2.37	8.90	$5.13E^{-7}$	**Subtilisin-like protease**
Solyc08g079920.1.1	-2.39	7.65	$5.61E^{-8}$	**Subtilisin-like protease**
Solyc09g097770.2.1	-2.59	11.39	$6.29E^{-4}$	**Cell wall protein**
Solyc05g005560.2.1	-2.69	8.18	$5.34E^{-6}$	**BURP domain-containing protein**
Solyc01g008620.2.1	-2.77	9.65	$4.54E^{-4}$	**Beta-1 3-glucanase**
Solyc08g079860.1.1	-2.78	8.80	$3.08E^{-7}$	**Subtilisin-like protease**
Solyc08g079840.1.1	-2.85	8.62	$5.61E^{-8}$	**Subtilisin-like protease**
Solyc08g079900.1.1	-2.92	9.74	$5.61E^{-8}$	**Subtilisin-like protease**
Solyc08g079870.1.1	-2.95	8.76	$5.61E^{-8}$	**Subtilisin-like protease**
Solyc09g007020.1.1	-3.03	11.09	$5.70E^{-8}$	**Pathogenesis-related protein**
Solyc08g079910.1.1	-3.06	8.11	$5.61E^{-8}$	**Subtilisin-like protease**
Solyc08g079880.1.1	-3.15	9.16	$5.61E^{-8}$	**Subtilisin-like protease**
Solyc09g007010.1.1	-3.37	11.95	$1.68E^{-3}$	**Pathogenesis related protein PR-1**

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Supplementary Table legends

Table 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) in greenhouse conditions (mean ± SE). Different letters indicate significant differences among graft combinations (n = 3, $P \leq 0.05$). * and ** indicate significant differences between SD/SP12 or SD/SP5 and SD/AC at $P \leq 0.05$ and $P \leq 0.01$, 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.