Impact of overexpression of 9-cis-epoxycarotenoid dioxygenase on growth and gene expression under salinity stress

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Highlights:

- Constitutive ABA overproduction reduces shoot and root growth and close stomata, under optimal conditions.
- Constitutive ABA overproduction reduces the percentage loss in shoot and root growth and increases the total root length, under salinity conditions.
- The differential growth response in ABA overproducing plants between optimal and suboptimal conditions is related to differentially altered growth regulatory gene networks between both conditions.
Abstract

To better understand abscisic acid (ABA)’s role in the salinity response of tomato (*Solanum lycopersicum* L.), two independent transgenic lines, sp5 and sp12, constitutively overexpressing the *LeNCED1* gene (encoding 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in ABA biosynthesis) and the wild type (WT) cv. Ailsa Craig, were cultivated hydroponically with or without the addition of 100 mM NaCl. Independent of salinity, *LeNCED1* overexpression (OE) increased ABA concentration in leaves and xylem sap, and salinity interacted with the *LeNCED1* transgene to enhance ABA accumulation in xylem sap and roots. Under control conditions, *LeNCED1* OE limited root and shoot biomass accumulation, which was correlated with decreased leaf gas exchange. In salinized plants, *LeNCED1* OE reduced the percentage loss in shoot and root biomass accumulation, leading to a greater total root length than WT. Root qPCR analysis of the sp12 line under control conditions revealed upregulated genes related to ABA, jasmonic acid and ethylene synthesis and signalling, gibberellin and auxin homeostasis and osmoregulation processes. Under salinity, *LeNCED1* OE prevented the induction of genes involved in ABA metabolism and GA and auxin deactivation that occurred in WT, but the induction of ABA signalling and stress-adaptive genes was maintained. Thus, complex changes in phytohormone and stress-related gene expression are associated with constitutive upregulation of a single ABA biosynthesis gene, alleviating salinity-dependent growth limitation.

Keywords

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

Salinity is one of the major limiting factors for crop productivity, causing land abandonment for agricultural purposes in arid and semi-arid areas throughout the world [1]. In aiming to develop more stress-tolerant plants, manipulating both metabolism and signalling of different plant hormones has been a main biotechnological target [2, 3]. It is clearly important to understand the effects of gene manipulation on whole-plant and crop physiology to check its agronomic interest. The plant hormone abscisic acid (ABA) is a good candidate for such genetic manipulation since it is involved in local and systemic responses to various abiotic stresses (drought, salinity, cold and high temperature stresses) and regulating plant water status [4, 5]. ABA is also involved in regulating developmental processes such as flower, fruit, root and seed development [6-8] some of which may be considered as stress-adaptive responses, mainly changes in root system architecture [9]. Tomato for the fresh fruit market is predominantly grown on rootstocks, and thus resistance to salinity stress can be potentially delivered through breeding improved rootstock genotypes [10]. A greater understanding of the genetic and molecular basis of resistance delivered through the root genotype will facilitate this breeding effort.

The first committed step in ABA biosynthesis in plants, catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) [11], is a target to manipulate endogenous ABA accumulation and to study its physiological effects. The tomato LeNCED1 gene is strongly up-regulated under water-stress in leaf and root tissues [12]. Overexpression of NCED1 in tomato and tobacco [13, 14] and NCED3 in Arabidopsis [15] and rice [16] increased ABA levels in different tissues and reduced transpiration in the absence of stress. Improved drought and salinity (survival) tolerance was observed in NCED
overexpressing tobacco, Arabidopsis and rice [13, 15], while increased biomass was reported in creeping bent grass (*Agrostis palustris*) grown under drought and high salinity [17].

Salinity rapidly (within a day) induces ABA accumulation in roots, xylem sap and leaves of the tomato plant [18, 19] and this hormone accumulation is associated with stomatal closure and growth inhibition. Physiological correlations in recombinant inbred tomato populations suggest a involvement of ABA in regulating leaf biomass in both the absence of stress, but also under salinity [2], although the underlying mechanisms remain an open question. In different plant species, ABA-deficient mutants had both positive and negative effects on growth, depending on the plant organ, timing of exposure and growing conditions [20-22]. Multiple studies indicate that salt-induced growth inhibition is more severe in ABA-deficient mutants [23-26].

Overexpressing *LeNCED1* in tomato using the strong constitutive chimaeric Gelvin superpromoter (*sp*) resulted in the “high-ABA lines” termed sp12 and sp5 (used in this study), which displayed moderately elevated ABA levels throughout the plants [14, 27]. Under well-watered conditions, *NCED* OE plants had similar ABA levels and stomatal conductance as moderately drought stressed WT plants [27]. In the case of well-watered sp5 plants, they also had a greater leaf area, and similar long-term biomass accumulation when compared to WT plants, and their significantly lower stomatal conductance with only a minor effect on assimilation rate greatly increased leaf water use efficiency [27]. It was proposed that any penalty in assimilation rate was compensated by improved leaf water status and turgor-driven growth, and antagonism of ethylene-induced epinastic growth inhibition [27]. However, young plant establishment was delayed in sp5, and stronger ABA accumulation in leaves and xylem
with the \textit{rbcS3C} promoter caused multiple negative phenotypes: photobleaching of young seedlings, interveinal leaf flooding, reduced chlorophyll and carotenoid content, and greatly reduced growth [28]. This suggests, in a crop improvement context, that the optimal rate of ABA biosynthesis in some environments may be above the naturally evolved rate when considering agronomic traits such as yield, water use efficiency and resistance to abiotic stress; however, exceeding the optimal amount does reduce growth.

Here we test the hypothesis that constitutive ABA overproduction alters the salinity response of tomato, and whether this is related to phytohormone levels and the associated ABA and stress signalling components before and during stress. Gas-exchange parameters, ionomic and hormone profiling, and the expression of a set of genes used as abiotic stress-responsive biomarkers in roots [29] were determined.

2. Material and methods

2.1. Plant material, germination and growth conditions

The two independent tomato transgenic lines sp5 and sp12 in the genetic background of the wildtype (WT) cultivar Ailsa Craig (AC) were previously reported [14]. These lines constitutively overexpress the \textit{LeNCED 1} gene [14] under the control of the Gelvin superpromoter (sp) and contain elevated levels of ABA compared to WT, with sp5 accumulating more ABA than sp12 [27]. 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. For all genotypes, seeds were sown in commercial vermiculite, watered with deionized water and kept at 26-28°C and 80-90% relative humidity in the dark until germination. After 2-3 true leaves had emerged, uniformly-sized seedlings were transferred to a hydroponic culture system in a controlled environment chamber. Plants
were floated in 20 L plastic black containers containing aerated half-strength modified Hoagland solution. A factorial design of three genotypes x two salt treatments x six replicates was performed and the six replicates were randomly distributed in each container. The environment was controlled to a 16/8 h day/night cycle with a photosynthetic photon flux density (PPFD) of 245 µmoles m⁻² s⁻¹. Day/night temperature was 25/18ºC and relative humidity was maintained in the range 40-60%. After one week within the hydroponic system, the plants were exposed to 0 (control treatment) or 100 mM of NaCl (salt treatment) added to the nutrient solution for 21 days. In both salt and control treatments, the nutrient solution was refilled daily and replaced twice every week.

Vegetative growth (shoot and root fresh weight, FW) was assessed and tissues sampled after 11 and 21 (end of the experiment) days of salinity treatment (DST¹). Shoots and roots were separated immediately and weighed to determine biomass. Young fully expanded leaves and young roots were immediately frozen in liquid nitrogen for hormonal and gene expression analysis. Mature leaves were weighed and stored in a 65ºC oven for at least 48 hours to dry them for ionomic analysis. To collect root xylem sap, control plants were detopped under the cotyledonary node and a short silicone tube fitted to the stump to collect spontaneously exuded xylem sap, which was removed with a pipette and placed in pre-weighed microcentrifuge tube. In salinized plants, xylem sap was collected by placing the roots in a Scholander-type pressure chamber and applying pneumatic pressure (0.2 - 0.8 MPa depending on the plant genotype). Leaves, roots and xylem sap samples were stored at -80ºC for further analyses.

¹ DST: Days of salinity treatment
2.2. *Plant water relations measurements*

Throughout the experiment, photosynthesis ($A^2$) and stomatal conductance ($g_s^3$) were measured in youngest fully expanded leaves using a CIRAS-2 (PP Systems, Massachusetts, USA) between 09.00 h and 12.00 h (considering that light were turned on at 08.00 h). CO$_2$ was set at ambient levels (400 ppm) and radiation matched the chamber conditions (245 µmol m$^{-2}$ s$^{-1}$ PPFD).

Leaf water potential of the youngest fully expanded leaf was measured by thermocouple psychrometry as previously described [30]. Discs of 8 mm diameter were punched from leaves, placed immediately on clean sample holders and then wrapped in aluminium foil to minimize water loss. After 20 discs had been collected (approximately 15 min), they were unwrapped and then loaded into C52 chambers (Wescor Inc., Logan, UT, USA), incubated for 3 h and then voltages were read with a microvoltmeter (model HR-33T; Wescor Inc., Logan, UT, USA). Voltages were converted into water potentials based on calibration with salt solutions of known osmotic potential.

2.3. *Plant hormone extraction and analysis*

**Trans**-zeatin ($t$-$Z$), indole acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), gibberellin $A_3$ (GA$_3$) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were extracted and analysed as described previously Albacete, Ghanem, Martínez-Andújar, Acosta, Sánchez-Bravo, Martínez, Lutts, Dodd and Pérez-Alfocea [18], 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 4°C. Solids

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2 $A$: Photosynthetic rate  
3 $g_s$: Stomatal conductance
were separated by centrifugation (20 000 g, 15 min at 4°C) and re-extracted for 30 min at 4°C with 1 mL of the extraction solution. Pooled supernatants were passed through Sep-Pak Plus C18 cartridge (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 40°C. 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 dark microcentrifuge tubes.

Ten µL of filtrated 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 were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (0, 1, 10, 50, and 100 µg L⁻¹).

2.4. **Ion extraction and analysis**

To quantify Ca, K, Mg, Na, P, S, Mn, B and Zn concentrations, 0.1 g of dried and ground plant material (leaf or roots) was weighed and digested in a HNO₃:HClO₄ (2/1, v/v) solution. Ion analysis of root xylem sap, leaf and root tissue samples were performed in an inductively coupled plasma spectrometer (ICP-OES, ThermoFisher ICAP 6000 Series).
2.5. RNA isolation, cDNA synthesis and real-time quantitative PCR

Sample collection and RNA extractions were performed as described elsewhere [29]. Briefly, total RNA from ~150 mg of frozen tomato roots from each genotype and treatment was extracted in triplicate using Tri-Reagent (Sigma-Aldrich, St. Louis, USA), and the first strand cDNA was synthesized from 1 μg purified RNA using the iScript Reverse Transcription Supermix (Bio-Rad, 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 (Table 1) as described before Ferrández-Ayela, Sánchez-García, Martínez-Andújar, Kevei, Gifford, Thompson, Pérez-Alfocea and Pérez-Pérez [29]. To avoid amplifying genomic DNA, forward and reverse primers were designed to hybridize across consecutive exons. Real-time quantitative PCR 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 confirmed its specificity.

Primer pair validation and relative quantification of gene expression levels were performed by using the comparative Ct method [31]. Data were represented as the relative gene expression normalized to the Ct value for the tomato housekeeping gene.
ACTIN2 (Solyc04g011500) as previously described [29]. 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 statistical differences between samples (P-value ≤ 0.05).

2.6. In vitro culture

To investigate root growth of young seedlings in more detail, surface-sterilized (washed in 5% NaOCl) tomato seeds of the WT and the sp12 line were germinated in vitro using nutrient solution [32] diluted 350 times and supplemented with 10 g L⁻¹ agar and 1% sucrose. Seedlings were transferred to control and salt (50 mM NaCl) conditions when the two cotyledons were developed (after 6 days for WT and 9 days for sp12). After 30 days of treatment, total root length (TRL⁴) was evaluated using WinRHIZO software (Pro 2016, Regent, Canada). Root exudates were collected in sterile tubes following centrifugation of the agar medium (20,000 g, 15 min at 4°C) and the supernatant used for hormonal analysis.

2.7. Statistical analysis

Data were subjected to 2-way analysis of variance (ANOVA) to test the main effects of genotype, treatment and their interaction. Analyses initially comprised all three genotypes, and then pairwise comparisons were made. Genotypic means were compared using Tukey’s test at 0.05 of confidence level. Correlation analyses determined relationships between different plant variables. All analyses were performed using SPSS for Windows (Version 22.0, SPSS Inc., Chicago, IL, USA).

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⁴ TRL: Total root length
3. Results

3.1. Plant growth

Plants grown for 21 days after reaching the 2-3 leaf stage were harvested. Under control conditions, \textit{LeNCED1} overexpression significantly decreased shoot biomass by 35-50% compared to the WT (Fig. 1A); for root biomass, sp5 plants showed a significant decrease of 47% compared to WT, but sp12 did not differ statistically to WT (Fig. 1B). Salinity reduced shoot and root growth by 70% and 40% respectively in WT plants, but in sp5 and sp12 the reduction was lower: 53% and 50% reduction in the shoot (\(P=0.007\)) and 14 and 27% reduction in roots, although this was not significant (Figs. 1A, B). Salinity increased root/shoot ratio, but there were no significant genotypic effects (data not shown). With salinity, all genotypes had statistically similar biomass (Figs. 1A-C). Thus \textit{LeNCED1} overexpression decreased plant growth under control conditions at this stage of plant development, but salinity had a smaller inhibitory effect on sp5 and sp12 growth than it did on WT growth. No differences in leaf water content were found between genotypes, irrespective of the salinity treatment (data not shown).

3.2. Leaf gas exchange

Compared to the WT, \textit{LeNCED1} overexpression had no statistically significant effect on photosynthetic rate under control or salinity conditions (Fig. 2A), but it significantly reduced stomatal conductance by 40-50% when both treatments were considered together (Fig. 2B). While salinity had the greatest effect on photosynthesis rate (\(P \leq 0.001\)), genotype had the greatest effect on stomatal conductance (\(P \leq 0.022\)), and leaf gas exchange of all genotypes responded similarly to salinity (no significant genotype × treatment interaction).
3.3. **Plant hormones**

**Abscisic acid**

Under control conditions, sp5 plants had significantly higher ABA concentrations in roots (by 1.3-fold at 21 DTS) (Fig. 3F), xylem sap (by 3.5-fold at 21 DTS, Fig. 3E) and leaves (by 1.6-fold at 11 DTS and 1.4-fold at 21 DTS, Fig. 3A, D), compared to the WT. In sp12, ABA concentrations were similar in roots (Fig. 3C, F), significantly higher in xylem sap at 11 DST (1.9-fold, Fig. 3B) and slightly higher in leaves (1.2-fold, Fig. 3A, D) compared to the WT. Salinity increased xylem sap (Fig. 3B, E) and leaf (Fig. 3A, D) ABA concentrations in all genotypes, but in roots ABA only significantly accumulated in sp5 after 11 DST (Fig. 3C and Table S3). While salinity-induced leaf ABA accumulation was similar in all genotypes (no significant genotype × salinity treatment interaction, Fig. 3A, D and Table S3), xylem sap ABA concentration only significantly increased in sp12 and sp5 at 11 DST (Fig. 3B); this was confirmed in the genotype × salinity treatment interaction in xylem sap ABA at 11 DST (Fig. 3B).

Overall, *NCED* OE provoked significant ABA accumulation in xylem and leaves in sp12 and sp5, but in the roots the additional ABA accumulation was specific to sp5 at 11 DST (Fig. 3C). Additionally, it was apparent that both sp12 and sp5 gave a stronger increase in xylem sap ABA concentration in response to salinity than WT, but this was restricted to 11 DST (Fig. 3B).

**Jasmonic acid**
Under control and salt conditions, there were no significant genotypic differences in root, xylem and foliar JA concentrations on either sampling time (Fig. 4A-F and Table S3). Salinity significantly increased xylem JA concentration after 11 DTS ($P \leq 0.001$, Fig. 4B), but not after 21 DTS (Fig. 4E). Salt treatment decreased root JA concentrations in all genotypes at 11 DST ($P \leq 0.041$) and 21 DST ($P \leq 0.002$) (Figs. 4C, F), but had no consistent effect on foliar JA concentrations (Fig. 4A, D). Overall the salinity-induced reduction in JA in the roots, independent of genotype, was the clearest observation.

**Salicylic acid**

Under control conditions, the sp5 line had significant increased xylem (11 DTS) and foliar SA concentration (21 DTS) compared to the WT. Salinity significantly decreased root SA concentrations, but increased xylem SA concentrations, while having no effect on foliar SA concentrations (Table S1 and S4). The highest root, xylem sap and leaf SA concentrations occurred in sp5 plants at 21 DST (Table S1).

**Gibberellic acid**

Under control conditions, xylem GA$_3$ concentrations were 2-fold higher in the *NCED* OE lines at 11 DTS, but in sp12 returned to WT levels at 21 DTS. Salinity had no significant effect on xylem GA$_3$ concentration (Table S4). Xylem GA$_3$ levels in sp5 were higher than in WT plants only at 21 DST (Table S1). This hormone was not detected in other tissues.

**1-Aminocyclopropane-1-carboxylic acid**
Under control conditions, ACC concentrations were significantly lower in sp12 (xylem) and sp5 (leaf and xylem) plants at 11 DST, compared to the WT (Table S1). Significant salt treatment effect was found only in root ACC concentrations ($P \leq 0.0001$ at 11 DST, $P \leq 0.001$ at 21 DST, Table S4). While salinized sp5 plants had the highest root ACC concentrations in both harvest points, sp12 had the highest xylem (11 DTS) and leaf (21 DST) ACC concentrations (2-fold) (Table S1).

**Cytokinins**

Under control conditions, sp5 had lower root concentrations of trans-zeatin ($t$-Z) than the WT, but significant differences occurred only at 21 DST (Table S1). Salinity increased xylem and leaf (only in sp12) $t$-Z concentrations (Table S1, Table S4), but decreased root $t$-Z concentrations in WT and sp12 roots after 21 DST.

**Indole-3-acetic acid**

Under control conditions, there were no significant genotypic effects on IAA concentrations (Table S1, S4). Salinity decreased root (AC and sp12) and leaf (sp12) IAA concentrations at 21 DST, while xylem IAA concentrations increased only in sp12 plants at the second harvest point (Table S1).

**3.4. Nutrients**

Salinity treatment increased leaf, xylem sap and root Na$^+$ concentrations by 55-, 200- and 44-fold respectively (averaging across both measurement times). Salinized sp5
plants had the lowest xylem Na\textsuperscript{+} concentrations at 21 DST, but significant differences were found only compared to sp12 plants (Table S2). In salinized plants, xylem sap Na\textsuperscript{+} concentrations significantly decreased in sp5 at 21 DST. K\textsuperscript{+} concentrations decreased in both leaf and roots, while they decreased xylem compared to control conditions (Table S2).

After 21 DTS, sp5 had the highest root Mg and Mn concentrations compared to the WT (Table S2). Roots of salinized sp5 consistently had the highest Fe concentrations (Table S2). Under control conditions, P and S concentrations did not differ among genotypes while salinized sp12 plants had significantly higher xylem P concentrations at 21 DST (Table S2).

3.5. In vitro total root length (TRL) and ABA concentration in root exudates

Under control conditions, TRL of sp12 was 2.5-fold less than the WT, while TRL of sp12 was more than double than that of the WT under saline conditions. Salinity decreased TRL of WT seedlings by 80\%, while TRL of sp12 roots was not affected (Fig. S1A). Under control conditions, ABA concentration in the growing medium surrounding the roots was higher in samples collected from sp12 (0.85 nM), than WT (0.005 nM) plates. Under salinity, ABA was only detected in WT exudates (8.3 nM) (Fig. S1B).

3.6. Root gene expression responses

Since NCED OE prevented salinity-induced root growth inhibition, the expression of a set of ABA, stress and root-development related genes was analyzed in this organ in the WT and the sp12 line under both control and salinity conditions.
ABA related genes

Under control conditions, the ABA-signalling related genes $WRKY70/WRKY6$, $ATHB12$ and $AREB1$ were significantly upregulated in sp12 roots compared to the WT. Additionally, salinity induced $ATHB12$ and $AREB1$ expression to a higher level in sp12 than in WT, but there was no difference for $WRKY70/WRKY6$ (Fig 5A, 6). WT and sp12 roots had similar expression of ABA-biosynthetic ($ZEP1$, $FLC/AAO$, $DXS$) and catabolic ($CYP707A$, ABA 8'-hydroxylase) genes (Fig. 5A, 6) under control conditions. In contrast, salinity upregulated those genes in WT roots (3 to 300-fold), while they remained unchanged in sp12 roots compared to control conditions. Thus, in comparison to WT, sp12 roots show enhanced expression of some ABA-signalling related genes under control and salinity and salinity conditions. However, the salinity-induced increase in expression of ABA biosynthesis and catabolism genes observed in WT, does not occur in sp12 (Fig. 5A, 6).

Stress-related genes

Under control conditions, the osmotic stress-related genes $TAS14$, $PIP1.2$, $PRO2/P5CS$, $KIN2$ and $MYB$ were significantly upregulated in sp12 roots compared with the WT (Fig. 5B, 6). Salinity upregulated the $PRO2/P5CS$, $KIN2$ and especially $TAS14$ genes in sp12 roots compared to control conditions, while $MYB$ was inhibited, and $PIP1.2$ was not affected. All these genes reached similar expression levels under salinity in both genotypes, except $PRO2/P5CS$ expression that was 35% lower in sp12 roots than in the WT (Fig. 5B, 6).

Ethylene-related genes
Under control conditions, the expression of the ethylene biosynthesis gene *ACS1A* (encoding 1-aminocyclopropane-1-carboxylate synthase 1) was 9-fold higher in sp12 than in WT. After salinity treatment, *ACS1A* expression was induced >100-fold in WT, and in sp12 it also increased to match the WT level. *JERF1* (jasmonate and ethylene response factor), a member of the ERF family, was expressed 3.5-fold more in sp12 than in WT under control conditions, and, upon salinity treatment, the WT increased expression to match the sp12 control level, but the sp12 level remained unchanged (Fig. 5C, 6). Thus, *NCED* OE increased expression of ethylene synthesis and signaling components under control conditions, but the expression become similar between the two genotypes under salinity treatment (Fig. 5C, 6).

*Auxin-related genes*

Under control conditions, the auxin-related genes *IAASGH3* (indole-3-acetic acid-amido synthase GH3) and *ARF6* tended to be upregulated in sp12 compared to WT roots, while *LAX2*, *DFL1* and *GH3.3* were not affected (Fig. 5D, 6). Under salinity, *IAASGH3* and *GH3.3* were the most highly expressed genes in both genotypes (500- and 60-fold, respectively). Among other auxin-related genes, *LAX2*, *DFL1* and *ARF6*, their expression did not increase significantly under salinity treatment, whereas it did in WT. Together, these observations suggest that *NCED* OE led to the removal of active auxins by conjugation (*IAAsGH3*) under control conditions, and to the prevention of the salinity-induced activation of auxin signalling observed in WT.

*JA-related genes*

Under control conditions, the JA biosynthetic and responsive genes *LOX* and *JAI* were down-regulated while *JA2* was strongly (70-fold) upregulated in sp12 roots compared to
WT (Figs. 5E, 6). Salinity reduced LOX expression in both genotypes and had no effect on the JA1 transcription factor, which was 50% down-regulated in sp12 compared to WT. In contrast, the JA2 transcription factor was strongly and similarly up-regulated (140-200-fold) in both WT and sp12 under salinity (Figs 5E, 6).

GA-related genes

Under control conditions, the GA biosynthesis gene GA20ox-1 was down-regulated, and the GA deactivation gene GA2ox-3 gene was upregulated (3-fold) in sp12 compared to WT roots (Fig. 5F, 6), suggesting that sp12 roots might have less GA, although GA was not present at detectable levels in roots of WT or sp12 (Table S1). Salinity upregulated GA2ox-3, but downregulated GA20ox-1 (7.5-fold) in WT plants. However, neither the expression of GA2ox-3 nor that of GA20ox-1 responded to salinity in sp12 (Figs. 5F, 6).

To summarise, NCED OE in the absence of stress (no added salinity) induced stress-adaptive gene expression responses related to some processes, i.e. ABA signalling, osmotic adjustment, ACC and JA synthesis and GA and IAA deactivation. In some of these cases, salinity treatment did not result in any further increases in gene expression in sp12, presumably because expression in the absence of stress was already high (i.e. JA2, KIN2). In other cases there was an additive effect, where gene expression was higher in sp12 in both control and salinity treatments (i.e. ATHB12, AREB1). However, NCED OE also prevented salinity-induced gene expression of ABA metabolism, IAA signalling and GA deactivation, suggesting that sp12 had constitutive mechanisms that led to avoidance (or lack of perception of) some aspects of salinity stress.

4. Discussion
Constitutive ABA overproduction via \textit{NCED} OE induced complex changes in root gene expression and plant hormone levels and ultimately biomass and root development (Fig. 7). It is important to understand how these changes may affect resistance to salinity stress.

4.1. \textit{LeNCED1} overexpression limits growth of young plants in the absence of imposed stress, but maintains shoot growth and enhances total root length under salinity stress

\textit{Control treatment}

Limited root and shoot growth of the \textit{NCED} OE lines under control conditions (Fig. 1) was likely due to the higher ABA concentrations which can act to reduce growth directly through signalling pathways [33], may limit photosynthesis by inducing partial stomatal closure (there was a non-significant reduction in assimilation under control treatment; Fig. 2A), may deplete protective xanthophylls, or may perturb water relations. Although, early seedling establishment until the four-leaf stage was delayed, previously sp5 plants compared to WT had increased leaf area and maintained their biomass accumulation when grown for 10 weeks [12], indicating developmental differences in response to elevated ABA. The study reported here was performed with younger plants that may be more sensitive to ABA-mediated growth inhibition, so it will also be important to determine growth responses to salinity in older plants.

\textit{Salinity effects}

Despite the reduction in biomass for sp12 and sp5 under control conditions, salinized plants achieved similar growth and photosynthesis than WT (Figs. 1, 2). Thus, the sp12 and sp5 plants gave a smaller growth reduction percentage comparing control and
salinity treatments. Remarkably, sp12 produced 2.5-fold more TRL than WT under salinity, thus root system development was much less sensitive to salinity in sp12. This is in agreement with previous work on ABA deficient mutants where basal ABA production was shown to be required to maintain leaf and root growth under both salinity [23, 26] and drought [8] conditions. Our study goes further to show that higher levels of ABA through transgenesis can reduce the impact of salinity on growth, particularly TRL (Fig. 1, S1), and this is an improvement in relation to the WT response.

4.2. The impact of LeNCED1 overexpression on ABA accumulation

Constitutive LeNCED1 gene expression increased leaf and especially xylem ABA concentrations in sp12 and sp5, and there was a stronger interaction between xylem ABA and salinity treatment in the sp12 and sp5 lines than in the WT (Fig. 3). Xylem ABA in recently detopped plants could have arisen partly through synthesis in the shoot (i.e. ABA imported before detopping), or from the root according to models of recirculation [34]. But grafting experiments clearly showed that root-synthesized ABA is not required for stomatal closure [35].

However, for roots, ABA concentration was not elevated in sp12 or sp5 in control treatment, nor did it increase under salinity in WT or sp12, but only in salinity-treated sp5 (Fig. 3, Table S3). This is surprising because in other studies the root ABA concentration was ~50% higher in sp12 roots compared to WT in both grafted whole plants and in root cultures[36], and 80% higher in roots from non-grafted whole plants [27]; indeed, the LeNCED1 gene expression was previously confirmed to be elevated 108-fold and 203-fold relative to WT in cultured roots of sp12 and sp5, respectively [36]. Salinity is also known to increase root ABA by 60-80% in other studies [26]. So,
in the present study there may have been unknown environmental interactions that prevented salinity and the NCED OE from causing additional accumulation of root ABA.

4.3. NCED OE prevents salinity-induced gene expression for ABA metabolism genes

ABA might regulate its own accumulation via feedback mechanisms that regulate catabolism via changes in the expression of CYP707A [37-39]. Also ABA is reported to stimulate expression of ABA biosynthesis genes in Arabidopsis by positive feedback [40]. As mentioned above, we found that, in sp12 roots, there was no accumulation of ABA relative to WT, excluding the possibility of feedback mechanisms mediated by ABA concentration in the root. In fact, expression of ZEP1, FLC/AAO and DXS were not significantly higher in sp12 than in WT roots under control or saline conditions (Fig. 5A), indicating no positive feedback. Nevertheless, surprisingly, the sp12 transgene prevented the induction of expression of ABA biosynthesis (ZEP1, FLC/AAO, DXS) and catabolism genes (CYP707A) that occurred under salinity in WT roots (Fig. 5A). We speculate that a change in distribution of ABA, an increase in the flux of ABA, or a difference in ABA content not detected at the 11 or 21 DST time points in sp12, may have triggered an unknown negative feedback signal or other adaptation that prevented the salinity treatment from activating these genes. Root, leaf and xylem sap Na⁺ concentration was elevated to a similar level in both sp12 and WT under salinity treatment (Table S2), so it is unlikely that stress avoidance was the reason for the absence of salinity-induced gene expression.
4.4. Salinity enhanced gene expression of ABA biosynthesis and catabolism genes, but ABA level remained the same

Arabidopsis CYP707A loss-of-function mutants had enhanced ABA levels and lower transpiration rates, with a similar phenotype to NCED OE lines including up-regulation of some ABA-inducible stress-related genes (TAS14, ATHB12, AREB1) under salinity [38]. These loss-of-function mutants were hypersensitive to exogenous ABA, presumably because of reduced catabolism of the applied ABA, while Pro35S:CYP707A OE plants were ABA-insensitive, consistent with their expected ABA catabolism. Thus, the large increase in CYP707A expression that we observed under salinity treatment in WT roots (Fig. 5A) would depress ABA levels. Furthermore, the salinity treatment induced gene expression for both ABA synthesis (ZEP1, FLC/AAO, DXS) and catabolism (CYP707A) in WT roots, and the ABA level remained the same, suggesting an increased flux (high synthesis and high catabolism), or a balancing of import/export of ABA provided a homeostatic mechanism.

4.5. Expression of ABA signaling-related genes is enhanced in non-stressed sp12 roots

Upregulation of various genes under control treatment (WRKY70/WRKY6, ATHB12 and AREB1) in sp12 suggests enhanced constitutive ABA signalling compared to WT plants (Fig. 5A). WRKY proteins have been associated with stomatal regulation and stress tolerance, and modulate gene expression in the ABA signalling pathway [41], with ABA, drought, salinity and AREB OE upregulating the WRKY70/WRKY6 gene [42]. Thus, WRKY70/WRKY6 could be a signaling intermediate involved in the reduction of stomatal conductance in sp12. ATHB12 is an ABA and abiotic stress inducible homeodomain-leucine zipper protein that negatively regulates stem elongation by down-regulating the GA20ox1 gene (Fig. 5F) and GA synthesis [43].

However,
ATHB12 overexpression also promotes both leaf and root growth through increased cell expansion and endoreduplication in Arabidopsis [44], and it is possible that ATHB12 could have a role in the enhanced leaf area as reported previously in sp5 plants [27].

4.6. Sp12 plants upregulate stress protection-related processes under control conditions

Several osmotic stress-related genes (PRO2/P5CS, TAS14, PIP1.2, KIN2, and MYB) were also upregulated in sp12 roots under control conditions compared to WT (Fig. 5B). These genes are induced by ABA, abiotic stresses and in AREB OE plants and they contribute to drought and salinity tolerance through proline (PRO2/P5CS), sugar and K+ (dehydrin TAS14) mediated osmoregulation [42, 45-47], CO2 transport facilitation (aquaporin PIP1.2), Ca2+ regulation (LEA protein KIN2), and stress-mediated ABA biosynthesis (MYB) [48]. Although these proteins may play a protective role in sp12 roots before and during the stress, both sp12 and WT plants had similar leaf water and osmotic potential (Fig. S2), and K+ and Ca2+ concentrations (Table S2). Constitutive expression of these genes may limit the growth of sp12 under control conditions, depending on the developmental stage and endogenous sensitivity to these factors.

4.7. Ethylene synthesis and/or signaling are induced in sp12 roots

Although ABA downregulates production of the growth inhibitor ethylene [8, 49, 50], the ACS1A gene was surprisingly induced under control conditions in sp12 roots (Fig. 5C). Nevertheless, ACC did not accumulate in sp12 roots (Table S1), likely due to its rapid conversion into ethylene or alternative conjugation pathways. While upregulation of the ethylene-responsive transcription factor JERF1 gene (Fig. 5C) suggested
enhanced ethylene signalling, ABA and salinity may also directly induce the *JERF1* gene [51-53]. Interestingly, *JERF1* overexpression before or during stress increased or maintained leaf and root growth of salinized plants by interacting with stress responsive (i.e. proline synthesis) and ABA biosynthesis genes [52-54]. Thus, constitutive induction of *JERF1* may enhance salinity tolerance in sp12.

Salinity significantly increased *ACS1A* gene expression in both WT and sp12 plants, consistent with enhanced ACC concentrations throughout the plant [18] (Table S1). Pronounced salinity-induced root ACC accumulation suggests that ACC may act as a root-to-shoot signal [55], although reciprocal grafting studies with transgenic plants in which ACC synthase is down-regulated [56, 57] are required.

### 4.8. Changes in auxin inactivation and signalling in sp12 are consistent with repression of lateral roots under control conditions while inducing them under salinity

Salinity reduces primary root growth and induces lateral root development to enhance resource capture while limiting salt acquisition, a hormonally regulated process in which auxin is key [1, 9]. While ARF-mediated transcription factors are required for lateral root formation [58], the *GH3* gene family encodes proteins that regulate auxin, jasmonic acid, and salicylic acid levels via amino acid conjugation for degradation/storage (auxins) or activation (jasmonates) [59, 60]. Interestingly, salinity induced auxin-related genes (*IAAsGH3, LAX2, DFL1, ARF6, GH3.3*) (Fig. 5D) in WT roots, suggesting that auxin conjugation (*IAAsGH3, DFL1* and *GH3.3*) increased root activity and potentially lateral root formation.

Constitutive ABA production (sp12) upregulated the auxin deactivation pathway (*IAAsGH3*) under control conditions, but downregulated other genes (*LAX2, DFL1, 
ARF6, but not IAAsGH3 and GH3.3) under salinity (Fig. 5D). Upregulation of IAAsGH3 and GH3.3 could limit root growth in sp12 (control) and WT (salinity) plants. However, greater root development of sp12 under salinity (Fig.S1A) can be explained by down-regulation of LAX2, DFL1 and ARF6, along with induced IAAsGH3 and GH3.3, suggesting that these genes have a limited role in auxin-mediated lateral root formation or that the IAASGH3 (SIGH3.3) is required for this process, as in Arabidopsis. Although ABA, IAA and salinity induce the GH3.3 (Solyc01g107390) gene in tomato [29, 61], its Arabidopsis homologue is required for adventitious root development by modulating JA catabolism downstream of the auxin signal [62]. Hence, further experiments are required to determine whether salinity stress and GH3.3 expression are linked, and whether this gene affects tomato root architecture.

ABA or abiotic stress also induces some MYB transcription factors involved in lateral root formation [63, 64]. Under control conditions, MYB gene induction was 2.5-fold higher in sp12, but salinity repressed MYB expression in both genotypes (Fig. 5B). Under control conditions, genotypic differences in total root length (Fig. S1A) were inversely related to MYB expression, but not under salinity where MYB down-regulation was related to enhanced root growth of sp12, but not WT plants (Fig. S1A). Similarly, elevated endogenous ABA and overexpression of MYB transcription factors PtrSSR1 and R2R3 inhibited lateral root emergence and plant growth under normal conditions in Arabidopsis and tomato, but improved salt tolerance [65-67]. Thus, MYB factors seem to integrate ABA level to regulate root development and sensitivity to salt stress.

4.9. Antagonistic ABA-JA interactions in sp12 roots

Firstly, the LOX and JA1 genes involved in JA biosynthesis and plant defense were downregulated in sp12 roots (Fig. 5E), probably due to ABA synthesis [68, 69].
Secondly, although JA synthesis/signalling is required for root ABA accumulation [70], the inverse response does not apply as genotype (and thus ABA status) did not affect JA levels (Fig. 4). In contrast, salinity consistently down-regulated the LOX gene and decreased root JA levels (Fig. 4C, F), while transiently increased xylem JA concentrations (Fig. 4B). Although root-to-shoot JA transport can induce stomatal closure in tomato [71], JA concentrations were not correlated with stomatal conductance (Table 2). Nevertheless, the NAC transcription factor JA2 is activated by JA, ABA, drought and salinity [29, 72, 73], and promotes stomatal closure by inducing expression of the ABA biosynthetic gene NCED1. Indeed, the JA2-NCED1 transcriptional module might act as a regulatory loop to monitor endogenous ABA status [72, 73], contributing to stomatal closure in sp12 under control conditions. However, full activation of the JA2-NCED1 module by dehydration requires a basal level of ABA, while transient accumulation of JA and SA are involved in ABA biosynthesis [74].

4.10. Salinity induced gene expression of GA deactivation in sp12 roots

Salinity induces the GA2ox-3 gene, encoding a putative GA2 oxidase-3 involved in GA catabolism [75] in tomato roots [29]. Moreover, it was also strongly induced in sp12 roots in control conditions, which may explain their reduced growth, even if root GA concentrations were not detected (Table S1). Limited salinity induction of this catabolic gene in sp12 is consistent with the relative maintenance or increase of root growth, compared to WT (Fig. 1, S1A). Conversely, the opposite response of the GA biosynthetic gene GA20ox1 supports the idea that GA metabolism and signalling
constitute an important ABA-mediated growth regulatory check-point in response to salinity, similar to processes involved in overcoming seed dormancy [76].

5. Conclusions

Based on these results, the additional ABA synthesized by NCED OE lines (Fig. 7) under control conditions closes stomata (ABA, JA and ethylene), reduces shoot and root growth (associated with GA and IAA deactivation) and activates osmotic-related responses (dehydrins and LEA proteins, proline, aquaporins, transcription factors). Under saline conditions, growth of the NCED OE lines is less affected than WT, and TRL outperforms WT. NCED OE appears to dampen the normal plant response of upregulating genes for ABA synthesis and catabolism, but maintains the induction of other stress-adaptive processes (dehydrins, aquaporins, JA2, JERF1, root growth). Further research is required to fully understand and exploit molecular responses of roots to salinity; this will inform strategies for engineering and selecting genotypes with optimum hormonal and signaling behavior under saline conditions.

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Figure legends

Figure 1. Mean +/- standard errors of shoot fresh weight (A), root fresh weight (B) and total fresh weight (C) of WT (AC) and NCED OE plants (sp12 and sp5) growing under control and salt conditions (100 mM NaCl) for 21 days. Different letters indicate significant differences among genotypes and treatments according to the Tukey test \((n = 6, P < 0.05)\). Results of two way ANOVA (p values reported) for genotype (G), treatment (T) and their interaction (G x T) are indicated in the top right of the panel. *, ** and *** indicate statistically significant difference at \(p<0.05\), \(p<0.01\) and \(p<0.001\), respectively.

Figure 2. Mean +/- standard errors of photosynthesis (A) (A) and stomatal conductance \((g_s)\) (B) of WT (AC) and NCED OE plants (sp12 and sp5) growing under control and salt conditions (100 mM NaCl) for 14 days. Different letters indicate significant
differences among genotypes and treatments according to the Tukey test \( n=6, \ P \ < 0.05 \). Results of two way ANOVA (p values reported) for genotype (G), treatment (T) and their interaction (G x T) are indicated in the top right of the panel. *, ** and *** indicate statistically significant difference at \( p<0.05 \), \( p<0.01 \) and \( p<0.001 \), respectively.

Figure 2

![Figure 2](image)

**Figure 3.** A Mean +/- standard errors of abscisic acid (ABA) concentrations in leaf (A, D), root xylem sap (B, E) and root (C, F) of the WT (AC) and NCED OE plants (sp12 and sp5) growing under control and salt conditions (100 mM NaCl) for 11 (A, B, C) and 21 (D, E, F) days. Different letters indicate significant differences among genotypes and treatments according to the Tukey test \( n=6, \ P \ < 0.05 \). Results of two way ANOVA for each time point (p values reported) are indicated in the top left of the panel. *, ** and *** indicate statistically significant difference at \( p<0.05 \), \( p<0.01 \) and \( p<0.001 \), respectively. DST= days of salt treatment.
Figure 4. Mean +/- standard errors of jasmonic acid (JA) concentrations in leaf (A, D), root xylem sap (B, E) and root (C, F) of the WT (AC) and NCED OE plants (sp12 and sp5) growing under control and salt conditions (100 mM NaCl) for 11 (A, B, C) and 21 (D, E, F) days. Different letters indicate significant differences among genotypes and treatments according to the Tukey test (n=6, \( P < 0.05 \)). Results of two way ANOVA for each time point (p values reported) are indicated in the top left of the panel. *, ** and *** indicate statistically significant difference at \( p<0.05 \), \( p<0.01 \) and \( p<0.001 \), respectively. DST= days of salt treatment.
Figure 5. Real-time PCR quantification of the expression of selected genes in roots of WT (AC) and NCED OE plants (sp12) growing under control and salt conditions (100 mM NaCl) for 21 days (a-f). Bars indicate the relative expression levels. Different lowercases letters indicate significant differences between WT (AC) and sp12 within control treatment, and different upercases letters indicate significant differences between WT (AC) and sp12 within salt treatment. * indicate significant differences between control and salt treatment within each genotype according to the Mann-Whitney U test (p<0.05).
Figure 6. Relative expression for the analysed genes of sp12 plants compared to WT (AC) plants under control (blue) and salt (red) conditions. Colour intensity indicates down-regulation (low intensity, -), unchanged (intermediate intensity, 0) and up-regulation (high intensity, +) gene expression.
Figure 7. Proposed model to explain growth and adaptive responses in NCED OE (sp12 line) plants through up (filled lines) and down (dashed lines) regulation of genes and physiological processes under control (blue and green color lines) and saline (red and green color lines) conditions. NCED OE plants respond to ABA in absence of stress by upregulating ABA, jasmonic acid (JA) and ethylene-related genes (WRKY6/WRK70, ATHB12, AREB1, JA2, ACS1A, JERF1) associated with stomatal closure, gibberellin (GA) and auxin homeostasis genes (upregulating GA2ox-3 and IAASGH3, inhibiting GA20ox-1) associated with growth limitation and activating osmotic-related responses (dehydrin TAS14, proline synthesis PRO2/P5CS, aquaporin PIP1.2, LEA protein KIN2, transcription factor MYB). Moreover, NCED OE decreases sensitivity of growth to saline stress by downregulating ABA metabolism (CYP707A, ZEP1, FLC/AAO, DXS) and alleviating GA (GA2ox-3) and auxin (ARF6, LAX2, DFL1) deactivation, but maintaining or inducing ABA signalling (ATHB12 and AREB1) and stress-adaptive
processes (dehydridin TA14, aquaporin PIP1.2, KIN2, JA2, JERF1). Specific genes in red indicate up (bold characters) and down (normal characters) regulation under salinity, compared to WT. Arrow and bar heads indicate positive and negative regulation, respectively.

Figure 7
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*Genes previously described in [29].
Table 2. Linear correlation coefficients between shoot fresh weight (SFW), root fresh weight (RFW), photosynthesis (A), stomatal conductance (g_s), abscisic acid (ABA) and jasmonic acid (JA) concentrations in leaf, root xylem sap and root of WT and NCED OE plants (sp12 and sp5) growing under control and salt conditions (100 mM NaCl) after 15 days of treatment. *, ** indicate that correlations are significant at p<0.05 and p<0.01, respectively.

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