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The tomato *res* mutant which accumulates JA in roots in non-stressed conditions restores cell structure alterations under salinity

José O. Garcia-Abellan[a,†], Nieves Fernandez-Garcia[a,†], Carmen Lopez-Berenguer[a], Isabel Egea[a], Francisco B. Flores[a,*], Trinidad Angosto[b], Juan Capel[b], Rafael Lozano[b], Benito Pineda[c], Vicente Moreno[c], Enrique Olmos[a] and María C. Bolarin[a]

[a]Stress Biology and Plant Pathology Dpt., CEBAS-CSIC, P.O. Box 164, 30100 Espinardo-Murcia, Spain

[b]Agro-Food Biotechnology Research Centre (BITAL), University of Almería, La Cañada de San Urbano, 04120 Almería, Spain

[c]Plant Biotechnology and In Vitro Culture Dpt., IBMCP-UPV/CSIC, Camino de Vera s/n, 46022 Valencia, Spain

*Corresponding author, e-mail: borjaflores@cebas.csic.es

[†]Both authors contribute equally to this work

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Jasmonic acid (JA) regulates a wide spectrum of plant biological processes, from plant development to stress defense responses. The role of JA in plant response to salt stress is scarcely known, and even less known is the specific response in root, the main plant organ responsible for ionic uptake and transport to the shoot. Here we report the characterization of the first tomato (*Solanum lycopersicum*) mutant, named *res* (*re*stored cell structure by *s*alinity), that accumulates JA in roots prior to exposure to stress. The *res* tomato mutant presented remarkable growth inhibition and displayed important morphological alterations and cellular disorganization in roots and leaves under control conditions, while these alterations disappeared when the *res* mutant plants were grown under salt stress. Reciprocal grafting between *res* and wild type (WT) (tomato cv. Moneymaker) indicated that the main organ responsible for the development of alterations was the root. The JA-signaling pathway is activated in *res* roots prior to stress, with transcripts levels being even higher in control condition than in salinity. Future studies on this mutant will provide significant advances in the knowledge of JA role in root in salt stress tolerance response, as well as in the energy trade-off between plant growth and response to stress.

Abbreviations – ANOVA, analysis of variance; COI1, coronatine insensitive 1; GC-MS, gas chromatography coupled to mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectrometry; JA, jasmonic acid; JAR1, jasmonoyl isoleucine conjugate synthase 1; JAZ1, jasmonate ZIM domain protein 1; LOXD, lipoxygenase D; LSD, least significant difference test; MeJA, methyl-jasmonate; MS, Murashige-Skoog medium; NaCl, sodium chloride; RH, relative humidity; SE, standard error; TEM, transmission electron microscopy; UBI, ubiquitin; WT, wild-type.

Introduction

Development of crops tolerant to stress is vital to meet the growing food demand through sustainable agriculture (Godfray et al. 2010), since traditional agriculture and conventional breeding cannot uphold this increasing demand, partly because of the increasing salt stress impact due to environmental and climatic changes (Shaik and Ramakrishna 2014). Indeed, increased salinization of arable land is expected to have devastating global effects, resulting in losses of 30% within the next 25 years, and up to 50% by the year 2050 (Bergougnoux 2014). Tomato is the seventh most important crop species, and its production as well as the area turned over to its cultivation has doubled during the last 20 years (Ichihashi and Sinha 2014). Moreover, this species has also become a plant model in agronomic research programs (Ranjan et al. 2012). The simplicity of its genetics with a relatively small genome (estimated to be approximately 900 Mb for the inbred tomato cultivar 'Heinz 1706' used in the recent sequencing of the tomato genome, The Tomato Genome Consortium 2012) has been a highly valued feature in choosing it as a model plant. However, the mechanisms that govern

responses to abiotic stresses in this horticultural species are not well characterized and only a very small number of genes playing a role in tomato tolerance to salinity have so far been identified (Pineda et al. 2012).

Plants are sessile organisms that need to adapt to a changing environment. The specific plant response to a particular stimulus, crucial for its survival, is mediated by plant hormones, and one of these is jasmonic acid (JA). The first step in JA biosynthesis, from alpha-linolenic acid, is catalyzed by 13-lipoxigenase (LOX). Once induced by environmental or developmental signals, JA can be further converted into numerous conjugates, including the bioactive agent jasmonoyl-isoleucine (JA-Ile) that is perceived by a receptor complex consisting of SCFCOI1 with binding sites for IP5 (Thines et al. 2007, Chini et al. 2009, Sheard et al. 2010). The currently accepted model of JA signaling is one where JAZ plays a role as repressor of expression of JA-responsive genes and its degradation via the proteasome releases this gene expression (Fonseca et al. 2009). Specifically JAZ degradation relieves the inhibition of MYC2 transcription factor, leading to activation of JA-responsive genes (Pauwels and Goossen 2011). Recent reports have identified other JAZ-interacting transcription factors that cooperate in the above scheme to determine the specificity of JA-mediated responses (Fernández-Calvo et al. 2011, Qi et al. 2011, Song et al. 2011), but not every molecular component involved in JA signaling has been identified. Indeed, some JA-responsive genes are COI1-independent, and loss of function of MYC2 does not completely impair JA sensitivity, which suggests the possible existence of unidentified components involved in JA signaling (Svyatyna et al. 2014).

Analysis of mutants, mainly from *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) deficient in JA biosynthesis or signaling, has been extremely useful for the dissection of JA functions (Wasternack 2007, Browse 2009, Chini et al. 2009). Root growth inhibition is one of the first phenotypical effects detected when JA is applied in plants, so JA-insensitive Arabidopsis mutants like *jar1* or *jai1* are similar in root length upon methyl-jasmonate (MeJA) treatment to untreated wild type (Wasternack 2007, Browse 2009). JA is considered a key regulator for expression of stress-responsive genes in virtually all plant species. There are numerous studies on JA-induced defense responses against insects and pathogens as well as mechanical wounding, but few on the responses against abiotic stresses (Wasternack and Hause 2013 and references therein). Recently, several studies have shown that JA positively regulates plant responses to freezing stress in *A. thaliana* (Hu et al. 2013); drought stress in rice (Seo et al. 2011) and other abiotic stresses (Grebner et al. 2013). Since JA regulates the expression of defense-related genes in plants, genetic engineering of plants to induce increased endogenous JA levels may provide a strategy to raise the activity of JA-dependent defense processes, as has been observed in tomato against insect attack (Chen et al. 2006, Yan et al. 2013). However, attempts to increase endogenous JA levels and thus JA-dependent resistance by overexpression of individual JA biosynthetic genes in tomato and other plant species have met with limited success, probably because JA levels are mainly controlled by substrate availability (Li et al. 2003, Stenzel et al. 2003). Thus, plants overexpressing genes coding for allene oxide synthase or

allene oxide cyclase did not exhibit high JA levels before wounding or other stimuli (Miersch et al. 2004), which ultimately demonstrates that JA biosynthesis is particularly regulated by the above-mentioned substrate availability (Stitz et al. 2011).

Plants must defend themselves effectively against biotic and abiotic stresses in order to survive in changeable environments, but this defense is energetically costly and as a consequence it is often accompanied by a significant growth inhibition. Thus, the activation of JA defense signaling severely restricts plant growth, representing a prominent example of growth-defense trade-off in plants (Mitra and Baldwin 2014). Moreover, JA-signaling pathway may be differently regulated in roots and shoots, which may in its turn cause differential responses in both organs, depending on where the initial JA signal was first perceived. In this regard, a great amount of research has been made regarding the role of JA-signaling pathway in shoot, while in root it is relatively less known (Erb et al. 2009, Grebner et al. 2013, Tytgat et al. 2013). Although knowledge of the JA-signaling pathway is important in both shoot and root, in some cases it can be of high priority in root. JA synthesis is critical for defense in *Arabidopsis* and maize (*Zea mays*) against *Pythium* species, which are soil-borne oomycetes (Staswick et al 1998, Vijayan et al. 1998, Yan et al. 2012). In tomato, it has been demonstrated that JA plays an important role in arbuscular mycorrhizal symbiosis (Lopez-Raez et al. 2010). However, the effect of the specific JA induction in root on the salt tolerance has not been practically investigated (Pedranzani et al. 2003).

The root has a key role in abiotic stress like salinity, since it is the first organ that enters in contact with salt and, consequently, that which is mainly responsible for its uptake and transport to the shoot of toxic ions. In order to advance in the understanding of salt tolerance mechanisms displayed by tomato we are screening mutants for identification of those affected in root phenotypes. Here we report the characterization of the recessive tomato (*Solanum lycopersicum*) mutant *res* (*re*stored cell structure by *s*alinity), affected in root development *in vitro* and with important leaf and root morphological alterations and cellular disorganization features *in vivo* under non-stress conditions. However, *res* mutant is able to restore root and leaf morphology and cell ultrastructure when plants are grown under saline conditions. Notably, *res* mutant accumulates JA in root but not in leaf under non-stressful conditions, with the transcript levels of genes involved in JA-signaling and biosynthesis pathways being even higher in *res* roots under control than under salt stress.

Material and methods

Isolation of the res mutant

Tomato (*Solanum lycopersicum* cv. Moneymaker) was used to generate a collection of mutants by using the enhancer trap vector pD991, which contains a constitutively expressed *nptII* marker gene conferring resistance against kanamycin (kan) antibiotic, and a reporter gene (*uidA*) that encodes for ß-glucuronidase (GUS) (Atares et al. 2011, Pineda et al. 2012), following the transformation method previously described (Gisbert et al. 2000). An *in vitro* screening for mutants affected in root

phenotype was performed on segregating progenies (T_1) from self-pollinated transformants (T_0), and the recessive *res* mutant was identified. The T_1 progeny segregated 3 Wild type (WT) : 1 Mutant for low root development in Murashige-Skoog (MS) medium (Murashige and Skoog 1962), denoting a single recessive mutation. Segregation analysis of the mutant was also analyzed in two lots of T_1 plants grown *in vivo*, obtaining again a phenotypic segregation of 3WT : 1Mutant. Furthermore, the monogenic and recessive inheritance pattern of the *res* mutant was also confirmed by evaluating T_2 offspring, where homozygous lines for the mutant phenotype were identified. A co-segregation analysis mutant phenotype : kanamycin (kan) resistance was carried out in the homozygous lines (T_2) by means of a germination test with seeds in MS medium (Duchefa) supplemented with 100 mg L^{-1} kan (Sigma-Aldrich). Since the mutant phenotype was not correlated with tolerance to kan, one homozygous line for the mutant phenotype but azygous for tolerance to kan was selected and used in the different characterization experiments. To confirm selection of the right line, an amplification by PCR of *nptII* and *uidA* genes in genomic DNA with specific primers was performed, using the tomato elongation factor 1α (*LeEF1α*, acc. AB061263) as endogenous gene (positive control) (Table S1). Also to check for the absence of reporter gene in the selected line, GUS histochemical determination was performed following Atares et al. (2011).

Phenotypic characterization in non-stress and salt stress conditions

For *in vitro* culture, seeds were surface-sterilized with 50% sodium hypochlorite and 3 drops of Triton X-100 (Sigma-Aldrich) for 30 min followed by 3 washes with sterile deionized water for 5, 10 and 15 min. The composition of the control growth medium used was ½ MS mineral salts (Duchefa) plus sucrose (10 g L^{-1}) (Panreac) and indole-3-acetic acid (1 mg L^{-1}) (Sigma-Aldrich). For salt treatments, the control medium was supplemented with the corresponding concentration of NaCl (50, 100 and 150 mM). Cultures were maintained under 16 h light / 8 h darkness photoperiod at a constant temperature of 25°C and light with a photosynthetic active radiation intensity of 100 µmol m^{-2} s^{-1} provided by fluorescent tubes (Sylvania GRO-LUX F36W/GRO –T8, Germany). Germination tests and seedling growth evaluation in terms of shoot and root weights were performed *in vitro* with mutant seeds germinated and seedlings grown in MS medium supplemented with 50 µM methyl-jasmonate (MeJA) (Sigma-Aldrich), using as controls of the experiments seeds and plantlets in MS medium from the *res* mutant and WT.

The *in vivo* phenotypic characterization of *res* was carried out in greenhouse, under conditions previously described (Garcia-Abellan et al. 2014). The salt stress experiments were also carried out in a controlled growth chamber with 16 h light / 8 h darkness photoperiod, with light of a photosynthetic photon flux density (400–700 nm) of 350 µmol m^{-2} s^{-1} at the plant level, provided by fluorescent tubes (Philips Master TL-D 58W/840 REFLEX, Holland), and 25°C and 50–60% of temperature and relative humidity (RH), respectively. Plants were grown with an aerated ½ strength Hoagland solution (Hoagland and Arnon 1950). Salt treatments (100 and 200 mM NaCl) were gradually applied to avoid

osmotic shock. Electric conductivity and pH were checked periodically and nutrient solution was changed once per week until plants have got 4–5 leaves and twice per week when plants were bigger than 6-leaves stage. Each sampling part of the plant material was divided in two subsamples; one was weighed and then dried to determine ion content while another was immediately frozen in liquid nitrogen and kept at –80°C for analyses of JA content and gene expression. At the end of the experiments, fresh weights of shoot and root were determined.

Reciprocal grafting

Wild-type (WT, untransformed tomato cv Moneymaker) and *res* mutant plants were grafted as previously described (Martinez-Rodriguez et al. 2008). Briefly, plants were obliquely cut under cotyledons in the rootstock and over the cotyledons in the scion, and both parts were joined with a grafting tube clip. During the first days, plants were covered with translucent plastic lids, thus the RH was increased until 90% in order to improve the grafting success. Both self-grafted plants of WT and mutant were used as controls. The grafted plants were grown in the same environmental conditions in the controlled-condition growth chamber, except for the photoperiod; after the first day, in which the grafted plants remained in dark conditions, the photoperiod was gradually increased over 3 days until the required final values were achieved (daylight length of 16 hours with a photon flux density of 350 μmol m^{-2} s^{-1}).

Microscopy analysis

Transmission electron microscopy (TEM)

Leaf sections (1 × 1 mm from the most recent fully expanded leaves) and root sections from the tip (1 x 2 mm) were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2.5 h. After three washes with the buffer, samples were post-fixed in 1% osmium tetroxide, in the same buffer, for 2 h. After this, three washes with phosphate buffer were performed. All fixed tissues were dehydrated in a graded series of ethanol (35, 50, 70, 96 and 100%), then infiltrated, first with propylene oxide and then with propylene oxide and Spurr's resin mixture. The samples were then immersed in Spurr's resin overnight at 4°C. The samples were finally transferred to flat embedding molds filled with Spurr resin and polymerized at 68°C for 24 h. Blocks were sectioned on a Leica EM UC6 ultramicrotome (Leica Mikrosysteme, Hernalser Hauptstraße, Vienna, Austria), collected on copper grids and stained with uranyl acetate followed by lead citrate. Sections were examined using a Philips Tecnai 12 transmission electron microscope (Philips, Eindhoven, The Netherlands) equipped with a CCD SIS MegaView III camera (1280×1280 pixel, 12 bit).

White light microscopy

Semi-thin sections (0.5–0.7 μm thick) of material prepared for TEM (see earlier) were cut with a Leica EM UC6 ultramicrotome. The sections were stained with toluidine blue: 2–5 min in 1% (w/v)

toluidine blue, in 1% borax solution, at 60°C. The sections were rinsed with water and air in a dust-free environment, before being mounted in DPX and observed with a Leica DMR light microscope (Leica Microsystems, Wetzlar, Germany).

The chemical reagents used in the microscopy process are from Electron Microscopy Sciences (Hatfield, USA).

Cation concentration analysis

The concentrations of Na^+ and K^+ were measured in plant material dried for 48 h at 80°C, milled to powder and digested in a concentrated HNO_3:$HClO_4$ (2:1 v/v) solution. In the digestion products Na^+ and K^+ were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) Thermo ICAP 6000 SERIES (Ionomics Platform from CEBAS-CSIC, Murcia, Spain) and results expressed as mmol kg^{-1} DW.

Chlorophyll content and fluorescence analysis

Chlorophylls content was analyzed by measuring chlorophylls fluorescence by means of a leaf chlorophyll meter SPAD-502 (Minolta, Kyoto, Japan). The Soil Plant Analysis Development (SPAD) units that the equipment renders are correlated with the plant chlorophylls content (Hoel and Solhaug 1998). Measures were taken in three different areas of the leaf and the averaged values were calculated. Chlorophylls fluorescence was analyzed by means of a portable Chlorophyll Fluorometer (Opti-Sciences, Hudson, NH, USA). This measurement gives us the maximal photochemical efficiency of photosystem II estimated as $F_v/F_m=(F_m–F_0)/F_m$, where the F_v/F_m was the ratio between the variable fluorescence and the maximal fluorescence. F_m is the maximal fluorescence intensity in leaves adapted to darkness during 30 minutes, induced by a far red light excitation source (3000 µmol m^{-2} s^{-1}) during 0,8 s. F_0 is the minimal fluorescence intensity due to the exposition of leaves to a actinic light source (400 µmol m^{-2} s^{-1}) (Maxwell and Johnson 2000).

Analysis of jasmonic acid and methyl jasmonate

Samples quantification were carried out at the Technical Services of the CSIC Institute in Seville (Instituto de la Grasa; http://www.ig.csic.es/index.php/contenido/ver/46/Servicio%20de%20Masas). The method used for the quantitation in plant tissue of JA and MeJA by gas chromatography – mass spectrometry (GC-MS) has been previously published (Rodriguez-Serrano et al. 2006, 2009).

Quantitative Real-time PCR analysis

For gene expression analysis, plant material was sampled, immediately frozen in liquid N_2 and kept under –78°C until analysis. Total RNA was extracted as described in Garcia-Abellan et al. (2014). Contaminating DNA was removed with RNAse-free DNase (DNA-free kit, Ambion) and RNA quality was assessed by electrophoresis on a denaturing agarose gel. Total RNA was quantified in a

GeneQuant II spectrophotometer (Pharmacia Biotech) and 5 µg were used for cDNA synthesis with First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-Time PCR was performed in a Rotor Gene 3000 cycler using Rotor-Gene® SYBR® Green PCR Kit following the manufacturer guidelines.

The expression levels of Lipoxygenase D (LOXD), Jasmonoyl Isoleucine Conjugate Synthase 1 (JAR1), Coronatine Insensitive 1 (COI1), Jasmonate ZIM Domain Protein 1 (JAZ1) and MYC2 genes were assessed by quantitative real-time qPCR using 1 µl of undiluted cDNA mixed with iQ SyBr Green Supermix (BioRad), and 0.45 µM of forward and reverse primers (Table S1) using assay conditions as previously described (Garcia-Abellan et al. 2014). All reactions were performed in triplicate with three different RNA extracts for each sample. Gene expression was quantified using the comparative method ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak 2008). Relative expression data were calculated from the difference in threshold cycle (ΔCt) between the studied genes and the internal control gene UBI (ubiquitin from tomato). To calculate the relative expression level the expression level in root of WT plants in absence of salt stress for 2 days was used as the calibrator sample (equal to 1).

Statistical analysis

Data were statistically analyzed using the SPSS 13.0 software package by one-way ANOVA and LSD or Student's *t* test (P <0.05). All data are given as mean ± SE (n = sample size). Significant differences between means were denoted by different lower case letters or asterisks.

Results

Identification and phenotypic characterization of *res* mutant

In the mutant population generated from the tomato cv Moneymaker (Pineda et al. 2012), one of our objectives was to identify mutants affected in root development, as root is the organ involved in the uptake and transport to the shoot of saline ions. First, the *in vitro* screening of this collection was carried out, where the *res* tomato mutant was identified. Subsequently, the selected lines were phenotyped *in vivo* in both conditions, control and salt stress. The segregating progeny (T_1) of the *res* mutant grown on MS medium segregated for root development, showing the mutant plants lower root growth than plants with WT phenotype, while apparently no phenotypic differences were found in the shoot (Fig. S1A). Thus, the weights were slightly lower in seedlings with mutant phenotype (1.7 ± 0.12 g) than in those with WT phenotype (2.4 ± 0.25 g). Subsequently, morphology and segregation analyses of the mutant were fulfilled *in vivo*, both in the transformed (T_0) and in the segregating (T_1) generations. T_0 plants exhibited similar plant morphology, growth rate and development characteristics to WT plants (Fig. S1B). In T_1 mutant plants exhibited slower shoot growth rate throughout their life cycle and chlorosis from the cotyledon stage, which progressed to leaf chlorosis as plant growth advanced (Fig. S1C). Mutation inheritance was first studied by growing 82 T_1 plants, of which 15 showed mutant phenotype and 67 presented WT phenotype, and the Chi-square test for

the expected ratio of 3 WT:1 mutant was 1.97 ($P > 0.5$). This was further confirmed in additional T_1 plants (98 plants) and the phenotypic segregation (79 WT : 19 mutant) was also concordant with a monogenic and recessive inheritance pattern of the mutant phenotype ($\chi 2 = 1.65$, $P > 0.5$)**.** Furthermore, samples were highly homogenous ($\chi 2 = 0.03$, $P > 0.5$) as indicated by the homogeneity analysis. The inheritance of the *res* mutation was also confirmed by evaluating T_2 offspring, since the reproductive development of *res* seems not to be affected by the mutation, producing flowers that are indistinguishable from WT ones and yielding fruits that, although smaller than WT, produce viable seeds (Fig. S1C).

By growing T_2 offspring, we identified homozygous lines for the mutant phenotype (all descendants having chlorosis from the cotyledon stage and very slow growth rate), hemizygous plants (segregating 3:1) and azygous plants (all descendants showing WT phenotype). A further verification of the recessive inheritance of the *res* mutation was performed by measuring total chlorophylls content in homozygous, hemizygous and azygous *res* plants and comparing these data with WT plants (Fig. S2). Azygous and hemizygous plants have similar chlorophyll content to WT plants, while a significant reduction in this content was observed in homozygous plants. Since *res* mutant comes from an insertional mutagenesis program applied in tomato with T-DNA and enhancer trapping, with *nptII* as marker gene (Atares et al. 2011, Pineda et al. 2012), we subsequently analyzed co-segregation of the mutant phenotype with resistance against kan conferred by *nptII*. We observed that *res* phenotype was not correlated with tolerance to kan and, therefore, the mutation was not tagged by a T-DNA insert. The following step was to identify among the T_2 generation homozygous lines for the mutant phenotype, in particular one homozygous line for the phenotype but azygous for tolerance to kan, a result that was confirmed by PCR on genomic DNA using specific primers to amplify the marker gene (*nptII*), as well as the reporter gene (*uidA*), which are characteristic components of the T-DNA cassette (Fig. S3A). GUS histochemical determination was also performed to check the identification of the T_2 line homozygous for the phenotype but azygous for the T-DNA insert by observing the absence of GUS staining (Fig. S3B). This line has been used in all experiments carried out for the characterization of the mutant.

The tomato *res* mutant exhibited phenotypic alterations in both shoot and root of plants grown *in vivo* (Fig. 1A). When plants were grown in hydroponic culture, the mutant exhibited a dwarf phenotype, with chlorotic leaves, as previously observed in T_1 progeny (Fig. 1B). But the most outstanding phenotypic alteration in the mutant was the impressive morphological alterations observed in the root (Fig. 1C), which had not been so clearly observed when plants were previously grown in inert substrate. Thus, *res* not only produced fewer and shorter roots, but also the root development was drastically altered. In order to elucidate whether the root was the organ responsible for the morphological alterations observed in the shoot or *vice-versa*, a reciprocal grafting experiment between WT and *res* was performed and the phenotypic response was compared with self-grafted

plants of each genotype. The graft-combination using WT as scion and *res* as rootstock shows that mutant root induced a severe reduction of shoot growth, with the negative effect being already evident 10 days after grafting (Fig. S4A). After 25 days the shoot weight of WT/*res* grafted plants was reduced by up to 60% with respect to the self-grafted WT plants. In contrast, grafting of mutant onto WT significantly increased shoot weight by up to 200% with respect to *res* self-grafted plants (Fig. S4B), although mutant leaves continued to be affected by chlorosis. These results suggest that root is mainly responsible for the shoot growth reduction in the mutant plant.

Salt response of *res* mutant is characterized by the disappearance of root and leaf morphological alterations and restoration of cell structure

The mutant showed salt stress tolerance when seedlings were grown *in vitro* at different NaCl levels, with the biggest differences between WT and *res* mutant being observed when the highest levels of NaCl were applied (Fig. S5A, B), when it is observed that the plant weights diverged the most, and were lower in WT than in *res* (Fig. S5B). *In vivo, res* plants grew much slower than WT, achieving important differences in plant weights under control conditions (Fig. S5C, D**).** However, when plants were grown at 200 mM NaCl for 15 days, WT plant growth was significantly reduced while *res* plants increased their shoot and root growth under salt stress, compared with control conditions**,** although the plant weight continued to be much lower in *res* mutant than WT (Fig S5D). Moreover, leaves from *res* became dark greenish after 15 days 200 mM NaCl, with a similar aspect to WT leaves (Fig. 2A), and the data of chlorophyll and fluorescence showed that mutant plants were able to achieve the normal levels of WT with salinity (Fig. S5E).

Microscopic studies were performed to dissect leaf anatomy of *res* vs. WT in control and salt stress (15 days 200 mM NaCl) conditions (Fig. 2B). The micrographs of transversal leaf sections from plants grown in unstressed condition revealed that cells in the leaf epidermis and the palisade parenchyma are totally disorganized in *res*, which makes it difficult to differentiate between palisade parenchyma and spongy parenchyma. The most striking feature at this level is that mutant leaves recovered the typical structure of WT plants when grown in saline conditions. Since the mutant is deficient in chlorophylls, we decided to further explore the ultrastructure of chloroplasts by TEM. WT leaves in the control condition showed well-organized chloroplasts with starch grains (Fig. 3). The grana stacks of chloroplasts were typically composed of 5–10 thylakoids, with a significant number of plastoglobuli (Fig. 3A, B). However, *res* chloroplasts clearly showed a disorganized ultrastructure, especially showing dilated thylakoids (Fig. 3C–F). When WT leaves were grown in saline conditions (200 mM NaCl), the ultrastructure of the WT chloroplasts was significantly altered (Fig. 4A, B). The most evident effect of salt treatment was the reduction of starch grain and dilatation of the thylakoid lumen in salt treatment. Interestingly, *res* chloroplasts under salt treatment recovered a similar ultrastructure to WT; some were reorganized, showing well-stacked grana similar to WT (Fig. 4C, D), while others presented altered grana with dilated thylakoids (Fig. 4E, F). Thus, *res* chloroplasts after

10 days of 200 mM NaCl had become indistinguishable from WT ones in salt stress condition.

In roots, the morphological alterations observed in *res* mutant under control condition also disappeared when plants were exposed to salinity (Fig. 5A, B). After 10 days at 200 mM NaCl, mutant roots had completely recovered a normal morphology: *res* root primordia were developed in truly secondary roots, instead of being arrested in this developmental stage, as occurred in control condition. A close observation of the *res* root tips revealed a thicker area in the control condition, which disappeared when the mutant was subjected to salt stress. At microscopic level, in *res* transversal root sections a patent alteration in the pericycle showing abundant anomalous root primordia and vascular bundles was observed in the control condition. In salt stress the *res* root section microscopic visualization revealed the recovery of the normal structure in the vascular cylinder, but still some primordia development was detected. It is also interesting that this root morphological transformation process in *res* seemed to be completely dependent on the salt stress condition, since *res* root alterations reappeared again in mutant plants grown 10 days in salt stress condition and afterwards for 5 days in the control condition (Fig. 5A). The micrographs of *res* root sections after passing from salt stress to control condition also confirmed the formation of new primordia in the pericycle, which could be responsible for the new root alterations macroscopically observed (Fig. 5B). These results indicate that salinity induces normalization of leaf and root morphology.

The salt tolerance of *res* mutant does correlate with increased accumulation of K^+

To physiologically characterize the response of the mutant to salinity we analyzed Na^+ and K^+ contents in roots and leaves, analyzing separately the developing or young leaves and the 3rd completely developed or adult leaves. At 200 mM NaCl, root and leaf K^+ concentrations of the salt-treated plants for 15 days were significantly higher in *res* plants with respect to WT, whereas no differences were found between both genotypes in control conditions (Fig. 6A). Na^+ concentrations were generally similar in mutant and in WT, except for a significant decrease in the young leaves of the salt-treated *res* plants, compared with WT ones (Fig. S6A). Under control conditions, no differences were found in the Na^+/K^+ ratio of WT and *res*, while this ratio was significantly lower in *res* compared with WT in all three plant organs analyzed under salinity, achieving a ratio of 2.1 in young leaves of *res* mutant, while in WT the ratio was 8.6 (Fig. S6B).

To corroborate this maintenance of K^+ homeostasis in *res* under salt stress, K^+ concentration was also analyzed in the reciprocal combinations of grafted plants between WT and *res*, before applying salt stress and after 15 days at 200 mM NaCl (Fig. 6B). Similar values were found in the different grafting combinations in non-stress conditions (data not shown). However, K^+ concentrations of root and young and adult leaves (3rd developed leaf) of salt-treated plants increased not only in *res* self-grafted plants but also in the grafted plants using WT as scion and *res* as rootstock.

The *res* tomato mutant accumulated high JA levels in root but not in leaf in absence of salt stress

The phenotype of arrested root growth and altered root architecture in *res* mutant under control conditions could be due to a hormonal alteration in *res* root, especially auxins and JA. Preliminary *in vitro* assays with WT and *res* mutant in medium without and with auxins and JA showed that there were only evident changes in root development of the mutant by JA, but not by auxins (data not shown). Thus, when WT and *res* seeds were germinated in MS medium without and with 50 μM MeJA, no significant differences were found in the germination rate of *res* and WT seeds in MS medium, while the germination percentage decreased significantly more in WT than mutant when MeJA was added to MS medium (Fig. S7A). Also the reduction of root and shoot growth in seedlings by treatment with MeJA was evaluated (Fig. S7B). MeJA treatment did not affect root weight in *res*, while WT suffered a notable reduction (72%). A similar trend was observed in shoot growth for WT and *res*, although the reduction percentage was lower in *res* mutant. These results together with the root alterations in *res* mutant suggested that *res* mutant may be accumulating JA without activation by a stressful condition.

To confirm this hypothesis, JA and MeJA were measured in both roots and adult leaves (the 1st and 2nd developed leaves) of WT and mutant plants grown without stress and after 15 days at 200 mM NaCl (Fig. 7A, B). The endogenous level of JA in root was more than 6-fold higher in mutant than in WT plants under control condition. Moreover, JA levels in mutant plants were maintained in both conditions, without and with salt stress application. Meanwhile, JA content significantly increased with salinity in WT roots, although it was significantly lower than that of mutant roots. In contrast, leaf JA content was lower in *res* than in WT under the control condition, while the opposite behavior was observed under salt stress. In this experiment, MeJA levels were also analyzed, as one of the most common reactions in JA metabolism consists of its methylation at the carboxyl group to yield MeJA. Root MeJA content was higher in *res* with respect to WT only under control conditions, while this was reduced more than 50% under salinity in *res* compared with WT. In leaf, reduction of MeJA content was observed in the mutant, compared with WT, without and with salt stress. Regarding the relative distribution of JA and MeJA, opposite tendencies were shown by WT and *res* mutant (Fig. 7C); while JA in WT was mainly metabolized to MeJA, in agreement with the higher percentages of MeJA (except for leaf in control condition), *res* exhibited higher contents of JA than MeJA under both situations, without and with stress, in both plant organs analyzed, roots and leaves.

Roots of the *res* mutant increase the gene expression of the biosynthesis and signaling JA pathways

The expression analysis of genes involved in the biosynthesis and signaling pathways of JA was performed in roots of *res* and WT plants grown in control and salt stress condition (200 mM NaCl) for 2 days (prior to the reorganization of the root morphology), and after 15 days of salt treatment (when

the process has been completed). Among the genes encoding enzymes in the JA biosynthetic pathway, we tested the gene involved in the first step of JA biosynthesis from α-linolenic acid, *Lipoxygenase D* (*LOXD*) (Fig. 8A). Under control conditions, the higher amount of transcript was found in the youngest *res* plants (2 days), while under salt stress the expression level of *LOXD* increased significantly in the mutant at the longest time of salt stress (15 days).

Within the signaling pathway, the target genes selected for this study are *Jasmonoyl Isoleucine Conjugate Synthase 1* (*JAR1*), *Coronatine Insensitive 1* (*COI1*), *Jasmonate ZIM Domain Protein 1* (*JAZ1*) and finally the gene coding for MYC2, the bHLH-type transcription factor that controls the final step in the molecular activation of JA-responsive genes. The expression of *JAR1* increased significantly after 2 days in *res* when compared with WT, in control as well as in salt stress conditions, but these differences were not maintained after 15 days (Fig. 8B). No changes in *COI1* expression were observed at any time point between both genotypes and conditions (data not shown). The most remarkable and significant changes were found in *JAZ1*, as its expression level was significantly higher in *res* mutant than WT after 2 days and especially after 15 days (Fig. 8C). Finally, the *MYC2* expression only increased in roots of youngest *res* plants compared with WT when grown under control conditions (Fig. 8D).

Discussion

One of the key signal molecules integrating the regulation of stress response and of plant development is JA. Some mutants overproducing JA were identified in *A. thaliana*, such as *cev1*, *cet* and *cex*, which presented reduced root length similar to JA-treated plants (Wasternack 2007), as observed in *res* plants grown *in vitro*. But several differences arising between the phenotypes observed in *A. thaliana* mutants overproducing JA and that of *res* suggest that this new tomato mutation targets a different gene modulating the JA-response. Thus, when *res* plants were grown *in vivo*, we observed important morphological alterations and cellular disorganization in its leaves, with chlorophyll loss and alteration of the chloroplast structure (Figs. 1, 3 and S2). This phenotype is, to a certain degree, similar to the alterations induced in tomato leaves when treated with the phytotoxin coronatine (Uppalapati et al. 2005), although *res* also showed very important alterations in root, with the pericycle and stele cells being totally disorganized (Fig. 5). Interestingly, when JA was analyzed in roots and leaves of *res* plants, we observed that the plant organ presenting higher JA basal level in *res* was the root but not the leaf (Fig. 7A, B). Furthermore, reciprocal grafting between WT and *res* as scion : rootstock revealed strong evidence that the mutant root inhibits the WT shoot growth, while the inverse combination of grafted plants (*res* as scion and WT as rootstock) did increase shoot growth of the *res* mutant (Fig. S4). These findings suggest that JA is synthesized in the root independently of the shoot, and the important alterations at the cellular level observed in the mutant shoot may be due to the root. The mutation responsible for the *res* phenotype has still not been located, since the mutated gene is not tagged by a T-DNA insert even if it has been identified in the

scrutiny of a collection of T-DNA insertional mutants of tomato (Pineda et al. 2012). The occurrence of somaclonal mutations without disruption of the gene by a T-DNA insertion has long been observed since the strategy of insertional mutagenesis started to be implemented in *A. thaliana* (Koncz et al. 1992, Van Lijsebettens et al. 1991). Subsequently, a significant number of somaclonal mutants have been detected in other insertional mutagenesis programs such as those of rice and the model legume *Medicago truncatula* (Izawa et al. 1997, Jeong et al. 2002, Tadege et al. 2008). Currently, strategies of positional cloning and whole-genome sequencing are being pursued to locate the mutation responsible for the *res* phenotype within the tomato genome. Among the JA overproducing mutants identified to date in *A. thaliana*, only the *cev1* phenotype is caused by a mutation in a gene predominantly expressed in root tissues (Ellis et al. 2002), and surprisingly the constitutive defense responses of the *cev1* resulted from a recessive mutation in the cellulose synthase *CeSA3* gene, involved in the cell wall biosynthesis. Therefore, it cannot be excluded that the *res* mutant might have a defect in cellulose biosynthesis, as occurs in the *cev1* mutant. In any case, the mutation responsible for the *res* phenotype could not be necessarily located among the genes directly involved in the JA biosynthesis and signaling pathways.

Under salt stress, *res* mutant increases the shoot and root weights under salinity, compared with control conditions (Fig. S5), although its plant growth continues to be low compared with WT plants. Pedranzani et al. (2003) analyzed the basal endogenous JA levels and their changes in response to salt stress in two tomato cultivars with different degrees of tolerance towards salinity, and observed higher endogenous JA levels in roots of the salt-tolerant tomato cultivar under control conditions. Moreover, it has been demonstrated that JA and related compounds were synthesized in the isolated roots of the salt-tolerant tomato cultivar, independently of the JA produced in the shoot (Abdala et al. 2003). In sum, the JA basal content in root could be critical in conferring salt stress tolerance in tomato. But what is even more exciting is that *res* plants were able to restore their phenotype and cell structure with salinity, at both the root and shoot levels (Figs. 2, 4 and 5). Thus, *res* root developed pericycle and stele cells similarly to WT roots when plants were grown under salt stress, which explains the macroscopic normalization of the mutant root development observed in salt stress (Fig. 5). Besides the root cell reorganization, salinity restores the leaf cell organization, including the chloroplast structure, which explains the remarkable recovery of the greenish color in leaves (Figs. 2 and 4). It is noteworthy that only in some cases of halophytes, where non-stressful conditions may represent a stress itself, may an increase in the photosynthetic activity with salinity occur (Redondo-Gomez et al. 2010). It is interesting to point out that the morphological alterations and ultrastructural disorganization of the mutant roots reappeared when *res* plants grown under salinity were transferred again to control conditions (Fig. 5), which suggests that the disappearance of the morphological alterations and cellular disorganization in *res* mutant is dependent on the salt stress.

Numerous studies have demonstrated the tolerance induced by JA to abiotic and biotic stress (Wasternack and Hause 2013), but most of them refer to JA biosynthesis in leaves, while in roots such

investigations are scarce. Recently, Grebner et al. (2013) compared the accumulation of JA and derivatives in leaves and roots after different stress treatments, such as wounding, osmotic stress and drought, and concluded that roots synthesize these compounds independently of the shoot, despite the low expression of several JA-biosynthetic enzymes in *A. thaliana* roots. On the other hand, Tytgat et al. (2013) came to the same conclusion when dissecting the molecular mechanisms underlying the interactions between above- and below-ground herbivores in *Brassica oleracea*. Furthermore, these authors found that the JA biosynthetic pathway was differently regulated in roots and shoots, which may in turn cause differential responses in both organs depending on whether the initial JA signal is perceived first in one or the other organ. In addition to JA content, it is important to take into consideration the flux of JA towards its methylated derivative MeJA. Once formed, JA is the substrate for several different types of derivatization reactions, including methylation (MeJA) and conjugation of JA to isoleucine by JAR1 to form JA-Ile, which represents the active form of JA (Wasternack and Kombrink 2010). Interestingly, *res* and WT show opposite tendencies regarding the relative distribution of JA and MeJA; while *res* shows markedly higher contents of JA than that of its derivative MeJA, JA is mainly metabolized to MeJA in WT (Fig 7C). In this regard, Pedranzani et al. (2003) observed that MeJA levels only increased with salinity in roots of the salt-sensitive tomato cultivar, but not in the salt-tolerant tomato one. According to the much higher percentage of JA vs. MeJA found in *res* roots under control conditions, the defense responses in *res* mutant should be already activated prior to stress application.

Since *res* roots are mainly responsible for the clear morphologic alterations observed in shoot, their physiological characterization would allow us to explain how changes in roots affect shoot responses, which is the subject of current intense research in plant science (Baluska et al. 2009, Fragoso et al. 2014). Under salinity, the accumulation of Na^+ induces a nutritional stress due to an altered nutrient uptake, especially of K^+, with the control of K^+ acquisition being a critical requirement for plant growth (Shabala and Cuin 2008, Wu et al. 2013). Interestingly, we found that K^+ concentrations were higher in leaves of *res* than in those of WT plants grown under salt stress, which suggests that *res* roots transport more K^+ to the shoot under salt stress condition (Fig. 6A). JA is known to integrate plant responses for defense against stress and processes related to plant development, but this phytohormone has been scarcely linked to nutritional stress, although nowadays there is more evidence on how hormonal signals are able to control the nutritional status of plants and vice-versa (Krouk et al. 2011). Thus, Armengaud et al. (2004) revealed an important role of JA in nutritional signaling, especially with respect to K^+, as JA-genes may respond to an early root-shoot signal rather than to a rise of shoot K^+ content *per se*. More recently the same authors (Armengaud et al. 2010) observed that a large part of the K^+-dependent transcriptome is absent or replaced in *A. thaliana coi1-16* mutant, an observation that supports a role for JA in the plant response towards alterations in K^+ supply. Moreover, it is worth noting the similar K^+ concentration rises found in the different parts of grafted-plants, young and adult leaves, and roots, when *res* was used as rootstock

(Fig. 6B), which shows the ability of *res* to regulate K^+ homeostasis. Finally, and taking into account that plants' ability to maintain low Na^+/K^+ ratios is a key trait of salt tolerance (Estañ et al. 2005), the high salt tolerance exhibited by *res* could, at least in part, be related to the maintenance of Na^+/K^+ homeostasis, as leaf Na^+/K^+ ratio was significantly lower in the mutant compared with WT (Fig. S6).

Jasmonates activate defense responses against abiotic and biotic stresses but this also implies an energetic cost for the plants throughout their life cycle. How plants are able to coordinate the fluctuating growth-defense dynamics is not well understood and remains a fundamental question (Yang et al. 2012). In this regard, *res* plants are a clear example of the high cost for plant growth that the constitutive activation of defense mechanism involves. Thus, the JA biosynthesis and signaling pathways in *res* are activated without stress intervention according to the induced expression of JA signaling genes in the root mutant under non-stress condition (Fig. 8). This response is opposite to the generalized behavior of plants prior to stress application, which maintains these expressions at basal levels under non-stressed conditions and only induces them after a signal of abiotic and biotic stress comes up (Shin et al. 2012). Since JA-induced defense in *res* is imposing significant energetic costs on plant growth, it would be interesting to attenuate this associated cost by means of down-regulating JA levels up to a point where a trade-off between growth and defense occurs, as pointed out by Mitra and Baldwin (2014), through redirecting JA flux towards inactive MeJA and creating a JA sink which diminishes JA-Ile biosynthesis and its associated-defense responses. In spite of the expression levels of JA signaling genes, which are generally lower under salt stress than under non-stress condition in *res* roots, when compared with WT, the expression levels of the JA-signaling genes continue to be higher in *res*, especially the level of *JAZ1* expression. Although many questions remain open regarding the targets, specificity, function and regulation of the different JAZ proteins (Pauwels and Goosens 2011), degradation of a JAZ protein by the ubiquitin-proteasome pathway leads towards transcriptional activation of its encoding gene (Chini et al. 2007, Thines et al. 2007) and, therefore, the profile of activated *JAZ* genes mimics the profile of the different degraded JAZ proteins. Recently, it was reported that NINJA, a transcriptional co-repressor of JA response genes that bridges target JAZ proteins to members of the TOPLESS co-repressor family (Pauwels et al. 2010), is indispensable for repression of JA signaling in roots and maintenance of normal root growth (Acosta et al. 2013). Furthermore, these authors showed that the constitutive JA-signaling activation in roots of *A. thaliana ninja* mutants can occur independently of MYC2 action, unlike aerial organs where MYC2 is required to activate normal JA signaling. In any case the role of MYC2 transcription factor in the activation of JA-regulated gene expression is widely recognized (Boter et al. 2004, Chini et al. 2007). This set of facts again demonstrates that the function of JA signaling genes may be different depending on the plant organ.

In conclusion, the high level of endogenous JA found in root of *res* mutant plants is associated to increased expression of genes from the JA-biosynthesis and signaling pathways, especially under non-stressful conditions. Therefore, *res* seems to be always in a state of 'alert' to confront stress, and the

remarkable growth inhibition it suffers represents the energy cost for this continuously activated defense state. A deeper knowledge of the processes involved in the balance plant growth inhibition versus tolerance or defense against stress could be very interesting in order to determine how much endogenous growth retardation would be acceptable to compensate for growth reduction caused by abiotic stress impact. In summary, the research on *res* could afford very significant advances in the knowledge of JA function in the trade-off plant growth versus salt stress – and other type of stresses – tolerance, a central question today in plant science research and a critical one from the agronomic point of view.

Author contributions

NFG, IE, FBF, EO and MCB designed research, JOGA, NFG, CLB, IE, RL, BP and VM performed experiments, JOGA, NFG, IE, TA, JC, VM and MCB analyzed data, JOGA, NFG, FBF, EO and MCB wrote the paper.

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References

Abdala G, Miersch O, Kramell R, Vigliocco A, Agostini E, Forchetti G, Alemano S (2003) Jasmonate and octadecanoid occurrence in tomato hairy roots. Endogenous level changes in response to NaCl. Plant Growth Regul 40: 21–27

Acosta IF, Gasperini D, Chételat A, Stolz A, Santuari L, Farmer EE (2013) Role of NINJA in root jasmonate signaling. Proc Natl Acad Sci USA 110: 15473–15478

Armengaud P, Breitling R, Amtmann A (2004) The potassium-dependent transcriptome of Arabidopsis reveals a prominent role of jasmonic acid in nutrient signaling. Plant Physiol 136: 2556–2576

Armengaud P, Breitling R, Amtmann A (2010) CORONATINE-INSENSITIVE 1 (COI1) mediates transcriptional responses of *Arabidopsis thaliana* to external potassium supply. Mol Plant 3: 390–405

Atares A, Moyano E, Morales B, Schleicher P, García-Abellán JO, Antón T, García-Sogo B, Pérez-Martín F, Lozano R, Flores FB, Moreno V, Bolarín MC, Pineda B (2011) An insertional mutagenesis programme with an enhancer trap for the identification and tagging of genes involved in abiotic stress tolerance in the tomato wild-related species *Solanum pennellii*. Plant

Cell Rep 30: 1865–1879

Baluska F, Mancuso S, Volkmann D, Barlow PW (2009) The 'root–brain' hypothesis of Charles and Francis Darwin: Revival after more than 125 years. Plant Signal Behav 4: 1121–1127

Bergougnoux V (2014) The history of tomato: from domestication to biopharming. Biotechnol Adv 32: 170–189

Boter M, Ruiz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 18: 1577–1591

Browse J (2009) The power of mutants for investigating jasmonate biosynthesis and signalling. Phytochem 70: 1539–1546

Chen H, Jones AD, Howe GA (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS Lett 580: 2540–2546

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666–671

Chini A, Boter M, Solano R (2009) Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signalling module. FEBS J 276: 4682–4692

Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. Plant Cell 14: 1557–1566

Erb M, Lenk C, Degenhardt J, Turlings TCJ (2009) The underestimated role of roots in defense against leaf attackers. Trends Plant Sci 14: 653–659

Estañ MT, Martinez-Rodriguez MM, Perez-Alfocea F, Flowers TJ, Bolarin MC (2005) Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. J Exp Bot 56: 703–712

Fernández-Calvo P, Chini A, Fernández-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, Pauwels L, Witters E, Puga MI, Paz-Ares J, Goossens A, Reymond P, De Jaeger G, Solano R (2011) The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell 23: 701–15

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol 5: 344–350

Fragoso V, Rothe E, Baldwin IT, Kim SG (2014) Root jasmonic acid synthesis and perception regulate folivore-induced shoot metabolites and increase *Nicotiana attenuata* resistance. New Phytol 202: 1335–1345

Garcia-Abellan JO, Egea I, Pineda B, Sanchez-Bel P, Belver A, Garcia-Sogo B, Flores FB, Atares A, Moreno V, Bolarin MC (2014) Heterologous expression of the yeast *HAL5* gene in tomato enhances salt tolerance by reducing shoot Na^+ accumulation in the long term. Physiol Plant 152:

700–713

Gisbert C, Rus AM, Bolarín MC, Lopez-Coronado JM, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast *HAL1* gene improves salt tolerance of transgenic tomato. Plant Physiol 123: 392–402

Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010) Food security: the challenge of feeding 9 billion people. Science 327: 812–818

Grebner W, Stingl NE, Oenel A, Mueller MJ, Berger S (2013) Lipoxygenase6-dependent oxylipin synthesis in roots is required for abiotic and biotic stress resistance of Arabidopsis. Plant Physiol 161: 2159–2170

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stn Circ 347: 1–39

Hoel BO, Solhaug KA (1998) Effect of irradiance on chlorophyll estimation with the Minolta SPAD-502 leaf chlorophyll meter. Ann Bot 82: 389–392

Hu Y, Jiang L, Wang F, Yu D (2013) Jasmonate regulates the INDUCER OF CBF EXPRESSION-C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 cascade and freezing tolerance in Arabidopsis. Plant Cell 25: 2907–2924

Ichihashi Y, Sinha N (2014) From genome to phenome and back in tomato. Curr Opin Plant Biol 18: 9–15

Izawa T, Ohnishi T, Nakano T, Ishida N, Enoki H, Hashimoto H, Itoh K, Terada R, Wu C, Miyazaki C, Endo T, Iida S, Shimamotol K (1997) Transposon tagging in rice. Plant Mol Biol 35: 219–229

Jeong D-H, An S, Kang H-G, Moon S, Han J-J, Park S, Lee SH, An K, An G (2002) T-DNA Insertional Mutagenesis for Activation Tagging in Rice. Plant Physiol 130: 1636–1644

Koncz C, Németh K, Rédei GP, Schell J (1992) T-DNA insertional mutagenesis in Arabidopsis. Plant Mol Biol 20: 963–976

Krouk G, Ruffel S, Gutiérrez RA, Gojon A, Crawford NM, Coruzzi GM, Lacombe B. (2011) A framework integrating plant growth with hormones and nutrients. Trends Plant Sci 16: 178–182

Li C, Liu G, Xu C, Lee GI, Bauer P, Ling HQ, Ganal MW, Howe GA (2003)The tomato *suppressor of prosystemin-mediated responses 2* gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. Plant Cell 15: 1646–1661

Lopez-Raez JA, Verhage A, Fernandez I, Garcia JM, Azcon-Aguilar C, Flors V, Pozo MJ (2010) Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. J Exp Bot 61: 2589–2601

Martinez-Rodriguez MM, Estañ MT, Moyano E, Garcia-Abellan JO, Flores FB, Campos JF, Al-Azzawi MJ, Flowers TJ, Bolarin MC (2008) The effectiveness of grafting to improve salt

tolerance in tomato when an 'excluder' genotype is used as scion. Env Exp Bot 63: 392–401

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence – a practical guide. J Exp Bot 51: 659–668

Miersch O, Weichert H, Stenzel I, Hause B, Maucher H, Feussner I, Wasternack C (2004) Constitutive overexpression of allene oxide cyclase in tomato (*Lycopersicon esculentum* cv. Lukullus) elevates levels of some jasmonates and octadecanoids in flower organs but not in leaves. Phytochemistry 65: 847–856

Mitra S, Baldwin IT (2014) Independently silencing two photosynthetic proteins in *Nicotiana attenuata* has different effects on herbivore resistance. Plant Physiol 148: 1128–1138

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497

Pauwels L, Goossen A (2011) The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell 23: 3089–3100

Pauwels L, Fernández Barbero G, Geerinck J, Tilleman S, Grunewald W, Cuéllar Pérez A, Chico JM, Van den Bossche R, Sewell J, Gil E, García-Casado G, Witters E, Inzé D, Long JA, De Jaeger G, Solano R, Goossens A (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464: 788–791

Pedranzani H, Racagni G, Alemano S, Miersch O, Ramırez I, Peña-Cortes H, Taleisnik E, Machado-Domenech E, Abdala G (2003) Salt tolerant tomato plants show increased levels of jasmonic acid. Plant Growth Regul 41: 149–158

Pineda B, García-Abellán JO, Antón T, Pérez F, Moyano E, García-Sogo B, Campos JF, Angosto T, Morales B, Capel J, Flores FB, Moreno V, Bolarin MC, Lozano R, Atarés A (2012) Tomato: Genomics approaches for salt and drought stress tolerance. In: Tuteja N, Gill SS, Tiburcio AF, Tuteja R (eds) Improving Crop Resistance to Abiotic Stress. Wiley-VCH Verlag GmbH & Co. KGaA, pp 1083–1118

Qi T, Song S, Ren Q, Wu D, Huang H, Chen Y, Fan M, Peng W, Ren C, Xie D (2011) The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. Plant Cell 23: 1795–1814

Ranjan A, Ichihashi, Y, Sinha NR (2012) The tomato genome: Implications for plant breeding, genomics and evolution. Genome Biol 13: 167

Redondo-Gomez S, Mateos-Naranjo E, Figueroa ME, Davy AJ (2010) Salt stimulation of growth and photosynthesis in an extreme halophyte, *Arthrocnemum macrostachyum*. Plant Biol 12: 79–87

Rodriguez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gomez M, del Rio LA, Sandalio LM (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. Plant Cell Env 29: 1532–1544

Rodríguez-Serrano M, Romero-Puertas MC, Pazmino DM, Testillano PS, Risueño MC, del Rio LA, Sandalio LM (2009) Cellular response of pea plants to cadmium toxicity: cross talk between

reactive oxygen species, nitric oxide, and calcium. Plant Physiol 150: 229–243

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108

Seo JS, Joo J, Kim MJ, Kim YK, Nahm BH, Song SI, Cheong JJ, Lee JS, Kim JK, Choi YD (2011) OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. Plant J 65: 907–921

Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. Physiol Plant 133: 651–669

Shaik R, Ramakrishna W (2014) Machine learning approaches distinguish multiple stress conditions using stress-responsive genes and identify candidate genes for broad resistance in rice. Plant Physiol 164: 481–495

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, He SY, Rizo J, Howe GA, Zheng N (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature 468: 400–405

Shin J, Heidrich K, Sanchez-Villarreal A, Parker JE, Davis SJ (2012) TIME FOR COFFEE Represses Accumulation of the MYC2 Transcription Factor to Provide Time-of-Day Regulation of Jasmonate Signaling in Arabidopsis. Plant Cell 24: 2470–2482

Song S, Qi T, Huang H, Ren Q, Wu D, Chang C, Peng W, Liu Y, Peng J, Xie D (2011) The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect Jasmonate-regulated stamen development in Arabidopsis. Plant Cell 23: 1000–1013

Staswick PE, Yuen GY, Lehman CC (1998). Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus *Pythium irregulare*. Plant J 15: 747–754

Stenzel I, Hause B, Maucher H, Pitzschke A, Miersch O, Ziegler J, Ryan CA, Wasternack C (2003) Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato – amplification in wound signalling. Plant J 33: 577–589

Stitz M, Gase K, Baldwin IT, Gaquerel E (2011) Ectopic expression of *AtJMT* in *Nicotiana attenuata*: Creating a metabolic sink has tissue-specific consequences for the jasmonate metabolic network and silences downstream gene expression. Plant Physiol 157: 341–354

Svyatyna K, Jikumaru Y, Brendel R, Reichelt M, Mithöfer A, Takano M, Kamiya Y, Nick P, Riemann M (2014) Light induces jasmonate-isoleucine conjugation via OsJAR1-dependent and -independent pathways in rice. Plant Cell Environ 37: 827–839

Tadege M, Wen J, He J, Tu H, Kwak Y, Eschstruth A, Cayrel A, Endre G, Zhao PX, Chabaud M, Ratet P, Mysore KS (2008) Large-scale insertional mutagenesis using the *Tnt1* retrotransposon in the model legume *Medicago truncatula*. Plant J 54: 335–347

The Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshly fruit evolution. Nature 485: 635–641

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J

(2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448: 661–665

Tytgat TOG, Verhoeven KJF, Jansen JJ, Raaijmakers CE, Bakx-Schotman T, McIntyre LM, van der Putten WH, Biere A, van Dam NM (2013) Plants know where it hurts: root and shoot jasmonic acid induction elicit differential responses en *Brassica oleracea*. PLoS One 8: e65502

Uppalapati SR, Ayoubi P, Weng H, Palmer DA, Mitchell RE, Jones W, Bender CL (2005) The phytotoxincoronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. Plant J 42: 201–217

Van Lijsebettens M, Vanderhaeghen R, Van Montagu M (1991) Insertional mutagenesis in *Arabidopsis thaliana*: isolation of a T-DNA-linked mutation that alters leaf morphology. Theor Appl Genet 81: 277–284

Vijayan P, Shockey J, Lévesque CA, Cook RJ, Browse J (1998). A role for jasmonate in pathogen defense of Arabidopsis. Proc Natl Acad Sci USA 95: 7209–7214

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100: 681–697

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot 111: 1021–1058

Wasternack C, Kombrink E (2010) Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. ACS Chem Biol 5: 63–77

Wu H, Shabala L, Barry K, Zhou M, Shabala S (2013) Ability of leaf mesophyll to retain potassium correlates with salinity tolerance in wheat and barley. Physiol Plant 149: 515–527

Yan L, Zhai Q, Wei J, Li S, Wang B, Huang T, Du M, Sun J, Kang L, Li Ch-B, Li Ch (2013) Role of tomato lipoxygenase D in wound-induced jasmonate biosynthesis and plant immunity to insect herbivores. PLoS One 9: e1003964

Yan Y, Christensen S, Isakeit T, Engelberth J, Meeley R, Hayward A, Emery RJN, Kolomiets MV (2012) Disruption of *OPR7* and *OPR8* reveals the versatile functions of jasmonic acid in maize development and defense. Plant Cell 24: 1420–1436

Yang DL, Yao J, Mei CS, Tong X-H, Zeng L-J, Li Q, Xiao L-T, Sun T-P, Li J, Deng X-W, Lee CM, Thomashow MF, Yang Y, He ZH, He SY (2012) Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proc Natl Acad Sci USA 109: E1192–E1200

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Figure legends

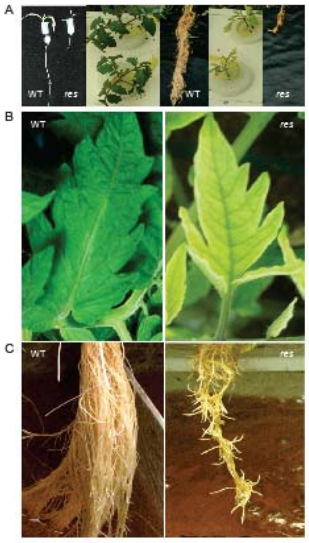

Fig. 1. The *res* mutant exhibits slower plant growth than WT and important morphological alterations when plants are grown *in vivo* under non-stress condition. (A) On the left, shorter root of *res* seedling compared with WT during germination. On the right, representative phenotypes of WT and *res* plants grown in hydroponic culture (1/2 Hoagland solution) for 30 days. (B) Detail of the leaf phenotype of *res* compared with WT, where the leaf chlorosis is patent in the mutant. (C) Details of the outstanding morphological alterations observed in roots of *res* compared with WT.

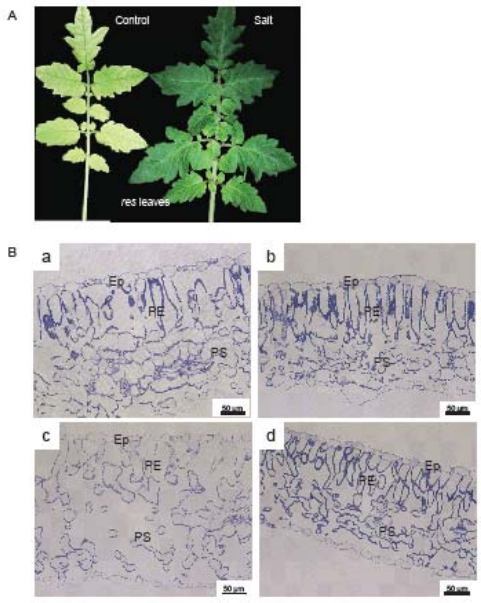

Fig. 2. Salt stress restores leaf morphological alterations and cell structure in *res* tomato mutant. (A) Leaf chlorosis of *res* observed in non-stressful condition (Control) disappeared when the mutant plant is subjected to 200 mM NaCl for 15 days (Salt). (B) The micrographs of transversal leaf sections of WT and *res* mutant under control and salt stress (200 mM NaCl for 15 days) show that the leaf anatomical alterations in *res* mutant observed in control condition disappear with salinity. Micrographs of transversal leaf sections of WT (a) and *res* (c) under control condition, and of WT (b) and *res* (d) under salt stress condition. Ep= epidermis; PE= palisade parenchyma; PS= spongy parenchyma.

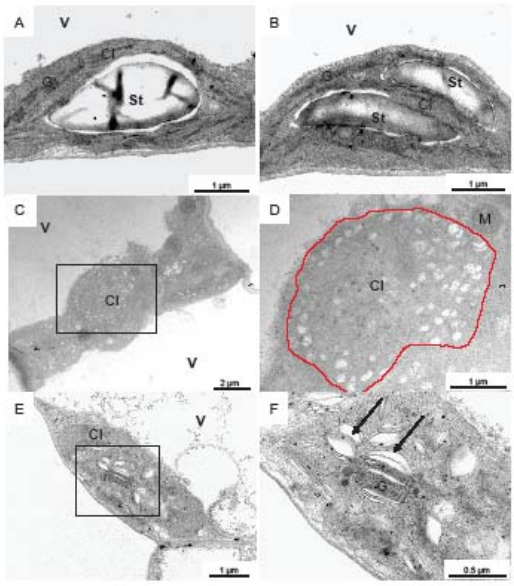

Fig. 3. Ultrastructure of chloroplasts from mesophyll cells grown in control conditions. (A) and (B) WT chloroplasts showing abundant starch grains. (C) Cytoplasm of *res* showing highly altered ultrastructure (D) Detail of chloroplast (square of picture C) from *res* showing altered structure (line is delimiting the chloroplast membrane). (E) Chloroplasts of *res*. (F) Detail of picture (E) showing dilated thylakoids (arrows). Cl= Chloroplast; G= Grana; M=Mitochondria; St= Starch Grain; V= Vacuole.

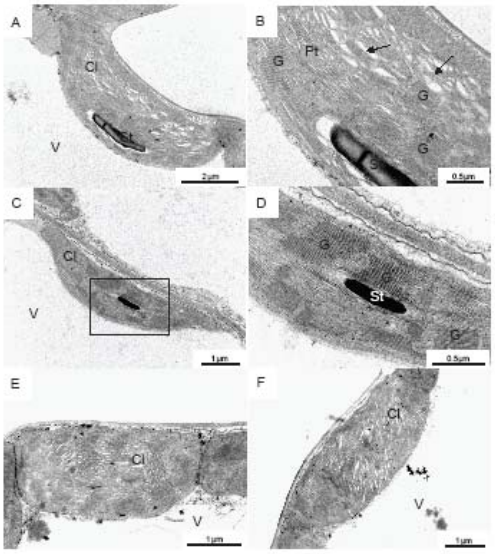

Fig. 4. Ultrastructure of chloroplasts from mesophyll cells grown in salt stress (200 mM NaCl for 15 days). (A) WT chloroplast. (B) Detail of figure (A) showing altered grana with dilated thylakoids (arrows). (C) *res* chloroplasts with lower content of starch grain. (D) Detail of figure (C) showing reorganized ultrastructure of the chloroplast. (E) and (F) *res* chloroplasts showing altered grana with dilated thylakoids. Cl= Chloroplast; G= Grana; M=Mitochondria; Pt= Plastoglobuli; St= Starch Grain; V= Vacuole.

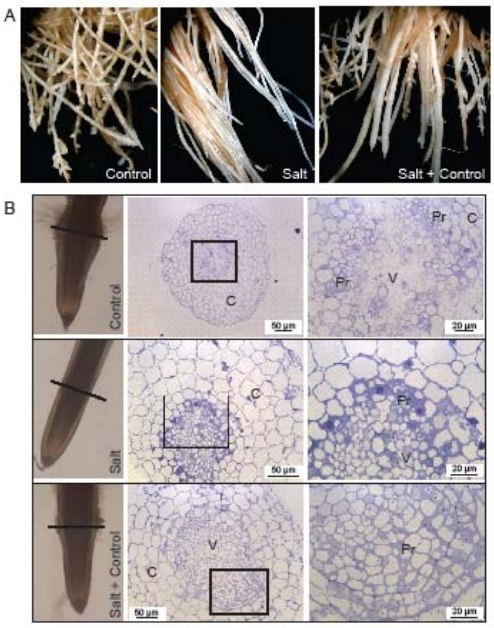

Fig. 5. (A) The morphological alterations and cellular disorganization observed in roots of the *res* mutant under non-stress conditions (Control) disappear when mutant plants are exposed to 200 mM NaCl for 10 days (Salt). The process is reversible, since when salt-treated plants were transferred to nutritive solution without salt for 5 days more, the morphological alterations appear again (Salt + Control). (B) Transversal root sections of *res* in Control, Salt and Salt + Control conditions and micrographs from these sections. On the left, the morphological aspect of *res* roots in each condition and the zone where the sections of the *res* roots tips were taken. On the center, micrographs of the root sections. On the right, details of previous micrographs (enclosed within the squares). C= Cortex; Pr= Root Primordium; V=Vascular cylinder.

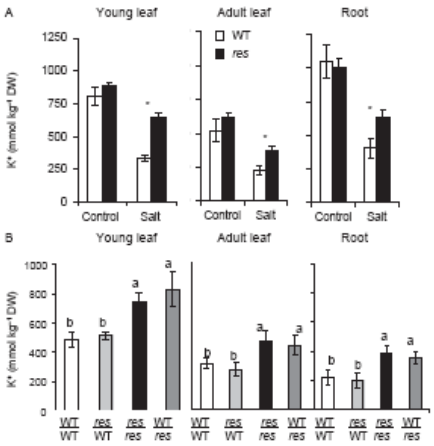

Fig. 6. The *res* mutant maintains higher K^+ levels than WT under salt stress. (A) K^+ concentrations in young and adult leaves, as well as in roots of WT and *res* plants under non-stress (Control) and at 200 mM NaCl for 15 days (Salt). (B) The self-grafted plants of *res* mutant (*res*/*res*) as well as the WT grafted onto mutant (WT/*res*) showed higher K^+ concentrations than the self-grafted WT plants WT/WT) and *res* grafted onto WT (*res*/WT) after 15 days of salt stress (200 mM NaCl). Plants were grown in hydroponic culture (1/2 Hoagland solution). Data are expressed as mean ± SE (n = 6). In (A) asterisks within each column indicate significant differences between WT and *res* (*t*-test, $P < 0.05$). In (B) values with different letters are significantly different as determined by LSD ($P \leq 0.05$).

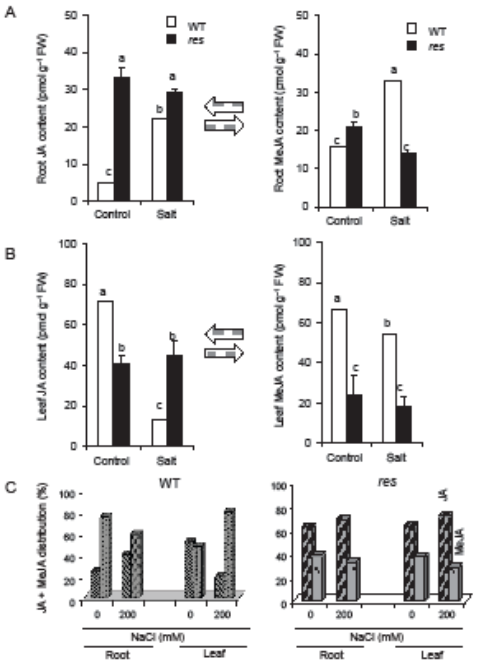

Fig. 7. The root of the *res* mutant accumulates JA before applying salt stress. Jasmonic acid (JA, graphic on the left) and Methyl Jasmonate (MeJA, graphic on the right) in roots (A) and leaves (B) of WT and *res* plants grown without (Control) or with 200 mM NaCl for 15 days (Salt). Data are expressed as mean ± SE (n = 6). Values with different letters are significantly different as determined by LSD ($P \leq 0.05$). (C) Relative distribution (expressed in %) of JA and MeJA in leaves and roots of WT and *res* plant, in control and salt stress conditions.

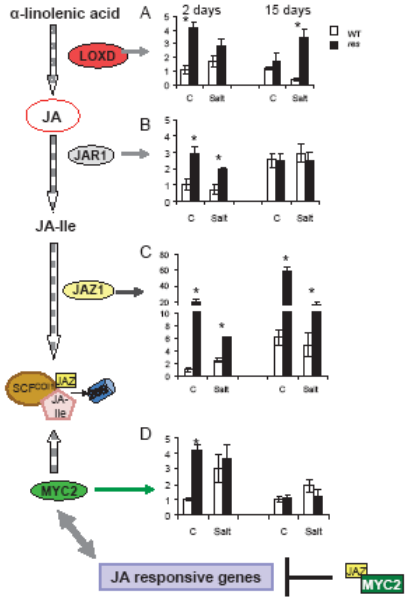

Fig. 8. Increased expression of genes from the JA biosynthesis and signaling pathways in root of the *res* tomato mutant compared with WT. Relative expression of *Lipoxygenase D* (*LOXD*) (A), *Jasmonoyl Isoleucine Conjugate Synthase 1* (*JAR1*) (B), *Jasmonate ZIM Domain Protein 1* (*JAZ1*) (C), and *MYC2* (D) genes quantified by real-time qPCR in root of WT and *res* plants grown under non-stress condition (C) and after 2 and 15 days of 200 mM NaCl treatment (Salt). The relative expression level was calculated by using as the calibrator sample the expression level in root of WT plants in absence of salt stress (C) for 2 days (equal to 1). Data are the mean ± SE (n = 6). Asterisks indicate significant differences between WT and *res* ($P < 0.05$).