

## RESEARCH PAPER

# Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.)

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## Abstract

Leaf senescence is one of the most limiting factors to plant productivity under salinity. Both the accumulation of specific toxic ions (e.g. Na<sup>+</sup>) and changes in leaf hormone relations are involved in the regulation of this process. Tomato plants (*Solanum lycopersicum* L. cv Moneymaker) were cultivated for 3 weeks under high salinity (100 mM NaCl) and leaf senescence-related parameters were studied during leaf development in relation to Na<sup>+</sup> and K<sup>+</sup> contents and changes in abscisic acid (ABA), cytokinins, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and the auxin indole-3-acetic acid (IAA). Na<sup>+</sup> accumulated to a similar extent in both leaves 4 and 5 (numbering from the base of the plant) and more quickly during the third week, while concurrently K<sup>+</sup> contents sharply decreased. However, photosystem II efficiency, measured as the  $F_v/F_m$  ratio, decreased from the second week of salinization in leaf 4 but only at the end of the third week in the younger leaf 5. In the prematurely senescent leaf 4, ABA content increased linearly while IAA strongly decreased with salinization time. Although zeatin (Z) levels were scarcely affected by

salinity, zeatin-riboside (ZR) and the total cytokinin content (Z+ZR) progressively decreased by 50% from the imposition of the stress. ACC was the only hormonal compound that increased in leaf tissue coincident with the onset of oxidative damage and the decline in chlorophyll fluorescence, and prior to massive Na<sup>+</sup> accumulation. Indeed, (Z+ZR) and ACC contents and their ratio (Z+ZR/ACC) were the hormonal parameters best correlated with the onset and progression of leaf senescence. The influence of different hormonal changes on salt-induced leaf senescence is discussed.

Key words: Abscisic acid, 1-aminocyclopropane-1-carboxylic acid, indole-3-acetic acid, plant hormones, salt stress, senescence, sodium chloride, tomato (*Solanum lycopersicum* L.), zeatin, zeatin-riboside.

## Introduction

Tomato (*Solanum lycopersicum* L.) is the world's most important vegetable crop in economic terms (Nuez *et al.*, 2004), and is considered moderately salt tolerant, with

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CK, cytokinin; CKX, cytokinin oxidase; CWIN, cell wall invertase; IAA, indole-3-acetic acid; NPQ, non-photochemical quenching; Z, zeatin; ZR, zeatin riboside.

yield reduced by 50% under a moderately saline regime of  $\sim 8 \text{ dS m}^{-1}$  (Subbarao and Johansen, 1994). Since available soil and water resources are becoming increasingly salinized, the negative impact on crop productivity is continuously increasing. Hence, efforts have been made during the last 50 years to improve the salt tolerance of tomato, but with little success due in part to insufficient knowledge about the mechanisms involved in limiting yield (Cuartero *et al.*, 2005). Since cellular events triggered by salinity, such as salt compartmentation, osmotic adjustment, changes in metabolic fluxes, and cell wall hardening, are connected to whole plant responses, such as growth reduction, changes in biomass allocation and phenology, leaf senescence, and finally plant death (Volkmar *et al.*, 1998), the candidate genes to increase salt tolerance are those involved in regulating such processes (Munns, 2005). Therefore, salt tolerance could be improved by avoiding or delaying senescence, allowing plant resource acquisition for a longer period, to generate new growth and maintain defence mechanisms such as ionic regulation to avoid the massive entry of toxic ions to the plant.

Plant senescence is an internally programmed degradation leading to cell death. Since chloroplasts appear to be the initial target of senescence, and chlorophyll breakdown is so prominent, chlorophyll loss and the associated yellowing of the leaves are commonly used as indicators of plant senescence (Noodén *et al.*, 1997). The onset and progression of leaf senescence are regulated by both environmental and endogenous factors and their interactions. Environmental cues include stress factors that adversely affect plant development and productivity, such as drought, waterlogging, high or low solar radiation, extreme temperatures, ozone and other air pollutants, excessive soil salinity, and inadequate mineral nutrition in the soil (Munné-Bosch and Alegre, 2004). Endogenous factors include age, reproductive development, and level of plant hormones. These environmental cues may accelerate leaf senescence by affecting those endogenous factors (Munné-Bosch and Alegre, 2004).

Salinity reduces plant productivity first by reducing plant growth during the phase of osmotic stress and subsequently by inducing leaf senescence during the phase of toxicity when excessive salt is accumulated in transpiring leaves (Munns, 2002a). Indeed, in spite of the positive effect of  $\text{Na}^+$  compartmentation into vacuoles to maintain turgor (Flowers, 2004), premature senescence caused by salinity seems to occur more quickly in glycophytic plants having a higher  $\text{Na}^+$  uptake (Fortmeier and Schubert, 1995; Munns, 2002a, b). However, as previously stated, growth inhibition and metabolic changes occurring during the osmotic phase of salinity are similar to those triggered by drought, which can also lead to premature senescence (Munns, 2002a, b). In turn, senescence induced by the osmotic component of salinity

probably follows the same physiological events as drought-induced senescence (Pic *et al.*, 2002). The initial reduction in shoot growth followed by accelerated senescence is probably related to hormonal signals generated in response to the stress. Much evidence suggests that cytokinins (CKs) are the major leaf senescence-inhibiting hormones, since senescence is delayed after the exogenous application of CKs (Van Staden *et al.*, 1988) or the overproduction of CKs in transgenic plants transformed with the CK biosynthetic genes, isopentenyl transferase (*IPT*) (Gan and Amasino, 1995; Cowan *et al.*, 2005; Rivero *et al.*, 2007) and zeatin *O*-glucosyltransferase (*ZOG1*) (Havlova *et al.*, 2008), and senescence is correlated with a decline in endogenous CK levels (Van Staden *et al.*, 1988; Singh *et al.*, 1992; Gan and Amasino, 1995). Leaf senescence can only be initiated when leaf CK levels fall below a threshold (Noodén *et al.*, 1997). Maintaining CK levels above this threshold inhibits transcriptional regulation of senescence-related genes and prevents the onset of senescence in the entire plant (Gan and Amasino, 1995).

A direct role in the regulation of drought-induced leaf senescence has also been demonstrated for abscisic acid (ABA), CKs, and ethylene. Although the role of ABA in phloem sugar transport remains controversial (Estruch *et al.*, 1989; Lacombe *et al.*, 2000), several studies revealed that enhanced ABA contents increased carbon remobilization from senescing leaves to grains in drought-stressed cereals (Yang *et al.*, 2002). In contrast, CK levels were positively correlated with leaf photosynthetic rate, and water and chlorophyll contents in tobacco (Rivero *et al.*, 2007), thus presumably preventing leaf senescence under stress (Munné-Bosch and Alegre, 2004). The involvement of ethylene in enhancing leaf senescence has been demonstrated by decreasing expression of genes involved in ethylene biosynthesis [1-aminocyclopropane-1-carboxylic acid (ACC) synthase or ACC oxidase], which delayed senescence under drought conditions in maize (Young *et al.*, 2004), under salinity in tobacco (Wi and Park, 2002), and in the absence of stress in tomato (John *et al.*, 1995). Although gibberellins and auxins have been suggested as putative regulators of leaf senescence (Zacarias and Reid, 1990; He *et al.*, 2002), further studies are necessary to provide direct evidence of a role for these compounds in the regulation of drought- and salt-induced leaf senescence. The auxin IAA is a putative senescence-retarding agent that decreases the expression of the *SAG12* senescence promoter (Noh and Amasino, 1999), probably through interactions with other hormones, such as ethylene, and metabolic fluxes (Schippers *et al.*, 2007). Studies at the molecular level have demonstrated that salicylates, jasmonates, and ethylene regulate expression of genes coding for transcription factors, which in turn regulate *SAG* expression, when plants are exposed to salt, osmotic, and cold stresses (Chen *et al.*, 2002; He *et al.*, 2002).

Although some studies have addressed how phytohormones were affected by salinity (reviewed by Naqvi, 1994; Munns, 2002a), there has been little attention paid to the role of changes in endogenous hormonal balance in the onset and progression of salt stress-induced leaf senescence in an important horticultural crop such as tomato. Therefore, the aim of this study was to examine the dynamic changes of selected endogenous hormones [CKs, ABA, ACC, and indole-3-acetic acid (IAA)] in relation to the salt-induced senescence of tomato leaves (*S. lycopersicum* L.). Such information is a necessary preliminary to modifying hormone contents *in vivo* with the aim of decreasing salt-induced senescence.

## Materials and methods

### Plant material and culture conditions

Seeds of tomato (*S. lycopersicum* L.) cv. Moneymaker were sown and seedlings allowed to grow in a germination chamber in trays filled with a perlite-vermiculite mix (1:3 v/v) moistened regularly with a half-strength modified Hoagland nutrient solution. Fourteen days after sowing, seedlings were transferred into a growth chamber and fixed on polyvinyl chloride plates floating on aerated half-strength modified Hoagland nutrient solution. The nutrient solution contained the following chemicals (in mM): 5 KNO<sub>3</sub>, 1 NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.5 MgSO<sub>4</sub>, 5.5 Ca(NO<sub>3</sub>)<sub>2</sub> and (in μM) 25 KCl, 10 H<sub>3</sub>BO<sub>3</sub>, 1 MnSO<sub>4</sub>, 1 ZnSO<sub>4</sub>, 0.25 CuSO<sub>4</sub>, 10 Na<sub>2</sub>MoO<sub>4</sub>, and 1.87 g l<sup>-1</sup> Fe-EDDHA. Solutions were refilled every 2 d and renewed every week.

Plants were grown in a growth chamber under a 16 h daylight period. The air temperature ranged from 25 °C to 28 °C during the day and from 17 °C to 18 °C during the night. Relative humidity was maintained at 70 ± 5% during the night and at 50 ± 5% during the day. Light intensity at the top of the canopy was ~245 μmol m<sup>-2</sup> s<sup>-1</sup> [photosynthetic photon flux density (PPFD)]. After 4 d of acclimatization in control conditions (18 d after sowing), the seedlings were exposed to 0 (control) or 100 mM NaCl added to the nutrient solution for 22 d. Three replications with eight plants per replication and salt treatment were used in this study for the measurement of different parameters. Two specific leaves per plant were chosen when salinity was imposed to monitor senescence and for subsequent biochemical determinations: a young expanding leaf (~100 cm<sup>2</sup>), identified as leaf number 4 (from the bottom), and leaf number 5. Leaf material from six plants was harvested for different analyses at 1, 9, 15, and 22 d of salt treatment.

### Chlorophyll fluorescence

Modulated chlorophyll fluorescence was measured in tagged and dark-adapted (30 min) leaves in 6–10 plants per treatment, using a chlorophyll fluorometer OS-30 (OptiSciences, Herts, UK) with an excitation source intensity of 3000 μmol m<sup>-2</sup> s<sup>-1</sup>. The minimal fluorescence intensity ( $F_0$ ) in a dark-adapted state was measured in the presence of a background far-red light to favour rapid oxidation of intersystem electron carriers. The maximal fluorescence intensities in the dark-adapted state ( $F_m$ ) and after adaptation to white actinic light ( $F_m'$ ) were measured by 0.8 s saturating pulses (3000 μmol m<sup>-2</sup> s<sup>-1</sup>). After the  $F_m'$  measurement, the actinic light (400 mol m<sup>-2</sup> s<sup>-1</sup>) was switched off, and the far-red light was applied for 3 s in order to measure the minimal fluorescence intensity in the light-adapted state ( $F_0'$ ). The maximum quantum yield of open photosystem II (PSII) ( $F_v/F_m$ ) and the non-

photochemical quenching (NPQ) were calculated as  $(F_m - F_0)/F_m$  and  $(F_m - F_m')/F_m'$ , respectively (Maxwell and Johnson, 2000).

### Oxidative damage

The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances (TBARs), mainly malondialdehyde (MDA), following the modified method of Heath and Parker (1968). Frozen leaf samples (0.25 g) were homogenized in a pre-chilled mortar with 5 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 12 000 g for 15 min at 4 °C. The assay mixture containing a 2 ml aliquot of supernatant and 2 ml of 0.67% (w/v) thiobarbituric acid was heated to 100 °C for 30 min and then rapidly cooled to 4 °C in an ice-bath. After centrifugation (10 000 g for 1 min at 4 °C), the supernatant absorbance was read (532 nm) and values corresponding to non-specific absorption (600 nm) were subtracted. The MDA concentration was calculated using its molar extinction coefficient (155 mM<sup>-1</sup> cm<sup>-1</sup>).

### Ion concentration

For K<sup>+</sup> and Na<sup>+</sup> quantification, leaf tissues were frozen with liquid nitrogen. After thawing, the samples were centrifuged for 10 min at 10 000 g and then for 5 min at 20 000 g to obtain the bulk tissue sap. Necessary dilutions were performed in order to measure K<sup>+</sup> and Na<sup>+</sup> concentrations, determined by using a Shimadzu AA-680 atomic absorption spectrophotometer (Shimadzu Ltd, Kyoto, Japan). All measurements were performed in three replicates.

### Hormone extraction and analysis

CKs [zeatin (Z) and zeatin riboside (ZR)], IAA, and ABA were extracted and purified according to the method of Dobrev and Kaminek (2002). A 1 g aliquot of leaf material was homogenized in liquid nitrogen and placed in 5 ml of cold (-20 °C) extraction mixture of methanol/water/formic acid (15/4/1, by vol., pH 2.5). After overnight extraction at -20 °C solids were separated by centrifugation (20 000 g, 15 min) and re-extracted for 30 min in an additional 5 ml of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, Millford, MA, USA) to remove interfering lipids and some plant pigments, and evaporated either to near dryness or until the organic solvent was removed. The residue was dissolved in 5 ml of 1 M formic acid and applied to an Oasis MCX mixed mode (cation-exchange and reverse phase) column (150 mg; Waters) pre-conditioned with 5 ml of methanol followed by 5 ml of 1 M formic acid. To separate different CKs (nucleotides, bases, ribosides, and glucosides) from IAA and ABA, the column was washed and eluted stepwise with the different appropriate solutions indicated in Dobrev and Kaminek (2002). ABA and IAA were analysed in the same fraction. After each solvent was passed through the columns, they were purged briefly with air. Solvents were evaporated at 40 °C under vacuum. Samples were then dissolved in mobile phase A, consisting of a water/acetonitrile/formic acid (94.9:5:0.1 by vol.) mixture for high-performance liquid chromatography (HPLC)/mass spectroscopy (MS) analysis. The analysis were carried out on a HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a μ-wellplate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray (ESI) interface. Prior to injection, 100 μl of each fraction extracted from leaf tissues was filtered through 13 mm diameter Millex filters with a 0.22 μm pore size nylon membrane (Millipore, Bedford, MA, USA). An 8 μl aliquot of each sample, dissolved in mobile phase A, was injected onto a Zorbax SB-C18 HPLC column (5 μm, 150×0.5 mm; Agilent Technologies), held at 40 °C, and eluted at a flow rate of 10 μl min<sup>-1</sup>.

Mobile phase A, consisting of water/acetonitrile/formic acid (94.9:5:0.1 by vol.), and mobile phase B, consisting of water/acetonitrile/formic acid (10:89.9:0.1 by vol.), were used for the chromatographic separation. The elution program maintained 100% A during 5 min, then a linear gradient from 0% to 6% B in 10 min, followed by another linear gradient from 6% to 100% B in 5 min, and finally maintained at 100% B for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analytical run. The UV chromatogram was recorded at 280 nm with the DAD module (Agilent Technologies). The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V, and a scan speed of 22 000 ( $m/z$ )  $s^{-1}$  from 50 to 500  $m/z$ . The nebulizer gas (He) pressure was set to 30 psi, whereas the drying gas was set to a flow of 6  $l\ min^{-1}$  at a temperature of 350 °C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik GmbH, Germany). For quantification of Z, ZR, ABA, and IAA, calibration curves were constructed for each analysed component (0.05, 0.075, 0.1, 0.2, and 0.5  $mg\ l^{-1}$ ) and corrected for 0.1  $mg\ l^{-1}$  internal standards: [ $^2H_5$ ]trans-zeatin, [ $^2H_5$ ]trans-zeatin riboside, [ $^2H_6$ ]cis,trans-abscisic acid (Olchemin Ltd, Olomouc, Czech Republic), and [ $^{13}C_6$ ]indole-3-acetic acid (Cambridge Isotope Laboratories Inc., Andover, MA, USA). Recovery percentages ranged between 92% and 95%.

ACC was determined after conversion into ethylene by gas chromatography using an activated alumina column and an FID detector (Cromatix-KNK-2000, Konik, Barcelona, Spain). ACC was extracted with 80% (v/v) ethanol and assayed by degradation with alkaline hypochlorite in the presence of 5 mM  $HgCl_2$  (Casas *et al.*, 1989). A preliminary purification step was performed by passing the extract through a Dowex 50W-X8, 50–100 mesh,  $H^+$ -form resin and later recovered with 0.1 N  $NH_4OH$ . The conversion efficiency of ACC into ethylene was calculated separately by using a replicate sample containing 2.5 nmol of ACC as internal standard, and used for correction of data.

#### Enzyme extraction and assay

To measure cytokinin oxidase (CKX, EC 1.5.99.12) activity, leaf samples were cut into small pieces, powdered with liquid nitrogen using a pestle and mortar, and extracted with a 1.5-fold excess (v/w) of 0.2 M TRIS-HCl buffer, pH 8, containing 1 mM phenylmethylsulphonyl fluoride and 3% Triton X-100. Tissue debris was removed by centrifugation at 12 000  $g$  for 10 min. The extract was loaded onto a Sephadex G-25 (50 × 2.5 cm) column equilibrated with 0.1 M TRIS-HCl, pH 8, to remove the low molecular mass fraction. The protein fraction was then used to assay CKX activity. The assay was performed according to the method described by Frébort *et al.* (2002), with some modifications. Samples were incubated in a reaction mixture (total volume 0.6 ml in an Eppendorf tube) of 100 mM reaction buffer (imidazole/HCl buffer, pH 6), 0.5 mM electron acceptor [2, 6-dichloroindophenol] and 0.5 mM substrate, for 0.5–12 h at 37 °C.

To measure cell wall invertase (CWIN, EC 3.2.1.25) activity, the enzyme extracts were prepared essentially as described in Balibrea *et al.* (1999). Fresh leaf tissue samples (100 mg) were frozen with liquid nitrogen and stored at –20 °C until analysis. Samples containing polyvinylpyrrolidone and Fontainebleau sand were homogenized in 1 ml of extraction buffer containing 50 mM HEPES-KOH (pH 7), 10 mM  $MgCl_2$ , 1 mM  $Na_2EDTA$ , 2.6 mM dithiothreitol (DTT), 10% ethylene glycol, and 0.02% Triton X-100. After centrifugation at 20 000  $g$ , the supernatant was discarded and the pellet was washed three times and re-suspended in 30 mM acetate buffer (pH 5). The amount of hexoses was determined through an enzyme-linked assay monitoring NADH formation at 340 nm, after adding 25  $\mu l$  of 0.6 M sucrose and incubating at

30 °C for 15 min. The proteins were analysed in the pellet after solubilization with 1 M NaCl, and the specific enzymatic activities were expressed as  $nkat\ mg^{-1}$  protein.

#### Statistical analysis

Data were subjected to an analysis of variance (ANOVA II) using the SAS software (SAS System for Windows, version 8.02). The statistical significance of the results was analysed by the Student–Newman–Keuls test at the 5% level.

## Results

### Chlorophyll fluorescence and leaf senescence-related parameters

The evolution of chlorophyll fluorescence and senescence-related parameters during salt stress is shown in Fig. 1. In leaf 4 of control plants, the maximum quantum efficiency of PSII ( $F_v/F_m$ ) was almost constant during the growing period, whereas in salt-stressed plants, these values sharply decreased below those of control plants 14 d after salinization (Fig. 1A). However, in the younger leaf 5, this salinity-induced decrease in  $F_v/F_m$  was delayed by 1 week (Fig. 1B).

From the first day of salt treatment, NPQ increased significantly in leaf 4, reaching a maximum by day 15 before declining during the last week (Fig. 1C) together with  $F_v/F_m$ . In control plants, NPQ increased as the leaves aged. In leaf 5, NPQ also increased during the first week of salinization but less than in the older leaf 4, and it reached control values on day 18 before strongly increasing on day 22, also coinciding with a significant decline in  $F_v/F_m$  (Fig. 1D).

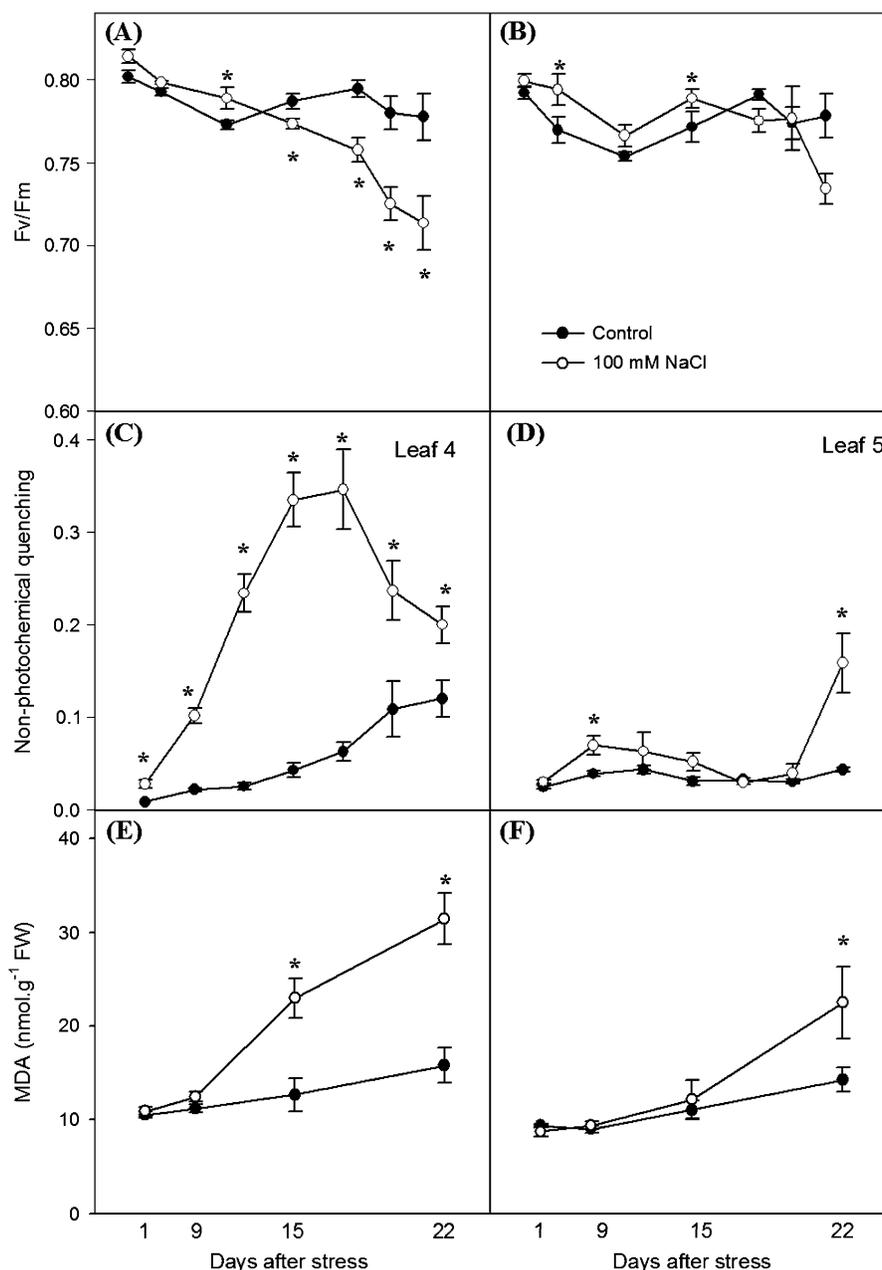
The level of lipid peroxidation slowly increased with age in both control leaves 4 and 5, but the salt stress significantly increased it from day 15 and 22, respectively (Fig. 1E, F), coinciding with the decrease in  $F_v/F_m$  (Fig. 1A, B).

### Sodium and potassium content

Sodium was accumulated to a similar extent in both leaves 4 and 5 (50–80 mM) during the first 2 weeks of salinization, and also a similar strong increase (until 150 mM) occurred at the end of the third week in both leaves (Fig. 2A, C). Compared with  $Na^+$ , salinity induced an earlier and stronger reduction in  $K^+$  content in both leaves 4 and 5 (Fig. 2B, D). In the younger leaf 5 of control plants,  $K^+$  content decreased during the first 2 weeks but recovered during the third week, probably due to a remobilization from older leaves such as the senescent leaf 4.

### Leaf hormonal profiling

**Cytokinins:** In leaf 4 of control plants, Z+ZR content decreased by 50% as the leaves aged (Fig. 3C). Although

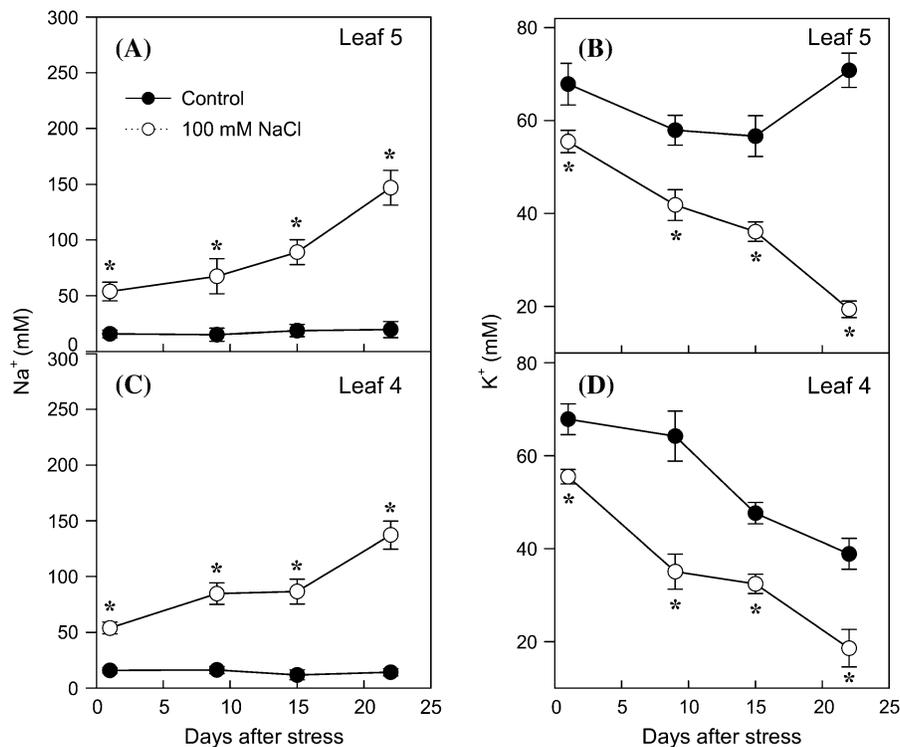


**Fig. 1.** Evolution of leaf senescence-related parameters. Maximum photochemical efficiency ( $F_v/F_m$ ) (A, B), non-photochemical quenching (NPQ) (C, D), and oxidative damage [malondialdehyde (MDA) accumulation] (E, F) in leaves 4 (A, C, E) and 5 (B, D, F) of tomato plants grown for 3 weeks on half-strength Hoagland medium in the absence (filled circles) or presence of 100 mM NaCl (open circles). Data are means of 10 plants  $\pm$  SE. Asterisks indicate significant differences between control and salinized leaves according to Student–Newman–Keuls test at  $P < 0.05$ .

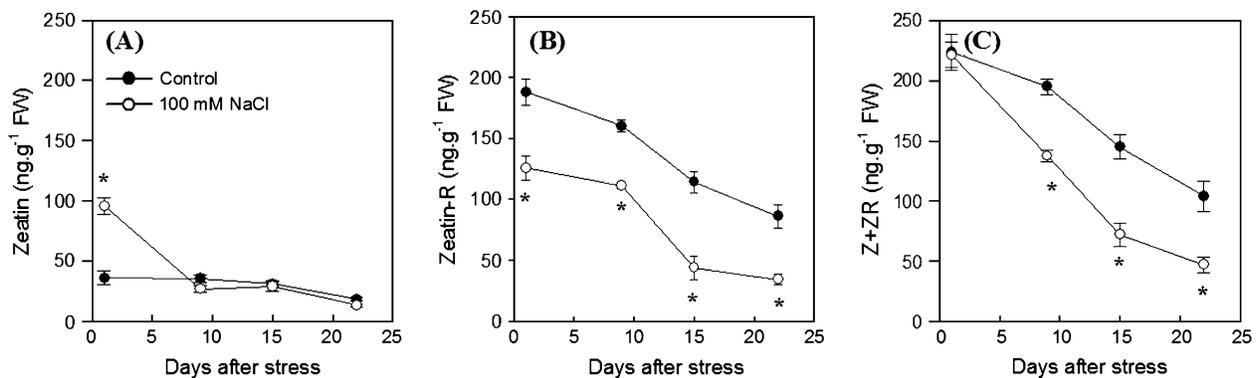
salinity increased leaf 4 Z content by 2-fold 1 d after salinization, for the remainder of experiment the values were only slightly lower (averaging 80% of the control) than control plants (Fig. 3A). However, salinity progressively decreased leaf ZR contents: by 30% 1 d after salinization and by 60% at the end of the experiment (Fig. 3B). Consequently the total (Z+ZR) CK content decreased with leaf development more dramatically under salinity. At the onset of the salt-induced leaf senescence (by day 15, according to the decreased  $F_v/F_m$  and MDA accumulation),

the Z+ZR CK content was  $\sim$ 50% lower than in the control leaves, and 70% lower than before salinization (Fig. 3).

**ACC, ABA, and IAA:** The concentration of the ethylene precursor ACC remained almost constant ( $\sim$ 5 ng g<sup>-1</sup> FW) in control leaves during leaf development, without any significant change that could be related to the onset of natural senescence (Fig. 4A). However, in leaf 4 of the salinized plants, the ACC levels increased during the second week. At the end of the experiment, the ACC



**Fig. 2.** Evolution of sodium (A, C) and potassium (B, D) concentrations in leaves 4 (C, D) and 5 (A, B) of tomato plants grown for 3 weeks on half-strength Hoagland medium in the absence (filled circles) or presence (open circles) of 100 mM NaCl. Data are means of six plants  $\pm$ SE. Asterisks indicate significant differences between control and salinized leaves according to Student–Newman–Keuls test at  $P < 0.05$ .



**Fig. 3.** Evolution of zeatin (Z) (A), zeatin-riboside (ZR) (B), and total cytokinins (Z+ZR) (C) contents during the development of leaf 4 in tomato plants grown for 3 weeks on half-strength Hoagland medium in the absence (filled circles) or presence (open circles) of 100 mM NaCl. Data are means of three plants  $\pm$ SE. Asterisks indicate significant differences between control and salinized leaves according to Student–Newman–Keuls test at  $P < 0.05$ .

content in salinized leaves was 12-fold higher than in the control leaves.

Leaf ABA content increased linearly with time in both control and salinized plants, but the increase was four times greater in salinized plants during the experiment (Fig. 4B).

Leaf IAA concentration increased between days 1 and 15 in both control and salinized plants, before decreasing to the initial levels by the end of the experiment. Salinity

dramatically decreased IAA content (by 80%) on the first day of salt treatment, and this percentage reduction was maintained almost unchanged during the experiment (Fig. 4C). A replicate experiment showed a 50% decrease in leaf IAA content after 3 d of salinization (data not shown).

*Endogenous hormonal ratios:* Although hormonal ratios, rather than absolute contents, could provide some insights

into the evolution of the salt-induced senescence, only two such ratios showed significant correlations with any indicator of senescence (Table 1). Thus, the (Z+ZR)/ABA ratio decreased linearly during leaf development under control conditions (from 5 at the start of salt treatment to 1 at the end of the experiment) (Fig. 5A). However, 1 d of salt treatment induced a similar effect as 3 weeks of development under non-stress conditions. After 3 weeks of salinization the (Z+ZR)/ABA ratio was close to zero.

The (Z+ZR)/ACC ratio decreased with age in control leaves (Fig. 5B). Salinity decreased this ratio from the first day of salinization and more drastically by day 15, coinciding with the onset of leaf senescence.

#### Effects of salinity on cytokinin dehydrogenase and cell wall invertase activities

CKX activity increased linearly as the leaves aged, irrespective of the growth conditions, but the activity was 2-fold higher in salinized plants by day 22 (Fig. 6A). Salt stress significantly decreased CWIN activity in leaf 4 from the first day of salt treatment (Fig. 6B).

#### Correlation analysis

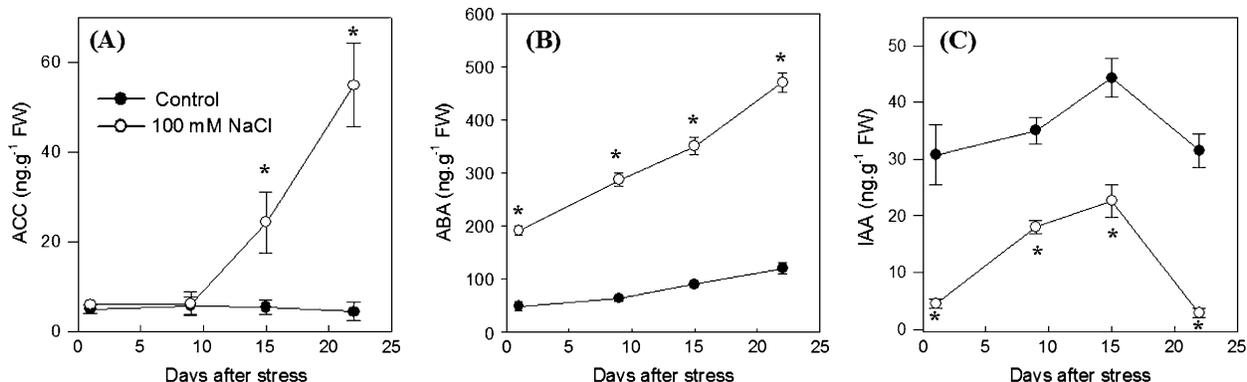
CKs (Z+ZR) and the ethylene precursor ACC contents and their ratio (Z+ZR/ACC) were the best hormonal parameters related to senescence in leaf 4 (Table 1). However, the influence of both hormones was opposite:

while  $F_v/F_m$  was positively correlated with the CK content and the (Z+ZR)/ACC ratio, a strong negative correlation was found with ACC contents. On the other hand, the evolution of the protective parameter NPQ was correlated positively with ABA but negatively with CK contents and their ratios with ACC and ABA. The indicator of oxidative stress MDA was inversely related to the indicator of PSII efficiency  $F_v/F_m$  ( $r = -0.84$ ,  $P \geq 0.01$ ). In turn, oxidative damage (MDA content) was positively related to ABA and especially to ACC contents, while a strong and inverse correlation was found with CK content and the (Z+ZR)/ACC ratio. Finally, while the contents of the major nutrient  $K^+$  were positively correlated with  $F_v/F_m$  and negatively with both NPQ and MDA in leaf 4, the contents of the toxic ion  $Na^+$  were only significantly correlated with the progression of the oxidative stress (Table 1).

#### Discussion

##### The onset of salt-induced leaf senescence seems independent of bulk leaf $Na^+$ accumulation

In leaf 4 of salt-stressed plants, a considerable decline in  $F_v/F_m$  resulting from a loss of PSII efficiency was evident after 15 d of salt treatment, indicating the onset of salt-induced senescence (Guamét *et al.*, 2002), while in the younger leaf 5 it was only observed after 22 d under

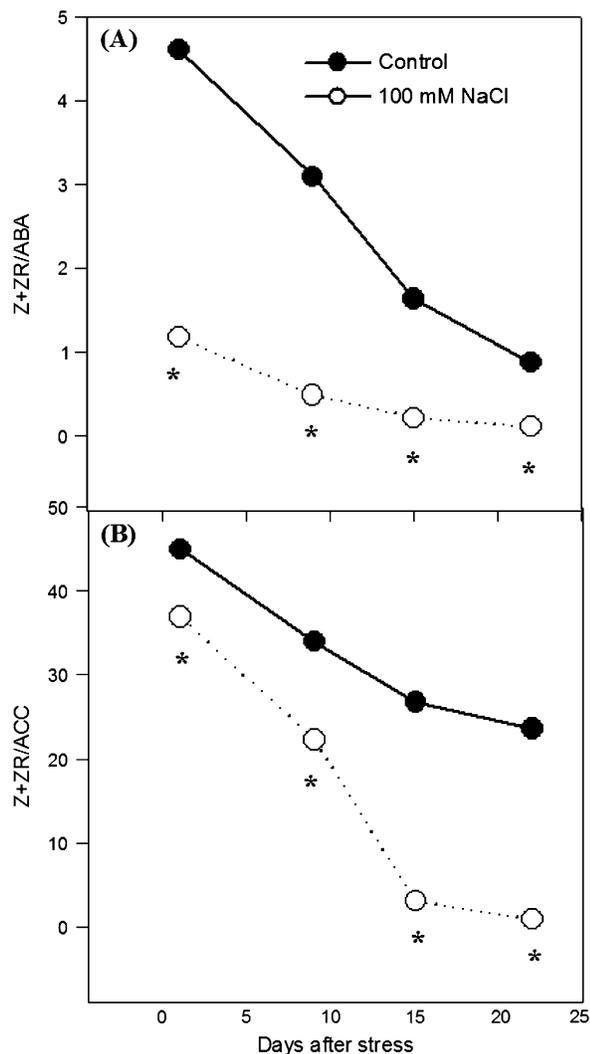


**Fig. 4.** Evolution of ACC (A), ABA (B), and IAA (C) contents during the development of leaf 4 in tomato plants grown for 3 weeks on half-strength Hoagland medium in the absence (filled circles) or presence (open circles) of 100 mM NaCl. Data are means of three plants  $\pm$ SE. Asterisks indicate significant differences between control and salinized leaves according to Student–Newman–Keuls test at  $P < 0.05$ .

**Table 1.** Linear correlation coefficients between senescence-related parameters ( $F_v/F_m$ , NPQ, MDA) and hormonal,  $Na^+$ , and  $K^+$  contents, and hormonal ratios in leaves developed on plants grown for 3 weeks on half-strength Hoagland medium in the absence or presence of 100 mM NaCl

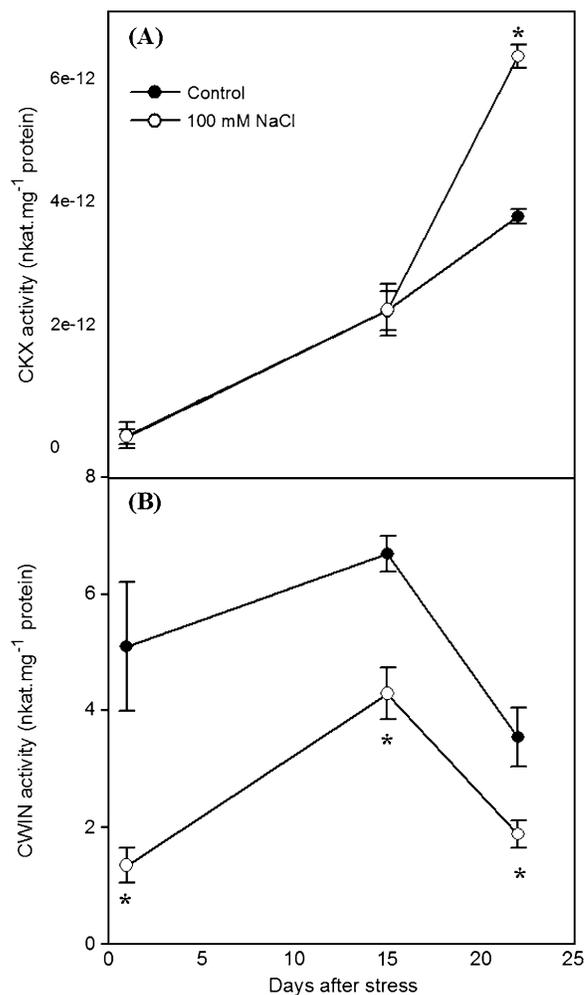
	Z+ZR	ACC	ABA	IAA	Z+ZR/ACC	Z+ZR/ABA	Z+ZR/IAA	IAA/ACC	ACC/ABA	$Na^+$	$K^+$
$F_v/F_m$	0.75*	-0.85**	-0.59	0.24	0.70*	0.41	0.21	0.20	-0.55	-0.56	0.70*
NPQ	-0.84**	0.63	0.77*	-0.30	-0.90**	-0.70*	-0.25	-0.56	0.10	0.66	-0.77*
MDA	-0.87**	0.96***	0.86**	-0.49	-0.91**	-0.61	-0.10	-0.58	0.46	0.80*	-0.84**

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 8$ .



**Fig. 5.** Evolution of some hormonal ratios: (Z+ZR)/ABA (A) and (Z+ZR)/ACC (B) during the development of leaf 4 in tomato plants grown for 3 weeks on half-strength Hoagland medium in the absence (filled circles) or presence (open circles) of 100 mM NaCl. Data are means of three plants  $\pm$ SE. Asterisks indicate significant differences between control and salinized leaves according to Student–Newman–Keuls test at  $P < 0.05$ .

salinity. Since  $\text{Na}^+$  accumulated to a similar extent in both leaf 4 and leaf 5, salt-induced senescence cannot be wholly explained by the putative toxic effect of this ion. Although the presumably higher  $\text{Na}^+$  accumulation in vacuoles of the younger and more actively growing leaf 5 could partially account for these differences (Flowers, 2004; Munns *et al.*, 2006), severe toxic levels ( $>100$  mM) were only found when massive  $\text{Na}^+$  influx occurred in both leaves during the last week of salinization. This could be related to a general failure of the control of  $\text{Na}^+$  exclusion at the root level and its translocation to the shoot, contributing to the late enhanced oxidative stress in leaf 4 and 5, as suggested by the positive correlation between  $\text{Na}^+$  contents and lipid peroxidation (measured as



**Fig. 6.** Evolution of the enzymatic activities for cytokinin dehydrogenase (CKX) (A) and for cell wall invertase (CWIN) (B) in leaf 4 of tomato plants grown for 3 weeks on half-strength Hoagland medium in the absence (filled circles) or presence (open circles) of 100 mM NaCl. Data are means of three plants  $\pm$ SE. Asterisks indicate significant differences between control and salinized leaves according to Student–Newman–Keuls test at  $P < 0.05$ .

MDA production). Nevertheless, the decrease in  $\text{K}^+$  content from the first day of salinization was more related than  $\text{Na}^+$  to the different senescence-related parameters (Table 1).

However, before the onset of the salt-induced senescence in leaf 4, the NPQ was strongly induced from day 1 of salt treatment. This parameter is considered a protective process minimizing the photooxidative damage resulting from the absorption of excess radiation in chloroplasts (Gilmore, 1997). Indeed, diminished capacity of NPQ in leaf 4 could be related to the oxidative damage by day 15 (Mytinova *et al.*, 2006) and with the subsequent decrease in  $F_v/F_m$  values and massive  $\text{Na}^+$  accumulation in both leaves.

Hence, other processes occurring from the first days of salinization, when the osmotic effect predominates, are

probably influencing the evolution of leaf senescence and the capacity of the plant to regulate the accumulation of toxic ions. These processes resulting in photooxidative damage and premature leaf senescence are common to several abiotic stresses such as cold, drought, and salinity (Hare *et al.*, 1997; Huner *et al.*, 1998), while the different responses found between leaves could be related to the different sink–source transitions during leaf development (Guamét *et al.*, 2002; Cowan *et al.*, 2005). Therefore, other endogenous factors influencing those processes, such as hormones, are likely to be influencing the onset and progression of the salt-induced senescence.

#### *Low cytokinin levels promote salt-induced senescence but are not the only signal*

The changing levels of major CKs in tomato (Z+ZR) was the hormonal parameter best related to  $F_v/F_m$ , NPQ, and MDA (Table 1). The involvement of cytokinins in the prevention of leaf senescence has been extensively studied in different plant species (Gan and Amasino, 1995; Balibrea *et al.*, 2004; He *et al.*, 2005; Guo and Gan, 2007; Rivero *et al.*, 2007). As a consequence, it is very likely that the large drop in CK contents (especially ZR) in salinized leaves (Fig. 3C) could contribute to the progression of senescence under stress. This decrease may be the result of less synthesis, reduced transport of root-synthesized CKs, and/or increased breakdown of CKs (Werner *et al.*, 2006; Havlova *et al.*, 2008). Initially, decreased transport of CKs from the roots could be responsible for the decrease in the leaves, as suggested by the decrease of both CKs in root xylem sap under salinity (data not shown). However, the strong decrease observed in leaf ZR content by day 15 of salt treatment can also be explained by an induction of CKX activity (Fig. 6A). The role of CKX could be the maintenance of an optimal level of CKs for growth adaptation to the stress conditions (e.g. shift in shoot to root assimilate partitioning) and/or resetting a CK signalling system to a basal level (Werner *et al.*, 2001; Roitsch *et al.*, 2003). In this regard, the concomitant decrease of the CWIN activity (Fig. 6B), a key factor in the control of senescence by CKs (Balibrea *et al.*, 2004), also argues in favour of the role of the low content of these hormones in the progression of salt-induced senescence (Gan and Amasino, 1995), but it is not a major signal triggering its onset (Werner *et al.* 2003; Munné-Bosch and Alegre, 2004).

#### *IAA and ABA are not the major signals for salt-induced senescence*

Although auxin effects on abscission and senescence were first reported >50 years ago (for a review, see Noodén, 1988), the involvement of this hormone in senescence is much less understood than that of ABA, ethylene, or CKs, and it is difficult to conclude that it antagonizes

senescence (Gan, 2004). In the present study, the highest IAA levels in salinized leaves coincided with the onset of senescence, suggesting it promoted this process. In rebuttal of this notion, IAA levels of salinized plants were 50% lower than in control leaves. Therefore, although a low IAA content could stimulate senescence on the basis that this compound has been generally seen as a senescence-retarding factor (Schippers *et al.*, 2007), it seems unlikely to be a primary factor in the onset of salt-induced senescence, as suggested by the lack of correlation with any of the related parameters (Table 1), and more work is needed to answer this question.

On first inspection, the increased leaf ABA concentration from the first day of salinization may be due to a transient loss of leaf turgor. However, later in the experiment leaf turgor was probably sustained by ionic uptake (Pérez-Alfocea *et al.*, 2000). Since leaf ABA accumulation during salinity occurred even when leaf turgor was sustained by root pressurization (Kefu *et al.*, 1991) and osmotic shock increased leaf ABA concentration 4-fold after 3 d even when leaf turgor was maintained (Gómez-Cadenas *et al.*, 1998), leaf ABA accumulation probably occurs due to root ABA synthesis and export. Although this hormone could certainly influence senescence, as suggested by the positive correlation with both NPQ and MDA, it can be rejected as a primary signal for the onset of this process because its early increase preceded both the decline in  $F_v/F_m$  and the MDA accumulation, and even a protective effect on senescence cannot be excluded. However, the first indirect but immediate effect of ABA on senescence may occur by ABA-induced stomatal closure (Dodds, 2005) decreasing photosynthesis. ABA could also provoke carbohydrate accumulation by putatively blocking sucrose export from mature leaves (Estruch *et al.*, 1989; Pérez-Alfocea and Larher, 1995), contributing to osmotic adjustment during the early (osmotic) phase of salinity (Balibrea *et al.*, 2000). Indeed, the role of ABA in the onset of leaf senescence in *Arabidopsis* appears to be coupled to changes in metabolic fluxes (Schippers *et al.*, 2007), although its effect on sugar transport is still controversial, as indicated above. Low ABA transport from the roots and high CK contents in the leaves (especially ZR) were postulated to delay natural senescence in stay-green maize phenotypes (He *et al.*, 2005). Moreover, while drought stress-induced senescence in tobacco was related to increases in both ABA and CKX activity and to a reduction in active CKs, the increase in CK content through the senescent-induced (*SAG12*) or constitutive (*35S*) expression of the *ZOG1* gene decreased CKX activity and delayed both ABA accumulation and senescence progression in lower leaves (Havlova *et al.*, 2008). An interaction between ABA and CKs is also plausible under salinity, since it has been described that ABA increases *CKX* gene expression (Brugière *et al.*, 2003). Hence, the

ABA increase in leaves could be partially responsible for the decreased CK contents, thus indirectly enhancing the salt-induced senescence. Moreover, since CKs counteract ABA-induced stomatal closure (Dodd, 2005) and inhibit the glucose repression response on photosynthesis (Moore *et al.*, 2003), a low (Z+ZR)/ABA ratio would enhance the non-stomatal photosynthetic inhibition under salinity (Balibrea *et al.*, 2000), as suggested by the negative correlation found with NPQ (Table 1).

However, the effect of ABA on the progress of senescence remains unclear and seems to depend on the plant species, growth conditions, and interactions with other hormones. In the ABA-deficient tomato mutant *sitiens*, salinity promoted leaf senescence more than in the wild type (Mäkälä *et al.*, 2003), presumably due to a higher ethylene evolution (Sharp *et al.*, 2000), while the higher incidence of leaf death was not explained by Na<sup>+</sup> accumulation (Mäkälä *et al.*, 2003). Moreover, it has also been proposed that ABA could provide a protective role against salinity and high intensity light stress in *Arabidopsis* (Cramer, 2002), which can be related to the induction of antioxidant defences (Hu *et al.*, 2006). On the other hand, various *Arabidopsis* mutants defective in either ABA biosynthesis or signalling have not shown measurable effects on the timing or progression of leaf senescence under different environmental stresses (Hensel *et al.*, 1993; Guo and Gan, 2007). Further studies are required to gain insights into the role of ABA in salt-induced leaf senescence.

#### *Increase in the ethylene precursor ACC coincides with F<sub>v</sub>/F<sub>m</sub> decrease and oxidative damage in salinized leaves*

Ethylene has long been considered a key hormone in regulating the onset of senescence (Zacarias and Reid, 1990), and is also suspected to be involved in salt-induced senescence (Munns, 2002a; Wi and Park, 2002). In the present study, leaf ACC concentration sharply increased after 15 d of salt treatment, coincident with the decline in both F<sub>v</sub>/F<sub>m</sub> and NPQ and with the appearance of oxidative damage, which indicate the onset of salt-induced leaf senescence in that leaf. Although increases in ACC, ethylene evolution, and the activity of the enzyme responsible for this conversion (ACC oxidase) are usually concomitant (Gómez-Cadenas *et al.*, 1998; Dodd, 2005; van der Graaf *et al.*, 2006), the relationship between increased ACC levels and onset of senescence should be considered with caution since leaf ethylene evolution was not directly measured. However, the relationship between ACC, ethylene, and senescence has been widely supported using physiological (Zacarias and Reid, 1990; Ella *et al.*, 2003) and genetic approaches. Leaf senescence has been delayed in either transgenic tomato plants with reduced ethylene biosynthesis (John *et al.*, 1995) or mutants with reduced sensitivity to ethylene (Zacarias and Reid, 1990;

Grbic and Bleecker, 1995). However, constitutive overproduction of ethylene in *Arabidopsis* and tomato plants did not cause precocious senescence, suggesting that ethylene alone is not sufficient to initiate leaf senescence (Grbic and Bleecker, 1995). It has been postulated that age-dependent factors are required for ethylene-regulated leaf senescence (He and Gan, 2002). In this regard, the prior decreases in CK content (Fig. 3) could sensitize the senescence process to ethylene. Indeed, the (Z+ZR)/ACC ratio was strongly correlated with the senescence-related parameters (Table 1), suggesting that both hormones are important in this process. It is also interesting to note that increased ACC concentration was prior (day 15) to massive Na<sup>+</sup> entry into the leaves (day 22) and it has been reported that ethylene receptors (e.g. NTHK1) affect sensitivity to salinity and modify Na<sup>+</sup>/K<sup>+</sup> homeostasis (Cao *et al.*, 2006). These data suggest that ethylene and its precursor ACC might play a role in the onset of salt-induced leaf senescence, as suggested by the strong correlation found with the decline both in F<sub>v</sub>/F<sub>m</sub> and in MDA production (Figs 1A, C, E and 4A, Table 1).

#### *Conclusion*

This comprehensive study on the changing levels of several major phytohormones during salt-induced leaf senescence of tomato provides new insights about the sequence of physiological events involved in this process that directly reduces crop productivity. Early events during the osmotic phase of salt stress promote leaf senescence prior to the massive accumulation of toxic ions. Indirect effects due to an initial ABA accumulation and IAA decrease, followed by a strong and continuous reduction in CK contents certainly favours the progression of senescence in salinized leaves, but it is the ethylene precursor ACC that is the major hormonal signal that is temporally correlated with the onset of the oxidative damage and the subsequent chlorophyll fluorescence decrease and, perhaps, the massive Na<sup>+</sup> accumulation. Exogenous or endogenous manipulation of these hormones could improve crop salt tolerance by delaying both senescence during the initial phase of salinity and the subsequent toxic effects. Since salinity induced simultaneous changes in several hormone groups (Figs 3, 4), dissecting the role of a specific hormone in salinity-induced senescence will require the use of transgenics, mutants, or other genetic variability to increase or decrease the level of the hormone of interest. Such experiments will aim to resolve whether it is the basal hormone level prior to the imposition of stress, or hormonal response to salinity that is important in mediating senescence or whether these variables are correlated. However, such experiments assume *a priori* that hormones are regulating senescence and measurement of hormonal relations in genetic material that differs in salinity-induced senescence may provide a complementary approach.

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