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The *aba3-1* Mutant of *Arabidopsis thaliana* Withstands Moderate Doses of Salt Stress by Modulating Leaf Growth and Salicylic Acid Levels

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Abstract The role of abscisic acid (ABA) and salicylic acid (SA) in salt stress tolerance was studied in *Arabidopsis thaliana* using mutants that show a defect in hormone biosynthesis or signaling. Plants were subjected to either control conditions (irrigated with nutrient solution) or a moderate salt stress (nutrient solution + 100 mM NaCl), and the response of the *aba3*, *abi4*, *sid2*, and *eds5* mutants (with defective ABA or SA biosynthesis/signaling) was compared to that of the wild type (WT). A particular phenotype was observed in the *aba3* mutant, which was characterized by reduced plant biomass and lower relative leaf water contents (RWC) under control conditions. However, salt stress reduced growth in the WT, *sid2*, and *eds5* mutants, and to a lesser extent in the *abi4* mutant, but not in the *aba3* mutant. An analysis of the hormonal balance of leaves revealed that altered SA levels may explain, at least partly, growth changes in the *aba3* mutant, under both control and salt stress conditions. The *aba3-1* mutant showed higher SA levels than the WT under control conditions and a drastic decrease in the levels of this plant growth regulator under salt stress, an aspect that was not observed in the WT. However, reductions in endogenous SA levels in *sid2* and *eds5* mutants did not result in increased growth either under control or salt stress conditions. Among the tested genotypes, the *aba3* mutant was the only one in which jasmonic acid (JA) levels did not increase in response to salt stress. It is concluded that although ABA deficiency can severely affect plant growth and water relations in *aba3* mutants, these plants modulate, among other processes, leaf growth and SA levels, which help them withstand moderate doses of salt stress.

Keywords Abscisic acid · Hormonal balance · Jasmonic acid · Plant growth · Salicylic acid · Salinity

Introduction

Salt stress is one of the main limiting conditions for plant growth and productivity (Chaves and others 2009). With increasing drought limitations and higher temperatures, salt stress is becoming an even more serious problem in many areas of the globe in the framework of global climate change (Engels and Jensen 2009; Pereira and others 2009). Acclimation of plants to salt stress requires complex and rapid adjustments in their physiological status at the cellular, tissue, and whole-plant levels. Salinity reduces growth by decreasing leaf water contents and by causing alterations in different metabolic activities of plants, including reductions in photosynthesis, alterations in nutrient homeostasis and solute accumulation, or a combination of all these factors (Vinocur and Altman 2005; Munns and others 2006; Chaves and others 2009). Tolerant plants respond to salt stress by osmotic adjustment, increasing soil water absorption by decreasing tissue water potentials, and through the activation of several other defense mechanisms to maintain growth or to withstand stress conditions once growth is impaired. Therefore, keeping an adequate balance of hormones and other plant growth regulators is essential in plant responses to salt stress (Hasegawa and others 2000). Furthermore, knowledge about stress signaling is also vital for continued

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development of rational breeding and transgenic strategies to improve stress tolerance in crops (Xiong and others 2002).

Because many kinds of stresses, including water, salt, and cold temperatures, induce abscisic acid (ABA) biosynthesis, this hormone is considered one of the most important regulators of plant responses to stress. Recent studies have demonstrated a pivotal role for ABA in the modulation of plant stress responses at the gene expression level and as a regulator of stomatal closure (Ramanjulu and Bartels 2002; Shinozaki and other 2003; Veselov and others 2008). The oxidative cleavage of xanthophylls 9-*cis*-violaxanthin and/or 9′-*cis*-neoxanthin to produce xanthoxin, which is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED), is considered a key step in ABA biosynthesis (Seo and Koshiba 2002). The maize *viviparous14* (*vp14*) mutant and the tomato *notabilis* mutant have been shown to be defective in *NCED* (Tan and others 1997; Burbidge and others 1999). After the cleavage of 9-*cis*-epoxycarotenoids, xanthoxin is converted to ABA in the cytosol. Three possible pathways have been proposed via abscisic aldehyde, xanthoxic acid, or abscisic alcohol (Seo and Koshiba 2002). *aba2* and *aba3* are typical *A. thaliana* mutants impaired in these later steps of ABA biosynthesis (Schwartz and others 1997). *A. thaliana aba3*, tobacco *aba1*, and tomato *flacca* mutants lack the activity of aldehyde oxidase isoforms because of a defect in generating the sulfurylated form of the molybdenum cofactor (MoCo), which is required for their activity (Leydecker and others 1995; Marin and Marion-Poll 1997; Schwartz and others 1997; Akaba and others 1998). In these mutants, exogenously supplied abscisic aldehyde is converted to abscisic alcohol, showing that abscisic aldehyde is reduced to abscisic alcohol and then oxidized to ABA via a shunt pathway (Rock and others 1991). This shunt pathway appears to be a minor source of ABA in WT plants but might play a significant role in these mutants (reviewed by Seo and Koshiba 2002). However, it is unknown how salt stress alters ABA biosynthesis in these mutants.

Aside from ABA, a number of studies suggest that salicylic acid (SA) also could be involved in plant response to abiotic stresses. However, whether it has a protective role in abiotic stress is still controversial. SA leads to H_2O_2 production that may result in acclimation or damage processes, depending on the amount of SA and H_2O_2 produced within the cell, or the SA doses used in preacclimation treatments (El-Tayeb 2005; Stevens and others 2006; reviewed by Horváth and others 2007). Endogenous SA levels have been shown to decrease with salinity (Wang and others 2001), and a negative effect of SA in plant tolerance to salt stress has been observed in studying transgenic *NahG A. thaliana* lines (Borsani and others 2001), which show SA deficiency due to degradation of this compound by a bacterial SA hydroxylase (Gaffney and others 1993). However, treatment with SA leads to ABA accumulation (Shakirova and others 2003) and reduced lipid peroxidation and membrane permeability (Gunes and others 2007) under salt stress, thus indicating a protective effect. Although we showed in a previous study that foliar ABA and SA levels increase in parallel during flowering in WT *A. thaliana* plants (Abreu and Munné-Bosch 2009), and that *NahG* transgenic lines and *sid2* mutants, which are impaired in SA biosynthesis by a defect in isochorismate synthase (Métraux 2002), display reduced ABA accumulation (Abreu and Munné-Bosch 2009), it is still unclear the possible interplay between ABA and SA in plants.

Because hormones are involved in plant responses to salinity, we hypothesized that (1) salt stress may lead to changes in endogenous levels of ABA, but also of SA and other plant growth regulators in leaves, and (2) mutants defective in ABA and SA biosynthesis or signaling may show alterations in the hormonal balance of leaves. With this goal we examined the salt-induced changes in stress indicators, growth, and endogenous levels of ABA, SA, jasmonic acid (JA), cytokinins, and auxins in WT plants and *aba3*, *sid2*, *abi4*, and *eds5* mutants of *A. thaliana*.

Materials and Methods

Plant Material and Treatments

Experiment 1

Seedlings of *A. thaliana* Columbia ecotype, including (1) WT (Col-0) plants, (2) the ABA biosynthesis mutant *aba3-1*, (3) the ABA insensitive mutant *abi4-1*, (4) the SA biosynthesis mutant *sid2-1*, and (5) the SA-deficient mutant *eds5-1* (*enhanced disease susceptibility 5*, previously named *sid1*) were compared. WT, *aba3-1*, and *abi4-1* mutants were purchased from the European *Arabidopsis* Stock Centre (NASC), and *sid2-1* and *eds5-1* mutants were kindly provided by Luis A. J. Mur (Institute of Biological Sciences, University of Wales). Seedlings were grown in separate pots containing a mixture of peat/perlite/vermiculite (1:1:1 by volume) in a constant-environment chamber (8-h photoperiod, 90–110 µmol quanta m^{-2} s^{-1}, air temperature 21–23°C). All potted plants were placed on trays and the different genotypes were mixed following a randomized distribution. When plants were 45 days old they were exposed to salt stress for 11 days by watering every 3 days with Hoagland solution plus 100 mM NaCl; control plants were irrigated at the same time with Hoagland solution without additional NaCl. Each time plants were watered with 1 l of the corresponding solution per tray, which contained around 45 plants, so that each salt-stressed plant received roughly 0.13 g NaCl in addition to that received by controls

every 3 days. Between 8 and 12 plants from different trays were measured per genotype and treatment at the end of the experiment.

Experiment 2

WT and *aba3-1* mutants were grown in the same conditions as in experiment 1 with the same treatments but were exposed to salt stress for 18 days. Sampling was performed on different days during the experiment: day 0 (before treatments) and days 6, 11, 15, and 18 from the start of treatments. Five to six plants, each one from a different tray, were measured per genotype and treatment.

In both experiments, plants remained in the vegetative (prereproductive) stage throughout the study. All measurements were made on whole-rosette leaves collected in the middle of the photoperiod, except for the F_v/F_m ratio, which was measured on attached leaves 1 h before lamps were turned on. The whole leaf rosette was frozen in liquid nitrogen and subsequently stored at −80°C until biochemical (chlorophyll and hormone) analyses. For analyses of leaf growth, water contents, and the F_v/F_m ratio, at least three leaves from different positions in the rosette were measured, and the average of all leaves in each rosette represents a replicate.

Stress Indicators

For the determination of the relative leaf water content (RWC), leaves were collected, immediately weighed (fresh weight), rehydrated for 24 h at 4°C in darkness (turgid weight), and subsequently oven-dried for 72 h at 60°C (dry weight). The RWC was determined as 100 × (fresh wt − dry wt)/(turgid wt − dry wt). Chlorophyll (Chl) levels were measured spectrophotometrically following extraction with 100% methanol, as described previously (Lichtenthaler and Wellburn 1983). The maximum efficiency of photosystem II photochemistry (F_v/F_m), an indicator of photoinhibitory damage to the photosynthetic apparatus (Werner and others 2002), was measured with a pulse-modulated fluorimeter mini-PAM (Walz, Effeltrich, Germany). The F_v/F_m ratio was calculated as $(F_m - F_0)/F_m$, where F_m and F_0 are the maximum and basal fluorescence yields, respectively, of dark-adapted leaves.

Growth Indicators

Rosette biomass was determined by weighing the whole rosette, and leaf biomass was determined as the average weight of at least three leaves per plant. The same leaves were scanned and their area was determined by image analysis using the Image J open software. The leaf mass area (LMA) ratio was calculated as the ratio of leaf biomass/leaf area.

Hormone Analyses

The extraction and analyses of ABA, SA, JA, indole-3-acetic acid (IAA), and the cytokinins zeatin (Z) and zeatin riboside (ZR) were carried out as described previously (Abreu and Munné-Bosch 2009), except that internal deuterated standards and UPLC–MS/MS (instead of HPLC–MS/MS) were used for analyses. Briefly, 100 mg of leaf samples were ground in liquid nitrogen and extracted with 1.5 ml methanol using sonication. After centrifugation, the supernatant was collected and the pellet was re-extracted with isopropanol:glacial acetic acid (99:1) to fully extract cytokinins. The two supernatants were dried completely under a nitrogen stream and redissolved in 150 µl methanol. Then, supernatants were combined, filtered through a 0.22-µm PTFE filter (Waters, Milford, MA, USA), and injected into the LC-MS/MS system. MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada). All the analyses were performed using the Turbo ion spray source in negative ion mode for ABA, SA, JA, and IAA and in positive ion mode for cytokinins. Internal standards (deuterium-labeled hormone analogs, including d_4-SA, d_6-ABA, d_5-JA, d_5-IAA, d_5-Z, and d_5-ZR purchased from OlChemIm, Olomouc, Czech Republic) were added to each sample immediately after grinding, thus allowing the calculation of specific recovery rates for each compound. Quantification by MS/MS using the MRM method was performed as described by Abreu and Munné-Bosch (2009). MRM acquisition was done by monitoring the following transitions: ABA, 263/153; SA, 137/93; JA, 209/59; IAA, 174/130; Z, 220/136; ZR, 352/220; d_6-ABA, 269/159; d_4-SA, 141/97.2; d_5-JA, 214/64; d_5-IAA, 179/135; d_5-Z, 225/141; and d_5-ZR, 357/225. The declustering potential and collision energy were optimized for each compound.

Statistical Analyses

Analysis of variance (ANOVA) was performed to examine statistical differences across genotypes and treatments. The DMS post-hoc test was applied to check for differences between genotypes. Differences were considered significant at a probability level of $P \leq 0.05$. These analyses were conducted using SPSS software (SPSS, Inc., Chicago, IL, USA).

Results

RWC and Growth of *aba3*, *abi4*, *sid2*, and *eds5* Mutants Relative to WT Plants

The comparative study of *aba3*, *abi4*, *sid2*, and *eds5* mutants relative to WT plants under moderate doses of salt

stress (100 mM NaCl) for 11 days revealed that RWC was similar in all genotypes, except for the *aba3* mutant, under both control and salt stress conditions (Fig. 1). The *aba3* mutant had a lower RWC than WT plants. Salt treatment caused a mild decrease in the RWC of all genotypes, with

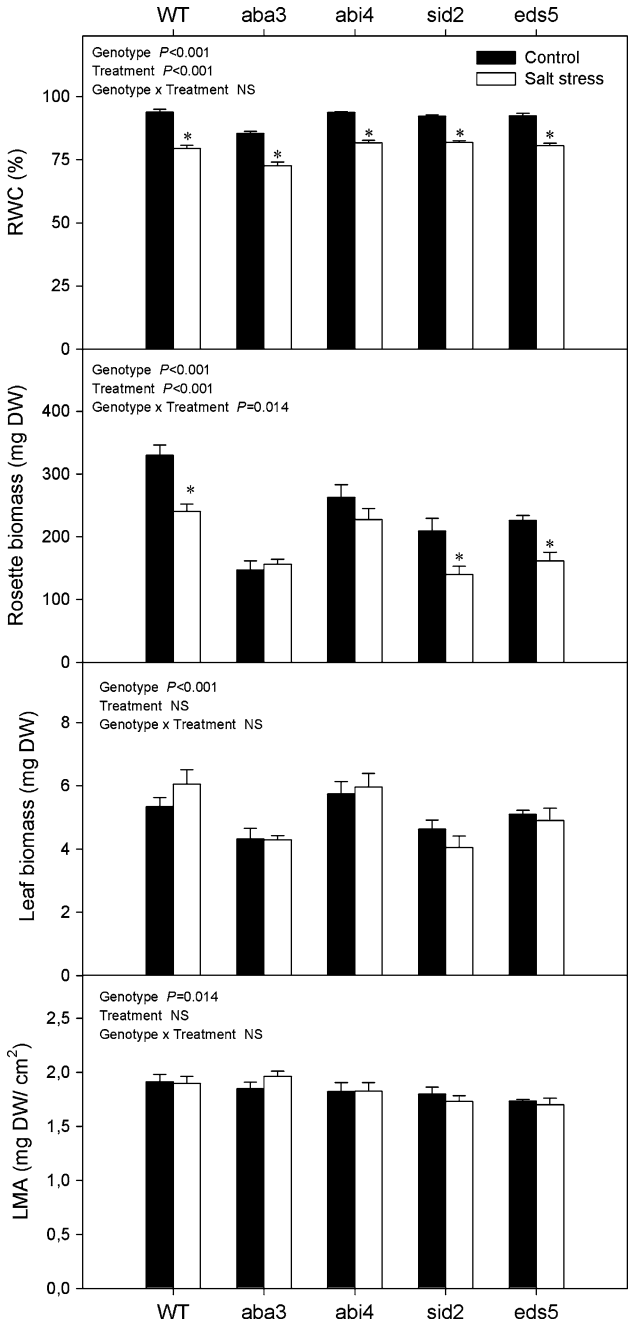

Fig. 1 Relative leaf water content (*RWC*), rosette biomass, leaf biomass, and leaf mass area (*LMA*) of wild type and *aba3*, *abi4*, *sid2*, and *eds5* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 11 days. Data represent the mean ± SE of 8–12 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant). *Asterisks* indicate significant differences between control and salt stress treatment within each genotype (one-way ANOVA, $P \leq 0.05$)

no differences between WT and *aba3*, *abi4*, *sid2*, and *eds5* mutants; however, *aba3* mutants showed smaller RWC values in comparison to WT plants under salt stress. Rosette biomass was affected in all mutants under control conditions, with the *aba3* mutant having the lowest biomass (with a 55% reduction relative to WT). Salt treatment reduced rosette biomass in WT, *sid2*, and *eds5* mutants, but not in *aba3* or *abi4* mutants. This effect was, however, particularly evident in *aba3* mutants and was caused by a lower leaf biomass under both control and stress conditions. Salt treatment did not significantly affect LMA in any genotype (Fig. 1). Chl *a*+*b* levels and the F_v/F_m ratio remained constant upon salt treatment in all genotypes (Table 1).

Hormonal Balance in *aba3*, *abi4*, *sid2*, and *eds5* Mutants and Changes upon Salt Treatment

ABA levels were altered in the *aba3* mutant, which had 55% lower endogenous concentrations than WT under control conditions. SA concentrations in *sid2* and *eds5* mutants were 51 and 53% lower than in WT, respectively (Fig. 2). In addition, *aba3* mutants presented altered endogenous SA concentrations, which were higher than those of WT plants, under control conditions. The hormonal response of mutants to salt stress did not differ from that of WT plants, with the exception of *aba3* mutants. ABA concentrations increased markedly in all genotypes upon salt treatment, but ABA levels in the *aba3* mutant remained lower than those of WT plants. SA concentrations decreased significantly in the *aba3* mutant with salt treatment, abolishing the differences with the WT found under control conditions. Salt stress did not alter SA concentrations in the other genotypes; differences between WT and *sid2* mutants were kept under salt stress. As with ABA, JA levels increased in salt-stressed plants, with the exception of *aba3* mutants, where JA remained unchanged compared to control conditions (Fig. 2). Upon salt treatment, IAA and Z

Table 1 Chlorophyll (*Chl*) a+b concentrations and F_v/F_m ratio for all genotypes under control or salt stress treatment for 11 days

	Chl a+b (mg/g DW)		F_v/F_m	
	Control	100 mM NaCl	Control	100 mM NaCl
WT	16.7 ± 5.2	13.7 ± 2.7	0.85 ± 0.01	0.85 ± 0.02
aba3	17.6 ± 4.5	18.2 ± 2.2	0.85 ± 0.02	0.86 ± 0.01
abi4	15.3 ± 2.3	17.4 ± 4.3	0.86 ± 0.01	0.84 ± 0.02
sid2	15.3 ± 3.5	18.1 ± 4.1	0.86 ± 0.01	0.85 ± 0.02
eds5	18.4 ± 3.7	14.2 ± 4.0	0.85 ± 0.02	0.85 ± 0.02

Data are mean ± SE of 8-12 individuals. No significant differences were observed between control and salt stress treatment in any of the genotypes (Student's *t*-test, $p \leq 0.05$)

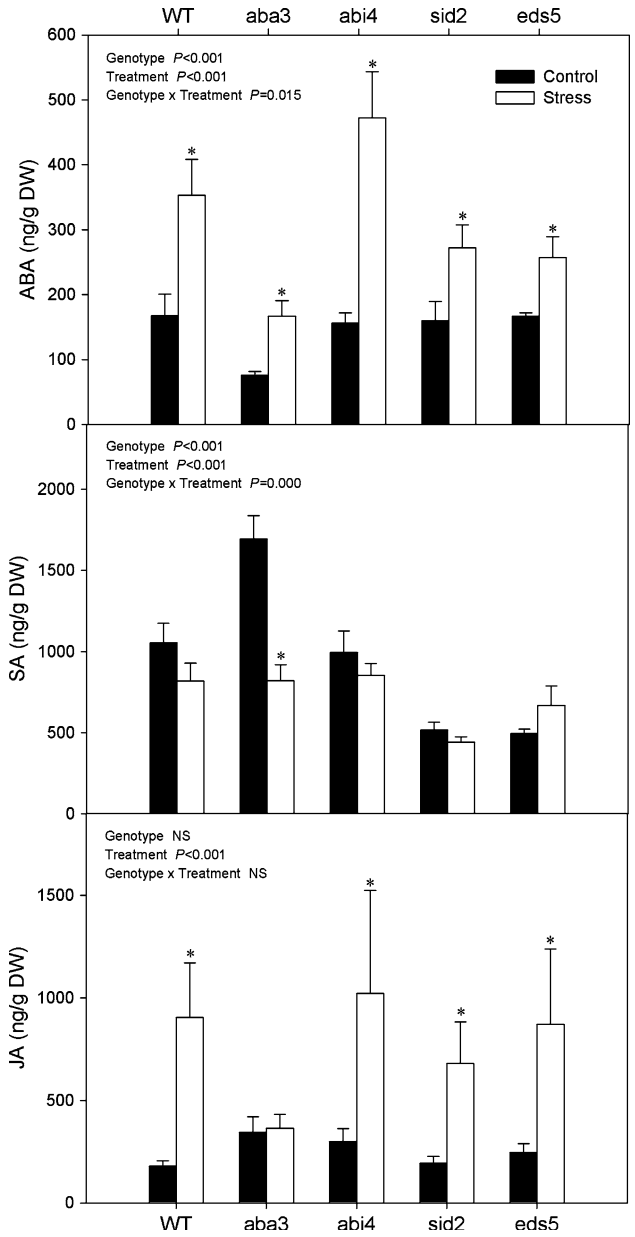

Fig. 2 Changes in the endogenous concentrations of abscisic acid (*ABA*), salicylic acid (*SA*), and jasmonic acid (*JA*) in the leaves of wild type and *aba3*, *abi4*, *sid2*, and *eds5* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 11 days. Data represent the mean ± SE of 8–12 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant). *Asterisks* indicate significant differences between control and salt stress treatment within each genotype (one-way ANOVA, $P \leq 0.05$)

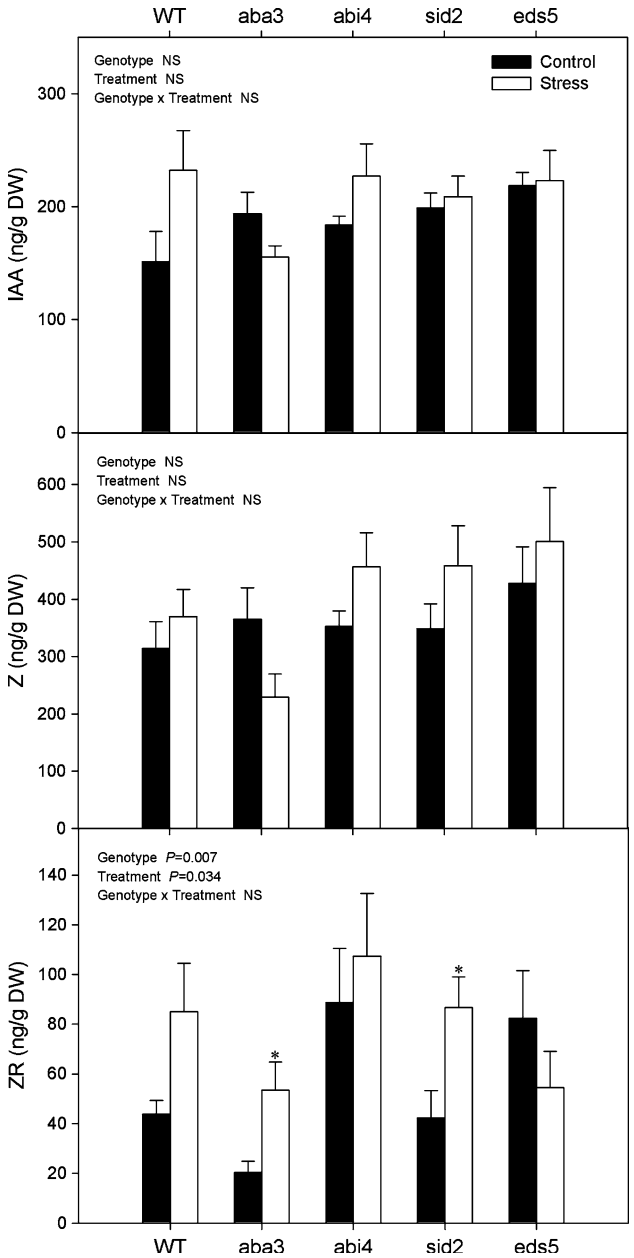

Fig. 3 Changes in the endogenous concentrations of indole-3-acetic acid (*IAA*), zeatin (*Z*), and zeatin riboside (*ZR*) in the leaves of wild type and *aba3*, *abi4*, *sid2*, and *eds5* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 11 days. Data represent the mean ± SE of 8–12 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant). *Asterisks* indicate significant differences between control and salt stress treatment within each genotype (one-way ANOVA, $P \leq 0.05$)

concentrations showed a general trend to keep constant or slightly increase in all genotypes except *aba3* mutants, in which the levels of these hormones tended to decrease. Under salt stress, *sid2* and *aba3* mutants showed significant increases in endogenous ZR concentrations, as in WT plants, although differences were not significant in WT plants (Fig. 3).

Time Course Evolution of RWC and Growth in the *aba3* Mutant

The dramatic decrease in growth observed in *aba3* mutants compared to the WT, and their growth maintenance together with an altered hormonal balance under salt stress, led

us to deepen the study of growth regulation in the *aba3* mutant under salt stress. With this goal, a second experiment was performed comparing WT and *aba3* genotypes under a longer treatment (18 days) and performing five samplings throughout the study. In this time course evolution experiment, RWC of *aba3* mutants remained below WT values throughout the study (Fig. 4). RWC fell at the early stages of salt stress in both *aba3* mutants and the WT but remained constant later; RWC of these mutants was always lower than that of WT but never fell below 70%. Differences in rosette biomass were found from the start of the experiment but became more marked after 6 days of treatment, from which WT plants experienced a sharp growth increase, whereas growth was arrested in the *aba3* mutant. The growth rate of this mutant under salt stress was lower than that of WT plants but similar to their rate under control conditions. Therefore, differences with WT plants became smaller with salt stress (Fig. 4). Leaf biomass in WT plants increased progressively under control conditions; leaf biomass of *aba3* mutants also increased, but values were always below those of the WT. Therefore, a lower leaf biomass of *aba3* mutants partly explained the lower rosette biomass. However, salt stress did not significantly affect leaf biomass in WT or *aba3* mutants (Table 2), which indicated that the lower rosette biomass of WT under salt stress was a result of a lower leaf number rather than a lower leaf biomass. There were no significant differences in LMA between *aba3* mutants and the WT under control conditions; however, salt stress significantly increased LMA in *aba3* mutants (Fig. 4, Table 2), indicating a reduction in leaf area in salt-stressed *aba3* mutants. Furthermore, Chl levels remained constant and the F_v/F_m ratio was maintained above 0.80 throughout the experiment in these mutants (data not shown).

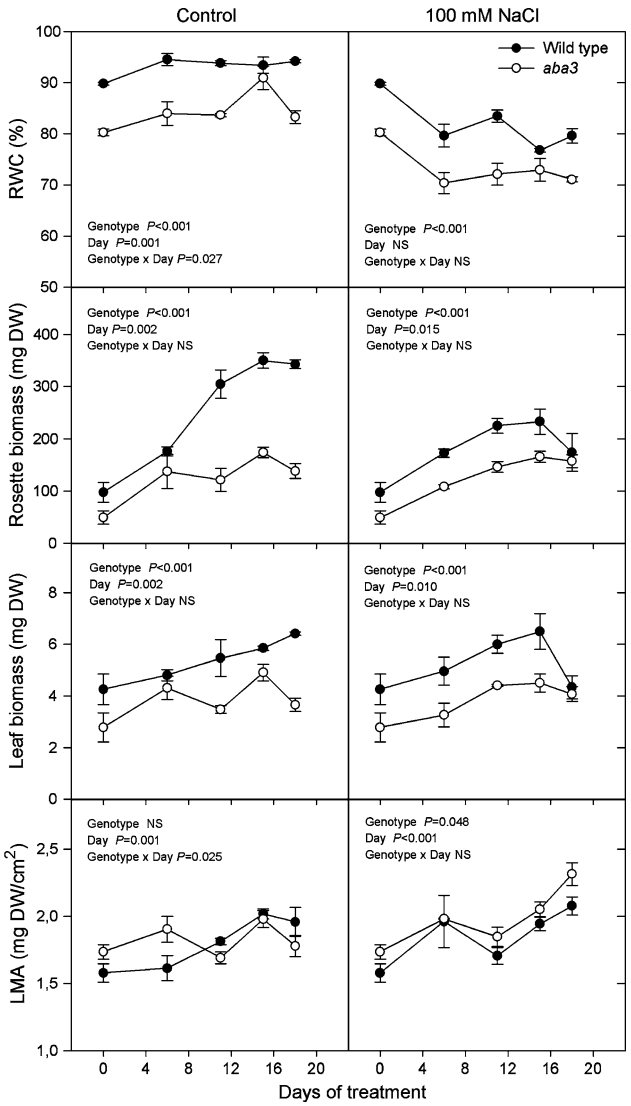

Fig. 4 Time course evolution of the relative leaf water content (*RWC*), rosette biomass, leaf biomass, and leaf mass area (*LMA*) of wild type and *aba3* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 18 days. Data represent the mean ± SE of 5–6 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant)

Time Course Evolution of Endogenous Hormone Concentrations in the *aba3* Mutant upon Salt Stress Treatment

Time course evolution of hormone concentrations was consistent with the differences observed between *aba3* mutant and WT plants in the first experiment. As shown in Fig. 5, ABA concentrations in the *aba3* mutant were lower than in the WT under both control and salt stress conditions. Differences increased upon salt treatment, because WT plants increased ABA concentrations since the start of salt treatment and *aba3* mutants were unable to enhance

Table 2 Results of ANOVA tests for parameters measured in experiment 2 comparing control versus salt stress treatment (100 mM NaCl) in wild-type (*WT*) and *aba3* mutants of *A. thaliana*

	WT	*aba3*
RWC	*	*
Rosette biomass	*	NS
Leaf biomass	NS	NS
LMA	NS	*
ABA (ng/g DW)	*	NS
SA (ng/g DW)	NS	*
JA (ng/g DW)	*	NS
IAA (ng/g DW)	NS	NS
Z (ng/g DW)	NS	NS
ZR (ng/g DW)	NS	NS

An *asterisk* indicates significant differences between control and salt stress within each genotype ($P \leq 0.05$)

NS not significant

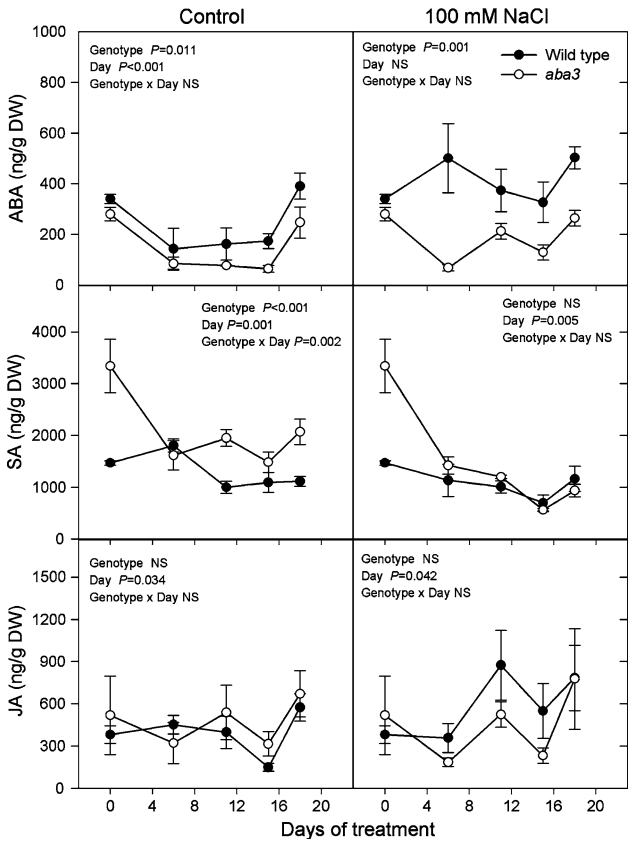

Fig. 5 Time course evolution of the endogenous concentrations of abscisic acid (*ABA*), salicylic acid (*SA*), and jasmonic acid (*JA*) in the leaves of wild type and *aba3* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 18 days. Data represent the mean ± SE of 5–6 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant)

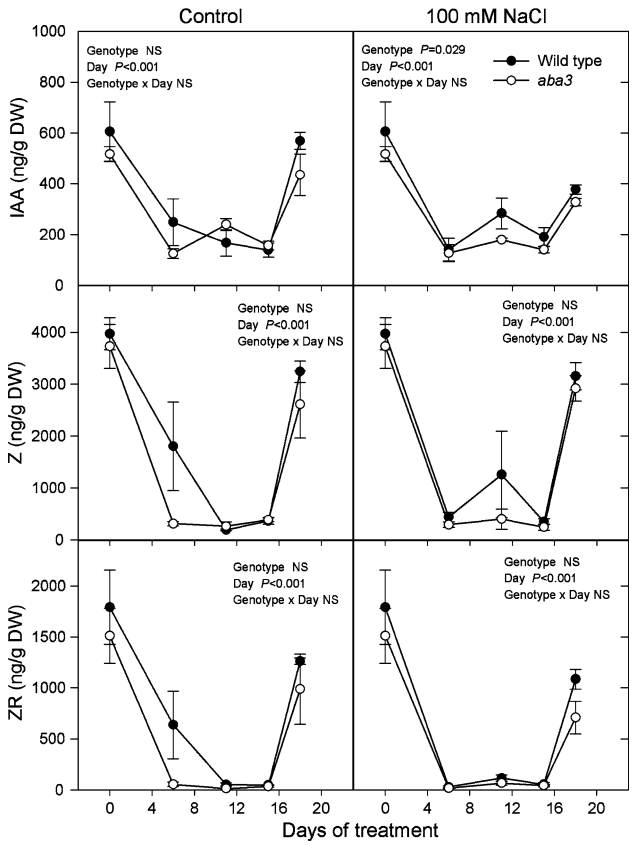

Fig. 6 Time course evolution of the endogenous concentrations of indole-3-acetic acid (*IAA*), zeatin (*Z*), and zeatin riboside (*ZR*) in the leaves of wild type and *aba3* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 18 days. Data represent the mean ± SE of 5–6 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant)

their ABA levels. SA concentrations were higher in *aba3* mutants than in the WT under control conditions, and salt treatment caused a decrease in the concentration of SA after 6 days of treatment in *aba3* mutants only (Fig. 5, Table 2). No differences in JA concentrations were found between *aba3* mutant and WT plants under control conditions, but it is remarkable that although JA showed a transitory increase in WT at days 11 and 15 of salt treatment, *aba3* mutants did not alter their JA concentrations with salt stress (Fig. 5, Table 2). The concentrations of IAA, Z, and ZR showed an initial decrease followed by similar levels along the experiment, with a final increase in both genotypes and treatments. The *aba3* mutant showed slightly but significantly lower IAA concentrations than the WT under salt stress (Fig. 6).

To better understand the hormonal response in *aba3* mutants, the amounts of hormones per plant (ng/rosette) were measured. Differences in ABA concentrations between genotypes increased on a rosette basis (Fig. 7). However, SA differences under control conditions disappeared, thus indicating a concentration effect due to a lower plant biomass in *aba3* mutants. For the same reason, lower SA levels per rosette were found in salt-stressed plants in comparison to control conditions. Differences in JA levels appeared under control conditions at the end of the experiment due to a higher rosette biomass of WT plants, and the higher accumulation of JA in salt-stressed WT plants compared to *aba3* mutants at days 11 and 15 became clearer and indicated an enhanced accumulation of JA in WT plants under salt stress (Fig. 7). Moreover, differences in IAA, Z, and ZR between WT and *aba3* mutants became apparent when hormone levels were expressed per rosette unit. WT plants had higher levels of these hormones per rosette under control conditions, especially at the end of the experiment (Fig. 8).

Discussion

A central role for ABA in abiotic stress tolerance such as drought, salt, and cold stress has been long recognized. Stomatal closure under drought or osmotic stress, which is

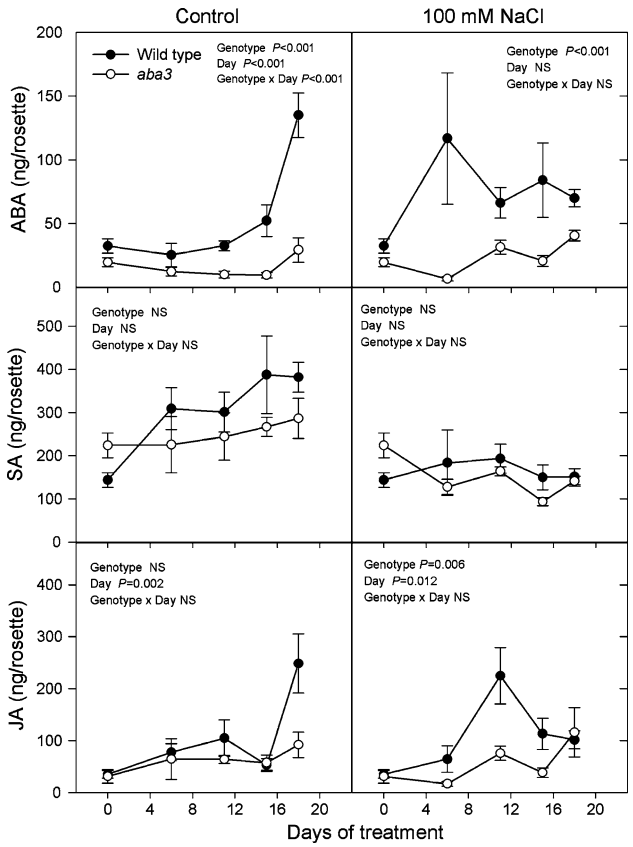

Fig. 7 Time course evolution of the endogenous levels of abscisic acid (*ABA*), salicylic acid (*SA*), and jasmonic acid (*JA*), given per rosette unit (ng hormone/rosette), of wild type and *aba3* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 18 days. Data represent the mean ± SE of 5–6 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant)

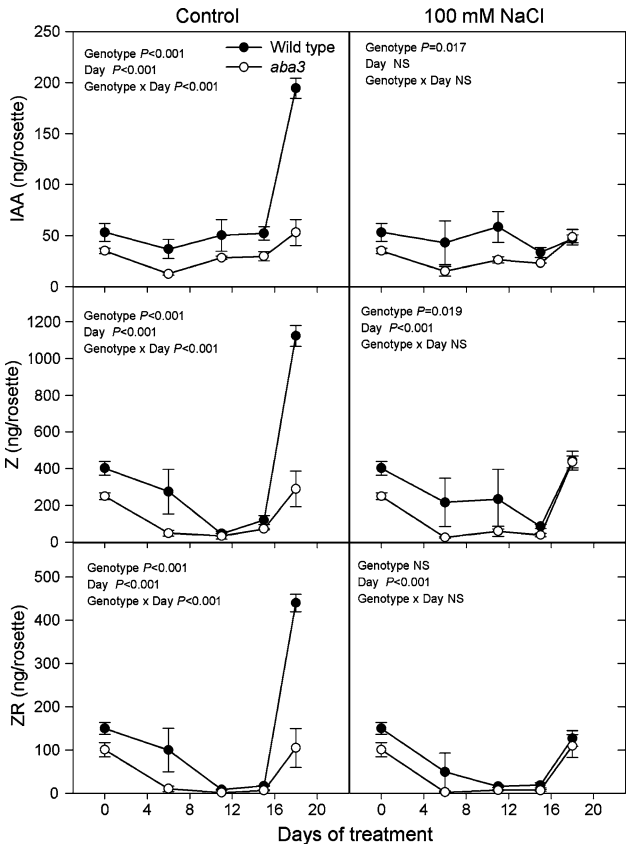

Fig. 8 Time course evolution of the endogenous levels of indole-3-acetic acid (*IAA*), zeatin (*Z*), and zeatin riboside (*ZR*), given per rosette unit (ng hormone/rosette), of wild type and *aba3* mutants of *A. thaliana* after exposure to control or salt stress treatment (100 mM NaCl) for 18 days. Data represent the mean ± SE of 5–6 individuals. Results of two-way ANOVA are shown inside panels ($P \leq 0.05$; *NS* not significant)

crucial to regulate plant water balance, is triggered by an ABA-induced increase in guard cells (Pei and others 2000). Growth arrest is a plant general response to stressful conditions and is considered a strategy to sustain metabolism under an impaired photosynthetic energy supply. ABA has been found to induce the expression of ICK1, an inhibitor of cyclin-dependent kinases (CDKs), which are emerging as key regulators of cell division. It has been hypothesized that ABA could be directly involved in cell cycle arrest under osmotic stress through this mechanism (Zhou and others 2003). In the present study, increased ABA levels and reduced growth were found in WT plants in response to salt stress, which are consistent with this view. However, our comparative study on different ABA and SA mutants revealed a particular phenotype for *aba3* mutants. ABA deficiency and a further partial inability to increase ABA levels under salt stress in *aba3* mutants did not imply higher growth, neither under control nor salt stress conditions. Indeed, *aba3* mutants grew less than WT plants under both control and salt stress conditions. We were therefore interested in examining the possible interplay between ABA and other hormones that might explain this phenotype, with a particular emphasis on the possible interaction between ABA and SA, two key regulators of plant responses to salt stress.

aba3 mutants were first identified as being impaired in the conversion of ABA-aldehyde to ABA (Léon-Kloosterziel and others 1996; Schwartz and others 1997). *ABA3* encodes a molybdenum cofactor (MoCo) sulfurase that catalyzes the generation of the sulfurylated form of MoCo, a cofactor required by aldehyde oxidase that functions in the last step of ABA biosynthesis in plants and expression of which increases in response to drought, salt, or ABA treatment (Xiong and others 2001). Interestingly, however, although *aba3* mutants are deficient in ABA, as shown in the present study; they can increase (though to a lower extent than WT plants) ABA levels under salt stress. Therefore, *aba3* mutants can increase ABA biosynthesis under salt stress by the activation of a shunt pathway that presumably involves the reduction of abscisic aldehyde to

abscisic alcohol followed by oxidation to ABA (Rock and others 1991). This shunt pathway is thought to have a minor role in WT plants but appears to play a significant role in these mutants, as has been previously suggested (Seo and Koshiba 2002).

It previously has been shown that SA accumulation in *A. thaliana* plants accelerates the developmental program at the expense of reducing plant growth and seed production, so that rapid reproduction is achieved at the cost of reducing seed yield in this short-lived species (Abreu and Munné-Bosch 2009). Moreover, recently it has been found that SA mediates inhibition of cell division and elongation in *siz1* mutants of *A. thaliana* through a downregulation of expression of *XTH8* and *XTH31*, which encode xyloglucan endotransglycosylase/hydrolases necessary for cell wall loosening and reorganization (Miura and others 2010). In *aba3* mutants, growth is severely impaired under control conditions; this was associated with increased SA accumulation in leaves, and reductions in SA accumulation under salt stress might help *aba3* mutants tolerate stress and maintain growth rates to a minimal level. Borsani and others (2001) found greater oxidative damage in WT than in SA-deficient *NahG* seedlings treated with 100 mM NaCl, suggesting that SA increased free radical generation during photosynthesis under salt stress. Recently, it has been found that *NahG* plants show a better adaptation to moderate salt stress due to higher contents of reduced glutathione and reduced ascorbic acid under stress (Cao and others 2009). Therefore, reduced growth and SA accumulation might be considered symptoms of stress, but at the same time they can help *aba3* mutants withstand moderate doses of salt stress by reducing transpiration losses at the whole-plant level and by triggering stress defense responses (Horváth and others 2007). However, reductions in endogenous SA levels in *sid2* and *eds5* mutants did not result in increased growth under either control or salt stress conditions. Therefore, it appears that although SA may contribute to modulating plant growth in *aba3* mutants, changes in the levels of this plant growth regulator alone may not necessarily lead to changes in plant growth. Interestingly, the behavior of *sid2* and *eds5* mutants is similar to that of WT under salt stress in terms of chlorophyll levels and the F_v/F_m ratio, thus indicating absence of oxidative damage to the photosynthetic apparatus. In contrast, it has been shown that SA-deficient *NahG* seedlings treated with 100 mM NaCl are more resistant to salt-induced oxidative stress than the WT (Borsani and others 2001). This discrepancy may be related to the fact that stress imposed on adult plants resulted in a moderate stress in the present study, whereas the same salt dose imposed to seedlings led to an increased degree of oxidative stress in the above-mentioned study. Furthermore, we can not rule out the possibility of a protective effect of catechol, which is known to accumulate in *NahG* transgenic lines which express a bacterial SA hydroxylase and convert SA into catechol (Nawrath and Métraux 1999).

Also of interest was the lack of a JA increase upon salt treatment in *aba3* mutants. Among the tested genotypes, the *aba3* mutant was the only one in which JA levels did not increase in response to salt stress. JA may activate the expression of several defense-related genes (Reymond and Farmer 1998); thus, lack of JA-related response may be attributed to a deficient defense mechanism, which might increase the susceptibility of these mutants to higher doses of salinity. Although the present study has shown that *aba3* mutants can withstand moderate doses of salt stress, it should be kept in mind that these mutants show a wilty phenotype and are very sensitive to high doses of salt stress (Sagi and others 1999; Xiong and others 2001). The fact that JA levels increased in WT plants and in *abi4*, *sid2*, and *eds5* mutants, but not in *aba3* mutants, upon salt treatment suggests that JA biosynthesis is under ABA control or may be at least partly associated with ABA-related processes occurring in salt-stressed plants, such as lipid peroxidation, which can increase as a consequence of stomatal closure and ROS formation in chloroplasts. Interestingly, the results indicate that this enhanced JA biosynthesis in salt-stressed plants is not triggered by *ABI4*, which encodes an ABA-regulated APETALA 2 domain transcription factor (Finkelstein and others 1998), because *abi4* mutants accumulated JA levels as did the WT upon salt stress treatment. Furthermore, it should be considered that the sulfurylated form of MoCo is required not only for the latest steps in the biosynthesis of ABA but also for IAA and purine biosynthesis (Sagi and others 1999; Zhong and others 2010). In the present study, it was shown that *aba3* mutants showed slightly but significantly lower IAA concentrations than the WT under salt stress, thus suggesting a complex interplay between ABA, SA, JA, and IAA in growth modulation in salt-stressed *aba3* mutants, an aspect that undoubtedly warrants further investigation.

It is also worthy to note that although *aba3* mutants showed altered SA levels under both control and salt stress conditions, ABA levels were not affected in *sid2* and *eds5* mutants relative to WT plants in any of the tested conditions. Wildermuth and others (2001) have mapped the SA-induction-deficient *sid2* mutation to a gene (*ICS1*) encoding isochorismate synthase. The level of SA after infection in *sid2* mutants is only 5–10% of the WT levels and resistance to fungal or bacterial pathogens is reduced, thus suggesting that higher plants produce significant amounts of SA from isochorismate, a biosynthetic pathway typical for bacteria (Métraux 2002). In the present study it was shown that this pathway is also important for SA biosynthesis under salt stress, because SA levels were half of those observed in WT plants under both control and salt

stress conditions. In addition, the SA-deficient mutant *eds5* (*enhanced disease susceptibility 5*, previously named *sid1*) behaved similarly to *sid2* mutants. Because SA accumulation was affected in ABA-deficient mutants but ABA accumulation was not altered in SA-deficient mutants, it is likely that ABA exerts control over SA accumulation in leaves.

Another important point is the biological significance of expressing hormone levels per dry weight (ng/g DW) and per plant (ng/rosette). When plants are found in an active growth period or plant growth is significantly altered by reductions in water availability, as occurs in plant responses to drought, salt, or osmotic stress, it is convenient to show hormone levels on a per-plant basis in addition to concentrations per dry weight, because changes in concentrations might simply reflect alterations in plant biomass. For instance, it was shown in the present study that when given on a rosette basis, differences in SA levels per dry matter disappeared. In contrast, IAA and Z levels per rosette were lower in *aba3* mutants than in the WT simply because of the reduced growth in the mutants. Therefore, modulation of cell growth is as important as hormone biosynthesis, conjugation, or degradation in the regulation of hormone concentrations in tissues.

In conclusion, *aba3* mutants modulate, among other processes, leaf growth and SA levels, which may help plants withstand moderate doses of salt stress. The present study suggests that growth reduction is a "necessary evil" to tolerate stress in *aba3* mutants, which optimize this response through an adequate hormonal balance.

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