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Auxin signaling participates in the adaptative response against oxidative stress and salinity by interacting with redox metabolism in *Arabidopsis*

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Abstract Auxin regulates gene expression through direct physical interaction with TIR1/AFB receptor proteins during different processes of growth and development in plants. Here we report the contribution of auxin signaling pathway to the adaptative response against abiotic stress in *Arabidopsis*. Phenotypic characterization of *tir1/afb* auxin receptor mutants indicates a differential participation of each member under abiotic stress. In particular, *tir1 afb2* and *tir1 afb3* mutants resulted more tolerant to oxidative stress. In addition, *tir1 afb2* showed increased tolerance against salinity measured as chlorophyll content, germination rate and root elongation compared with wild-type plants. Furthermore, *tir1 afb2* displayed a reduced accumulation of hydrogen peroxide and superoxide anion, as well as enhanced antioxidant enzymes activities under stress. A higher level of ascorbic acid was detected in *tir1 afb2* compared with wild-type plants. Thus, adaptation to salinity in *Arabidopsis* may be mediated in part by an auxin/redox interaction.

Keywords *Arabidopsis thaliana* · Auxin signaling · Abiotic stress · Reactive oxygen species · Antioxidants

Abbreviations

ROS	Reactive oxygen species
MV	Methyl viologen
H_2O_2	Hydrogen peroxide
O_2^-	Superoxide anion
AA	Ascorbate
DHA	Dihydroascorbate
CAT	Catalase
APX	Ascorbate peroxidase

Introduction

The phytohormone auxin regulates a wide variety of plant developmental programs through the action of TIR1/AFB receptors (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005), Aux/IAA transcriptional repressors (Overvoorde et al. 2005) and ARF transcription factors (Guilfoyle and Hagen 2007).

TIR1 (Transport Inhibitor Response 1) and its paralogs, AFB1, AFB2 and AFB3 (Auxin signaling F-Box 1, 2 and 3) are the F-box subunits of SCF E3-ubiquitin ligase complex and function as auxin receptors (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005). Auxin binding to $SCF^{TIR1/AFB}$ results in the targeted ubiquitination and degradation of Aux/IAA proteins (Gray et al. 2001; Dharmasiri et al. 2005b). Aux/IAA degradation promotes activation of ARF transcription factors and the consequent expression of auxin-responsive genes (Hagen and Guilfoyle 2002). Genetic studies show that *TIR1* and *AFB* genes function in a partially

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redundant way to mediate auxin response. The stepwise reduction in *TIR1* and *AFB* gene dosage causes a progressive decrease in auxin response and increasingly severe defects in development (Dharmasiri et al. 2005b).

On the other hand, plants are frequently exposed to diverse stresses throughout their life cycles. Environmental conditions seem to have a direct impact on hormone levels. Plants can integrate intrinsic developmental programs and quickly adjust growth to changing situations by influencing hormone homeostasis (Wolters and Jürgens 2009). Recent evidences indicated that auxin signaling is down-regulated under different biotic stresses (Kazan and Manners 2009). The recognition of conserved bacterial flagellin from *Pseudomonas syringae* by *Arabidopsis* plants activates the expression of the microRNA miR393 which then, down-regulates the expression of the auxin receptor genes *TIR1*, *AFB2* and *AFB3*. This leads to the suppression of auxin-responsive gene expression reducing the disease symptoms. Furthermore, supporting the role of auxin signaling in bacterial pathogenesis, the overexpression of a TIR1 paralog which is partially refractory to miR393 enhances susceptibility to *P. syringae* (Navarro et al. 2006). However, the role of auxin signaling pathway during abiotic stress remains largely unknown. Key elements in the adaptative response to abiotic stress seem to be hormone-induced changes in growth and morphology, as well as stress-affected gradients of reactive oxygen species (ROS) and antioxidants (Potters et al. 2009).

In this work, we demonstrate the participation of TIR1/AFB auxin receptors in response to oxidative treatments and salinity. *Arabidopsis* plants appear to use an auxin-redox interconnection during the adaptative response to oxidative and salt stresses.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana mutants *tir1*-1 *afb1*-3 (Savaldi-Goldstein et al. 2008), *tir1*-1 *afb2*-3 (Savaldi-Goldstein et al. 2008) and *tir1*-1 *afb3*-4 (Parry et al. 2009) are in the Columbia (Col-0) ecotype.

Seeds were surface-sterilized in 30% commercial bleach and 0.02% Triton X-100 for 15 min, rinsed four times with sterile H_2O and stratified at 4°C for 2 d in the dark. Then, they were plated on ATS medium plus 1% sucrose and 0.8% agar (Wilson et al. 1990) and placed vertically in the growth chamber at 23°C under 120 µmol photons m^{-2} s^{-1} with 16:8 h light:dark cycles. Alternatively, for superoxide anion (O_2^-) and ascorbate (AA) measurements, stratified seeds were grown on organic substrate and vermiculite (4:1) and placed in the growth chamber for 14 d. Then, seedlings were subjected to stress treatments.

Germination and growth measurements

Approximately 50 seeds per line were sown on filter paper, wetted with sterile H_2O, KCl (150, 200 mM) or NaCl (150, 200 mM) and stratified at 4°C for 2 d in the dark. Then, seeds were transferred into the growth chamber for 2 d. The percentage of germination was scored according to Boyes et al. (2001).

For root elongation measurements, seeds were grown vertically on ATS medium plus 1% sucrose and 0.8% agar supplemented with 10 nM methyl viologen (MV) or 50 mM NaCl. Root elongation was measured after 7 d of treatment using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij).

Cell death measurements

Seven-d-old seedlings were transferred to liquid ATS medium supplemented with or without 20 mM H_2O_2 for 1.5 h. Cell death was analyzed as optimized by Arce et al. (2010). Briefly, seedlings were stained with 0.5% Evan's blue solution (Sigma) for 30 min. Stained roots were cut 1 cm above the apical tip and incubated in 100 µl 100% dimethyl sulfoxide (DMSO) at 65°C for 30 min. For a quantitative estimation of cell death the colorant specifically retained in death cells was eluted and quantified spectrophotometrically at 565 nm (UltrospecTM 1100).

Chlorophyll content

Ten-d-old seedlings were transferred into liquid ATS medium supplemented with 250 mM NaCl for 3 d. Leaves (0.5 g) were ground in liquid N_2 and the powder was extracted with 80% acetone for 30 min in the dark. Plant extracts were centrifugated at 10,000*g* for 20 min. The chlorophyll content was measured spectrophotometrically at 652 nm (UltrospecTM 1100) according to Tossi et al. (2009).

Hydrogen peroxide level

Ten-d-old seedlings were transferred into liquid ATS medium supplemented with 1 µM MV or 250 mM NaCl for 8 and 24 h, respectively. Seedlings (0.5 g) were ground in liquid N_2 and extracted with H_2O for 30 min in the dark, followed by centrifugation at 10,000*g* for 20 min. H_2O_2 was quantified according to Bellincampi et al. (2000) based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (*o*-cresolsulfonephthalein 3′,3″-bis(methylimino)diacetic acid; sodium salt).

Superoxide detection

Leaves from 14-d-old plants were treated with 50 μM MV plus 5×10^{-3}% silwet L-77 for 1.5 h. Leaves were stained with 0.2% NBT in 10 mM potassium phosphate buffer pH 7.5 for 30 min as described by Jabs et al. (1996). Leaves were incubated in 96% ethanol overnight for bleaching. Photographs were taken with a camera mounted on a magnifying glass (Nikon SMZ800). Quantitative analysis was conducted by scanning the pixels of blue-stained spots and the entire leaf area using Matrox Inspector 2.2 software (Matrox Electronics Systems, Ltd.). Generation of O_2^- was expressed as the percentage of pixels in the stained area versus total number of pixels in the whole leaf.

Ascorbate measurements

Fourteen-d-old plants were watered with 250 mM NaCl twice with a 3 d interval. AA level was measured by High-performance liquid chromatography (HPLC) according to Bartoli et al. (2006) with minor changes. Leaves (0.5–1 g) were ground in liquid N_2 and the powder was extracted in 6% trifluoracetic acid (TFA) followed by centrifugation at 13,000*g* for 5 min. Supernatants were passed through a C-18 column (Bond Elute, Varian). Samples were filtered and injected onto an HPLC system (Shimatzu LC-10Atvp solvent delivery module) equipped with a C-18 column (Varian Chromsep 10034.6 mm) and detected at 265 nm (Shimatzu UV–Vis SPD—10Avp detector). AA was separated isocratically using 100 mM phosphate buffer pH 3.0 as a running solution at a flux of 0.5 ml min^{-1}. Total AA was measured after reducing dehydroascorbate (DHA) by mixing 1 volume of the sample obtained after the C-18 column and 1 volume of 100 mM K_2HPO_4, in the presence of 5 mM di-thiothreitol (DTT). The reaction was incubated for 10 min. Then 0.2 volumes of 6% TFA were added to the mixture and AA was measured. DHA was calculated as the difference between total and reduced AA.

Catalase and ascorbate peroxidase activities

Ten-d-old seedlings were transferred to liquid ATS medium supplemented with 250 mM NaCl for 24 h. Tissue samples (0.5–1 g) were ground in liquid N_2 and the powder was extracted in 4 volumes of 50 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA, 1% polyvinylpyrrolidone (PVP-40) and 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by centrifugation at 10,000*g* for 20 min. For ascorbate peroxidase (APX) activity, 1 mM AA was also added to the extraction buffer. Catalase (CAT) activity was measured by following the decomposition of H_2O_2 at 240 nm ($\varepsilon = 40\ M^{-1}\ cm^{-1}$) according to Rao et al. (1996). The reaction was initiated by adding H_2O_2. APX activity was measured following the H_2O_2-dependent oxidation of AA at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$), as described by Amako et al. (1994). Total proteins were determined according to Bradford (1976) with bovine serum albumin as a standard.

RNA preparation and northern blot analysis

Ten-d-old seedlings were transferred to liquid ATS medium supplemented with 250 mM NaCl for 12 or 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen). RNA was separated on a 1.5% agarose gel containing 1.2 M glyoxal (Sigma) and then transferred to Hybond-N+ nylon membranes (Amersham Biosciences, USA). RNA was fixed to the membrane by UV light. The RNA on membranes was hybridized to ^{32}P-labelled DNA probes in 0.5 M Na_2HPO_4, 7% SDS, and 10 mM EDTA pH 7.2 at 65°C for 16 h. Each ^{32}P-labeled specific DNA probe was produced from each corresponding PCR product using the *Prime-a*-Gene labeling system (Promega). Expressed sequence tags (EST) clones, *APX1* (At1g07890), *Zat12* (At5g59820), *GST1* (At1g02930) were obtained from the Arabidopsis Biological Resource Center (ABRC). Membranes were washed twice in 2X and once in 1X saline sodium citrate (SSC) containing 0.1% SDS at 42°C for 15 and 30 min, respectively. Membranes were exposed to Imaging plate (Fujifilm) screens and signals were detected by Phosphoimager software (Storm 840, Amersham Biosciences). RNA blots were carried out three times with independently isolated RNA.

Statistical analysis

The values shown in each figure are mean values ± SE. Approximately, 20 seedlings were processed per line in each experiment. The data were subjected to analysis of variance (one-way ANOVA) and post hoc comparisons were done with Tukey's multiple range test at $P < 0.05$ level. The statistical software program used was SigmaStat 3.1.

Results

Differential response of *tir1 afb1*, *tir1 afb2* and *tir1 afb3* to MV and H_2O_2

To study the role of auxin signaling in *Arabidopsis* plants growing under stress conditions, *tir1/afb* mutants were analyzed. Single *tir1* and *afb2* mutants subjected to oxidative and salt treatments did not show phenotypic differences compared with wild-type plants (Online Resource 1). Thereafter, the phenotypic characterization under abiotic stress was performed by using double loss-of-function

mutants previously described (Savaldi-Goldstein et al. 2008; Parry et al. 2009).

The elongation of primary root represents a very rapid, sensitive and quantitative parameter to evaluate stress-induced growth inhibition (Fujibe et al. 2004). Auxin receptor mutants, *tir1 afb1*, *tir1 afb2* and *tir1 afb3* were exposed to oxidative conditions mediated by MV, an inductor of oxidative injury through O_2^- production in plants. Firstly, the susceptibility of wild-type seedlings to MV was determined in a dose–response assay. The concentration of 10 nM MV was the minimum required to generate 50% of root growth inhibition (data not shown). At this concentration, *tir1 afb2* and *tir1 afb3* seedlings exhibited higher percentage of primary root elongation than wild-type (Fig. 1a, b). However, no differences were observed between *tir1 afb1* and wild-type seedlings. In the absence of MV, all lines showed similar values of root elongation (Fig. 1a). Subsequently, a different oxidative agent, such as H_2O_2 was assayed. Seven-d-old plants grown on ATS agar medium were transferred to 20 mM H_2O_2. Root cell death was estimated by staining with the vital dye Evan's blue. In this case, *tir1 afb2* and *tir1 afb3* evidenced a reduced extent of cell death damage, resulting approximately 30 and 20%, respectively less than wild-type (Fig. 1c). Untreated seedlings did not evidence cell death damage.

tir1 afb2 is tolerant to salt stress

In order to characterize the response of double mutants to salinity, root growth was measured in seedlings subjected to NaCl treatment. A slight but sustained increase of primary root elongation was observed in *tir1 afb2* seedlings compared with wild-type under 50 mM NaCl (Fig. 2a). However, *tir1 afb1* and *tir1 afb3* mutants did not show any differences. To further analyse the effect of NaCl on a different physiological process, the germination rate was measured. Germination rates were statistically different between *tir1 afb2* and wild-type under different doses of NaCl (Fig. 2b). At 150 mM, *tir1 afb2* evidenced an increase of 15% of germination rate compared with wild-type. A similar tendency was also observed at 200 mM NaCl. To test whether NaCl-effect is associated to osmotic stress rather than sodium toxicity, KCl was used as a non-toxic osmolyte. Again, *tir1 afb2* exhibited a higher germination rate compared with wild-type seeds but at elevated concentration of KCl (200 mM) suggesting that salt tolerance is probably not due to osmotic stress tolerance (Fig. 2b).

Chlorophyll content is one of the principal parameters affected in leaves when plants are grown under salinity. Therefore, chlorophyll was measured in *tir1 afb2* and wild-type leaves after 250 mM NaCl-treatment. The chlorophyll content was higher in *tir1 afb2* compared with wild-type (Fig. 2c).

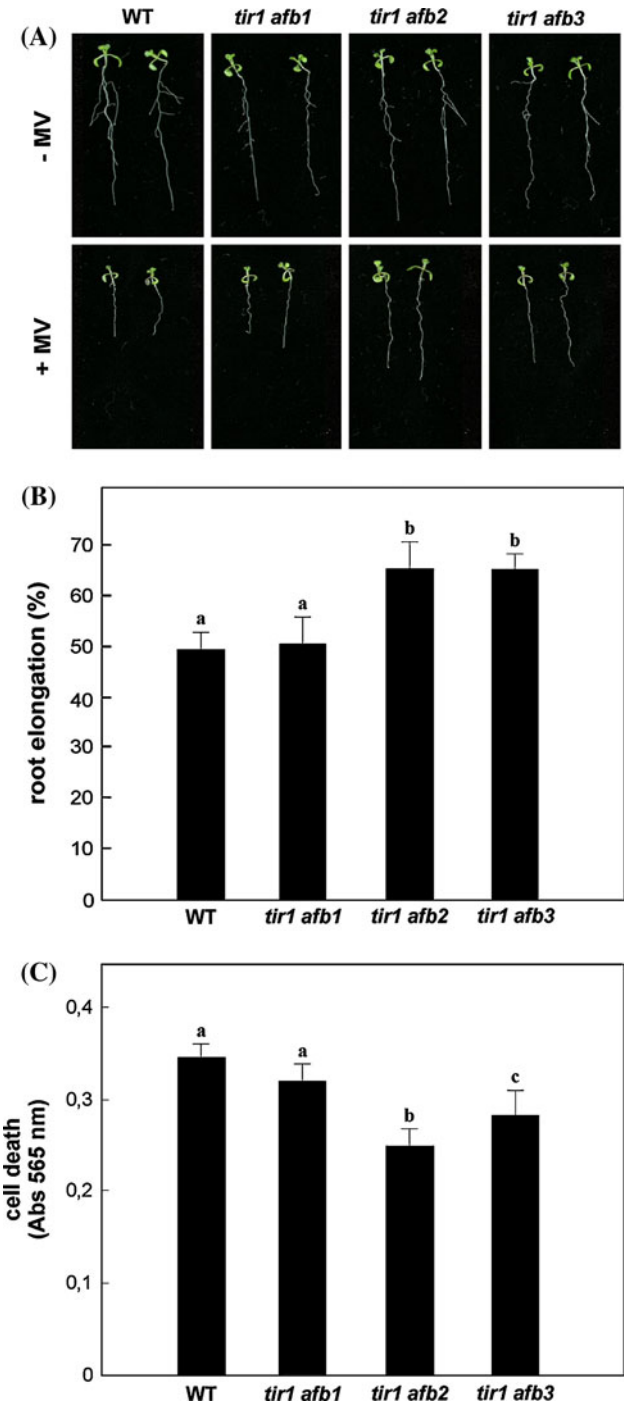
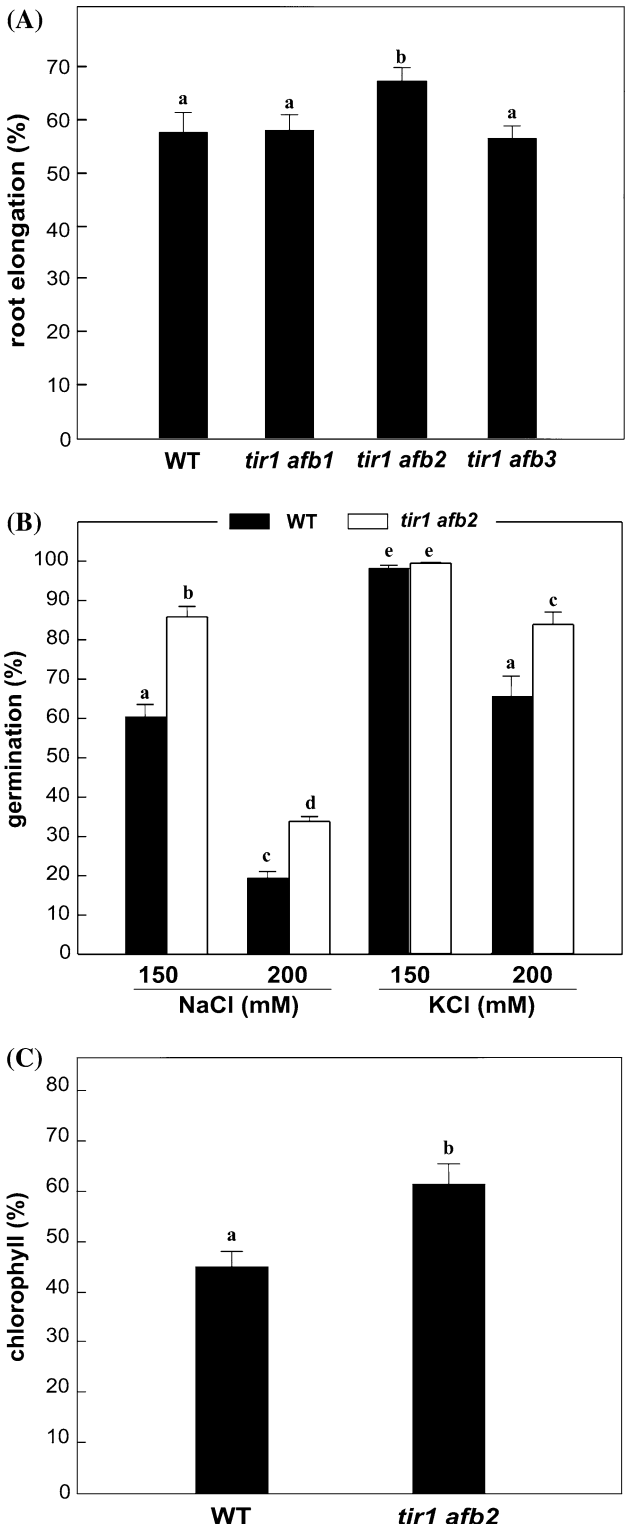


Fig. 1 Differential susceptibility of *tir1 afb1*, *tir1 afb2*, *tir1 afb3* and wild-type (WT) to oxidative stress. **a** Seedlings were grown on ATS agar medium in the absence (*upper panel*) or presence (*lower panel*) of 10 nM MV for 7 d. **b** Primary root elongation was expressed as percentage of untreated seedlings for each genotype. **c** Quantification of cell death in primary roots after H_2O_2 treatment. Seedlings were treated with 20 mM H_2O_2 for 1.5 h and stained with Evan's blue solution. The colorant retained in death cells was measured spectrophotometrically at 565 nm. Data are mean values (±SE) of five independent experiments. *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test)



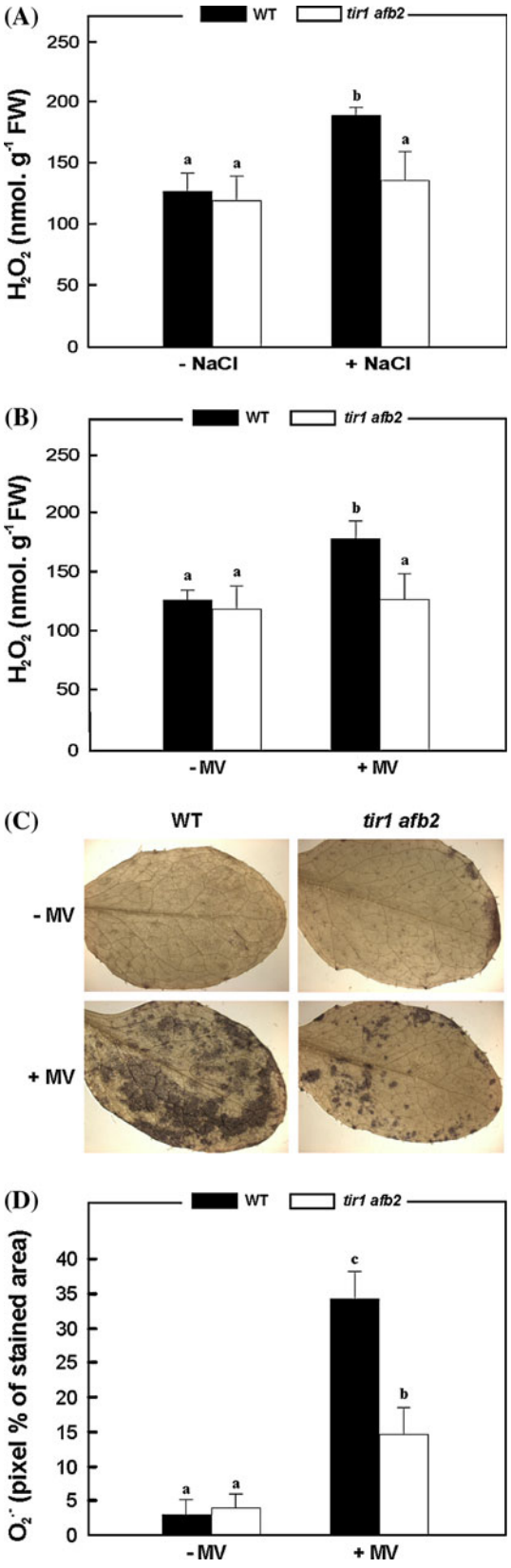
◀ **Fig. 2** Susceptibility of *tir1 afb1*, *tir1 afb2*, *tir1 afb3* and wild-type (WT) to salinity. **a** *tir1 afb1*, *tir1 afb2*, *tir1 afb3* and WT seedlings grown on ATS agar medium supplemented with 50 mM NaCl for 7 d. Primary root elongation was expressed as percentage of untreated seedlings. **b** Seeds from *tir1 afb2* and WT were grown under the concentrations of NaCl or KCl indicated. Germination was scored after 48 h and expressed as percentage of untreated seedlings. **c** Ten-d-old seedlings were transferred to liquid ATS medium supplemented with 250 mM NaCl for 3 d. Chlorophyll content was measured spectrophotometrically and expressed as percentage of untreated seedlings. Data are mean values (±SE) of at least five independent experiments. *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test)

tir1 afb2 displays a reduced accumulation of ROS upon NaCl and MV treatments

The balance of ROS and antioxidant compounds appears to be key elements in the adaptative response to abiotic stress (Mittler 2002). To explore the underlying mechanism that may act in *tir1 afb2* seedlings during their growth under adverse conditions, H_2O_2 level was monitored in 10-d-old seedlings upon exposition to stress. At 250 mM NaCl *tir1 afb2* seedlings showed a slight but significant decrease in H_2O_2 accumulation compared with wild-type (Fig. 3a). Similar results were obtained when *tir1 afb2* seedlings were subjected to oxidative stress mediated by 1 μM MV (Fig. 3b). In order to evaluate O_2^- accumulation in MV-treated leaves, in situ O_2^- detection by NBT assay was performed. The intense dark formazan precipitate evidences the reduction of NBT by O_2^-. In this case, NBT staining was lower in MV-treated *tir1 afb2* than in wild-type leaves (Fig. 3c, d).

Antioxidant metabolism is enhanced in *tir1 afb2*

In order to alleviate deleterious effects of ROS, plants employ defense systems that include non-enzymatic antioxidant compounds such as AA and glutathione and ROS-scavenging enzymes. We hypothesized that the reduced levels of ROS in *tir1 afb2* mutant plants under stress could be explained by an induction of the antioxidant metabolism. Then AA and its oxidized form (DHA) levels were measured in 250 mM NaCl-treated soil grown plants. *tir1 afb2*-treated leaves revealed two-fold higher AA level than wild-type. However, in the absence of NaCl such difference was not detected (Fig. 4). To get further insights on *tir1 afb2* antioxidant metabolism under salinity CAT and APX activities were measured. Ten-d-old plants grown on ATS agar medium were transferred to liquid ATS medium containing 250 mM NaCl for 24 h. Upon NaCl-treatment, an increase of 30 and 15% of CAT and APX activities, respectively, was detected in *tir1 afb2* compared with wild-type (Fig. 5a, b). In the absence of NaCl, APX activity was also increased in *tir1 afb2* seedlings. Finally, the expression of antioxidant-related genes, such as glutathione S-transferase 1 (G*ST1*), cytosolic ascorbate peroxidase 1 (*APX1*) and a zinc finger transcription factor *Zat12* was examined (Fig. 5c). *GST1* and *Zat12* were transiently up-regulated in *tir1 afb2*. At 12 h after NaCl treatment, GST1 and Zat12 transcript levels increased 2.5 and 2.0 fold,



◀ **Fig. 3** ROS accumulation in *tir1 afb2* under oxidative stress and salinity. Ten-d-old *tir1 afb2* and WT seedlings were grown on ATS agar medium and transferred to liquid ATS medium supplemented with 250 mM NaCl for 24 h (**a**) or 1 μM MV for 8 h (**b**). After treatments, H_2O_2 level was measured. **c** Leaves from 14-d-old *tir1 afb2* and WT plants were grown in soil and treated with 50 μM MV for 1.5 h. In situ O_2^- accumulation was detected by NBT staining. Representative photographs are shown. **d** O_2^- accumulation expressed as the percentage of pixels in stained area/total leaf area from images shown in (**c**). Data are mean values (±SE) of five independent experiments. *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test)

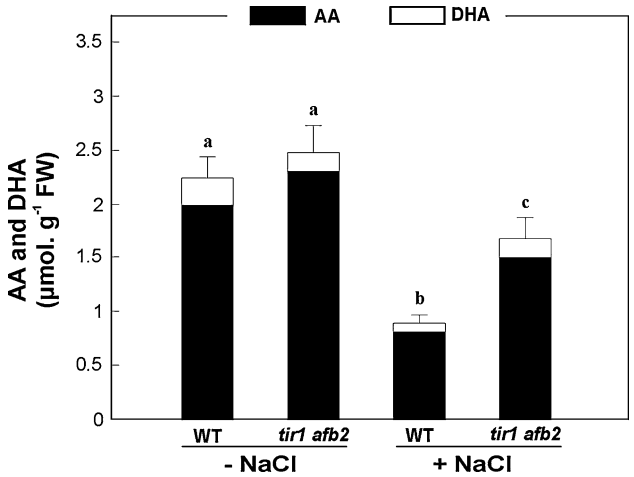
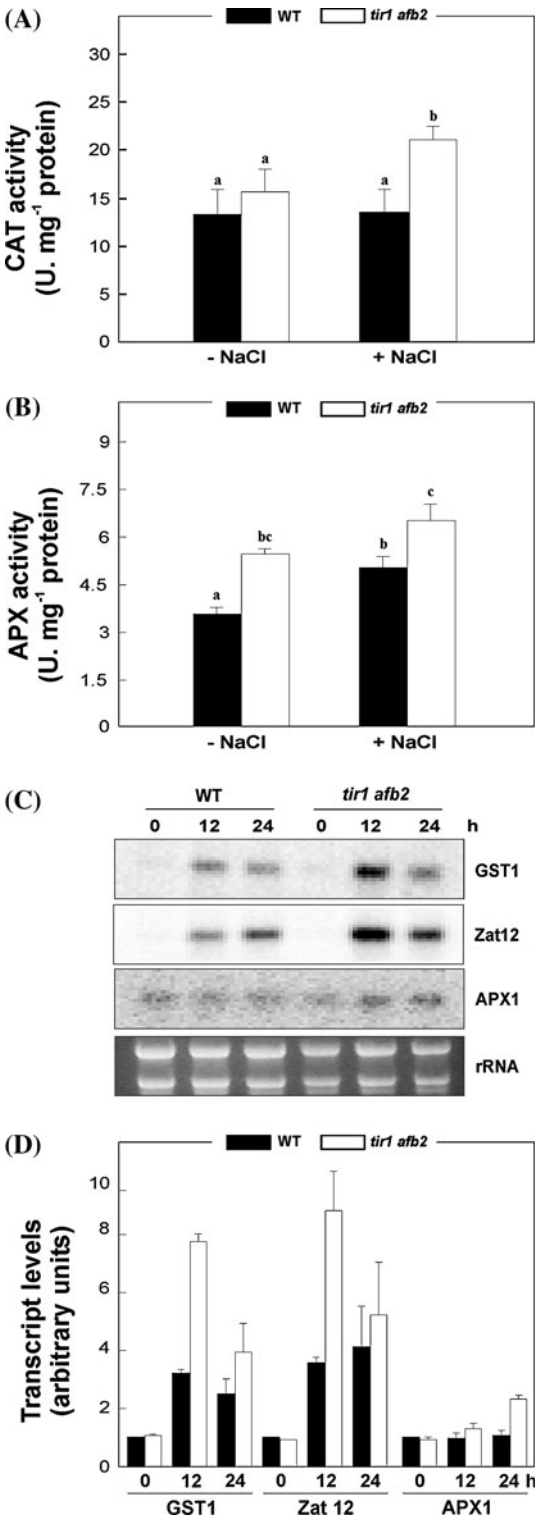


Fig. 4 Ascorbate level in *tir1 afb2* under stress. *Tir1 afb2* and wild-type (WT) plants grown in soil were watered with 250 mM NaCl. Seven d after initial treatment, plants were harvested and AA and DHA levels were analyzed by HPLC**.** Data are mean values (±SE) of three independent experiments. *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test)

respectively compared with wild-type. *APX1* gene was also up-regulated upon NaCl treatment.

Discussion

In this work, we report that auxin signaling participates in the adaptative response against oxidative stress and salinity by interacting with redox metabolism in *Arabidopsis*. By a genetic approach, TIR1- and AFBs- deficient seedlings give the opportunity to investigate how auxin signaling contributes to plant's ability to grow under changing environments. The phenotypic analysis of the single mutants, *tir1* and *afb2* did not show differences respect to wild-type plants under oxidative and salt stresses, presumably due to the functional redundancy between members of TIR1/AFB family (Dharmasiri et al. 2005b). Therefore, a set of double mutants, *tir1 afb1*, *tir1 afb2* and *tir1 afb3* was here characterized. Double mutants, *tir1 afb2*



◀ **Fig. 5** Antioxidant activities in *tir1 afb2* under stress. Ten-d-old *tir1 afb2* and wild-type (WT) seedlings were grown on ATS agar medium and transferred to liquid ATS medium supplemented with 250 mM NaCl for 24 h. **a** and **b** CAT and APX activities, respectively. Data are mean values (±SE) of five independent experiments. *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test). **c** Expression analysis of *GST1* (Glutathione S- Transferase 1, At1g02930), *Zat12* (Zinc-Finger Protein 12, At5g59820) and *APX1* (Ascorbate Peroxidase 1, At1g07890) after 12 and 24 h of treatment. Ethidium bromide staining was used to ensure equal loading in the gel. **d** Densitometric analysis from RNA gel blot as indicated in (**c**). rRNA, ribosomal RNA

and *tir1 afb3* showed higher tolerance to oxidative stress compared with wild-type plants judge by reduced inhibition of root growth and cell death upon MV and H_2O_2 treatment, respectively. Most importantly, *tir1 afb2* also showed tolerance to salinity, an abiotic stress produced by a natural factor. According to the previously assigned roles during auxin-mediated root growth (Parry et al. 2009), TIR1 and AFB2 receptors are more implicated in oxidative and salinity stress responses than other members of such auxin receptor protein family. Data from microarray analysis indicate that auxin responsive genes are repressed by abiotic stresses such as, wounding, oxidative, and selenium treatments in *Arabidopsis* (Cheong et al. 2002; Scarpeci et al. 2008; Van Hoewyk et al. 2008). In addition, Sunkar and Zhu (2004) reported that miR393 which has emerged as a key regulator of *TIR1, AFB2* and *AFB3* is induced by cold, dehydration, NaCl and ABA treatments in *Arabidopsis*. All these findings suggest that the suppression of auxin signaling may be a plant strategy to enhance tolerance to abiotic stress, as has been previously reported for biotic stress (Navarro et al. 2006). In support of our hypothesis, *tir1 afb2* showed significantly reduced ROS accumulation, higher antioxidant enzymatic activities as well as enhance levels of AA. Previously, Pignocchi et al. (2003) provided evidence that an ascorbate oxidase (AO) enzyme, which catalyses the oxidation of AA to DHA is induced in auxin-treated tobaco plants. Thus, we speculate that the higher AA level in *tir1 afb2* may be due to a reduced AO activity. Simultaneously with our results, Bashandy et al. (2010) reported that the disruption of redox pathways interferes with auxin-mediated processes in *Arabidopsis*. Indeed, the cross-talk between auxin and redox-status is emerging as a new regulatory node by which plants control growth and developmental processes including acclimation responses to environmental stresses. Nevertheless, its precise mechanism remains to be elucidated. Thereby, the possibility of an auxin cross-talk with other hormones should be also considered. In *Arabidopsis* roots, auxin enhances giberellic acid (GA)-induced degradation of DELLA proteins (Fu and Harberd 2003). DELLA proteins confer tolerance to salt stress by reducing ROS accumulation through the up-regulation of genes encoding for ROS detoxification (Achard et al. 2008). Consequently, down-regulation of auxin signaling in *Arabidopsis* plants may contribute to abiotic stress tolerance by reducing ROS through the accumulation of DELLA proteins. Likewise, other hormones may also regulate redox homeostasis integrating developmental and environmental signals.

Understanding hormonal cross-talk is thus essential to elucidate how plants control redox metabolism under stress. Future research on these versatile signaling switches will reveal innovative ways that plants adopt to get survival to highly dynamic environments.

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