Complex Signaling Network in Regulation of Adenosine 5′-Phosphosulfate Reductase by Salt Stress in Arabidopsis Roots[1][W]

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Sulfur-containing compounds play an important role in plant stress defense; however, only a little is known about the molecular mechanisms of regulation of sulfate assimilation by stress. Using known Arabidopsis (*Arabidopsis thaliana*) mutants in signaling pathways, we analyzed regulation of the key enzyme of sulfate assimilation, adenosine 5′-phosphosulfate reductase (APR), by salt stress. APR activity and mRNA levels of all three APR isoforms increased 3-fold in roots after 5 h of treatment with 150 mM NaCl. The regulation of APR was not affected in mutants deficient in abscisic acid (ABA) synthesis and treatment of the plants with ABA did not affect the mRNA levels of APR isoforms, showing that APR is regulated by salt stress in an ABA-independent manner. In mutants deficient in jasmonate, salicylate, or ethylene signaling, APR mRNA levels were increased upon salt exposure similar to wild-type plants. Surprisingly, however, APR enzyme activity was not affected by salt in these plants. The same result was obtained in mutants affected in cytokinin and auxin signaling. Signaling via gibberellic acid, on the other hand, turned out to be essential for the increase in APR mRNA by salt treatment. These results demonstrate an extensive posttranscriptional regulation of plant APR and reveal that the sulfate assimilation pathway is controlled by a complex network of multiple signals on different regulatory levels.

During their lifetime, plants are exposed to a variety of biotic and abiotic stresses. The common feature of the stresses is the generation of reactive oxygen species (ROS), which are potentially damaging to cell structures and components. Detoxification of ROS is essential to limit oxidative stress and a key mechanism for this is the ascorbate-glutathione cycle, which plays a pivotal role in defense (Noctor and Foyer, 1998). Therefore, glutathione synthesis is often induced under stress conditions, including salt stress, and the capacity for its synthesis is correlated with tolerance to various stresses (Noctor et al., 1998; Kocsy et al., 2001, 2004; Ruiz and Blumwald, 2002; Mittova et al., 2003).

Glutathione (GSH) is a tripeptide composed of Glu, Cys, and Gly. Its synthesis is primarily dependent on availability of the constituent amino acids (Strohm et al., 1995). Because Cys is the final product of assimilatory sulfate reduction, there is a tight link between the demand for GSH and the rate of sulfate reduction (Lappartient and Touraine, 1997; Vauclare et al., 2002; Kopriva and Rennenberg, 2004). Sulfate assimilation provides reduced sulfur for synthesis of the amino acids Cys and Met, many coenzymes and prosthetic groups (such as iron-sulfur centers, thiamine, lipoic acid, etc.), and a variety of secondary compounds. Sulfate taken up into plant cells has to be activated first by adenylation to adenosine 5′-phosphosulfate (APS) catalyzed by ATP sulfurylase. APS is reduced to sulfite by APS reductase (APR), the electrons are provided by GSH, and sulfite is subsequently reduced to sulfide by ferredoxin-dependent sulfite reductase. Sulfide is incorporated into the amino acid skeleton of *O*-acetyl-Ser, synthesized from Ser and acetyl-CoA by Ser acetyltransferase, by *O*-acetyl-Ser (thiol) lyase (OASTL) to form Cys, which is the primary donor of reduced sulfur for all subsequent biosynthetic reactions (for review, see Leustek et al., 2000; Kopriva, 2006).

Sulfate assimilation is highly regulated in a demand-driven manner (Lappartient and Touraine, 1996; Leustek et al., 2000; Kopriva and Rennenberg, 2004; Kopriva, 2006). The pathway is induced when GSH concentration is reduced by high demand (e.g. in detoxification of heavy metals or protection against cold and osmotic stress; Brunner et al., 1995; Lee and Leustek, 1999; Kocsy et al., 2004), by blocking its synthesis with enzyme inhibitors (Hartmann et al., 2004) or during sulfate starvation (e.g. Nikiforova et al., 2003). On the other hand, a surplus of reduced sulfur compounds by fumigation with H_2S or by feeding thiols represses the pathway (Westerman et al., 2001; Vauclare et al., 2002). The key regulatory steps of sulfate assimilation are the transport of sulfate into the cells and the reduction of APS to sulfite by APR (Vauclare et al., 2002). Until now, however, we had a very limited knowledge

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of the molecular regulation of sulfate assimilation. The recently discovered sulfur-responsive SURE element and SLIM1 transcriptional regulator are the first cis- and trans-factors associated with regulation of the pathway in the promoter of the high-affinity sulfate transporters SULTR1;1 and SULTR1;2, respectively (Maruyama-Nakashita et al., 2005, 2006). Both sulfate transporters and APR seem to be regulated at the transcriptional level because, on most occasions, changes in activity correlate with changes in mRNA levels. In Arabidopsis (*Arabidopsis thaliana*), APR activity and the mRNA levels for its three isoforms undergo a diurnal rhythm with the highest level at the beginning of the light period (Kopriva et al., 1999). They are reduced during nitrogen deficiency (Koprivova et al., 2000) and by treatment with thiols (Vauclare et al., 2002), and increase upon treatment with *O*-acetyl-Ser and carbohydrates (Hesse et al., 2003). In addition, a posttranslational regulation of APR was shown to be responsible for rapid modulation of activity during oxidative stress (Bick et al., 2001).

We are interested in the molecular mechanism of regulation of sulfate assimilation. Here, we describe a genetic approach to identify components in the regulation of APR by salt stress. To address the regulation in its whole complexity, we determined not only mRNA levels, but also APR protein accumulation and enzyme activity. We found that APR is regulated at different levels by a complex network of multiple signals. We provide evidence for a novel translational regulation of APR and for involvement of GAs in transcriptional regulation of the corresponding genes.

RESULTS

Regulation of APR by Salt Stress

We searched the GENEVESTIGATOR Arabidopsis microarray database to identify abiotic stress conditions that strongly induce APR mRNA levels (Zimmermann et al., 2004). Exposure to 150 mM NaCl appeared to be the most suitable treatment because the mRNA levels of the APR isoforms increased 2- to 3-fold in the leaves and 6- to 10-fold in the roots within 6 h (Supplemental Fig. S1). However, in our experimental system, treatment of hydroponically grown Arabidopsis with 150 mM NaCl did not affect the transcript levels for the three APR isoforms in the leaves within 5 h, whereas they were increased in the roots 3- to 5-fold (Fig. 1, A and B). In leaves, no effect of the salt treatment on APR activity was measured up to 8 h after salt treatment. On the other hand, after 5 h, there was a significant, approximately 3-fold higher APR activity in salt-treated roots compared to controls (Fig. 1, C and D). The activity was still elevated at 8 h, but only by 80%. It has to be noted here that the decrease in APR activity in control plants was observed consistently in all time course experiments performed and can be explained by the well-characterized diurnal rhythm of APR (Kopriva et al., 1999). The increased APR activity in the Columbia-0 (Col-0) ecotype correlated with increased concentration of total GSH after 5 h of salt treatment (Table I). The transcript of the salt-regulated gene *RD29A*, used as a positive control, was also highly induced in these experimental conditions (Fig. 3E). Because the APR activity was not regulated

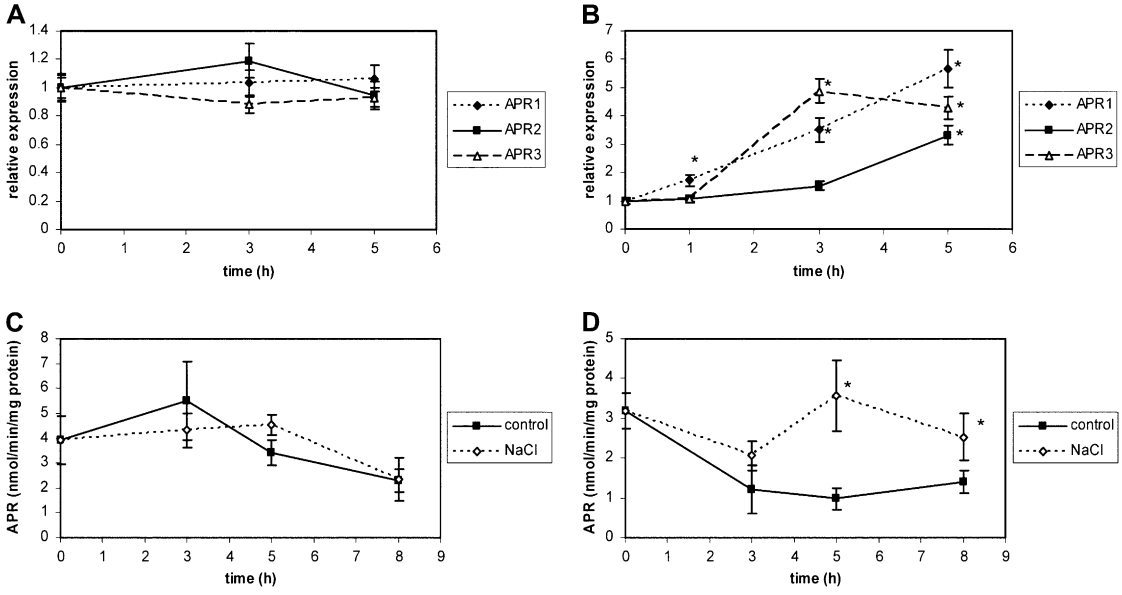

Figure 1. Regulation of APR by salt. Arabidopsis plants (Col-0) grown in hydroculture were treated with 150 mM NaCl. Relative mRNA levels of the three APR isoforms were determined by semiquantitative RT-PCR in shoots (A) and roots (B). Data are presented as ratios of mean transcript levels of four independent RNA preparations from treated versus untreated plants. APR activity was measured in shoots (C) and roots (D). Means ± SDs of three independent plants are displayed. Values indicated by asterisks are different from controls at $P \leq 0.05$. All experiments were repeated at least twice with similar results.

Table I. *GSH content in Arabidopsis roots treated with salt*

GSH was determined in roots of control and salt-treated plants by HPLC. Mean values ± SEM from three to four independent plants are presented. Asterisks mark values significantly ($P \leq 0.05$) higher than in control samples.

Genotype	Total GSH	
	Control	Salt
	nmol g FW^{-1}	
Col-0	371 ± 44	544 ± 12*
L*er*	294 ± 167	686 ± 79*
npr1-2	430 ± 57	614 ± 32*
ein2-1	209 ± 30	203 ± 36
jar1-1	207 ± 13	220 ± 35
tir1-1	429 ± 25	650 ± 54*
gai	310 ± 33	895 ± 102*

in the shoots, we focused our attention on the roots only. Because the activity in roots showed a maximal increase 5 h after the beginning of the salt treatment and declined thereafter, most subsequent experiments were performed at this time point only.

Because salt treatment induces accumulation of ROS, we tested how APR responds to treatment of the roots with hydrogen peroxide (H_2O_2). Indeed, diaminobenzidine (DAB) staining revealed formation of H_2O_2 in the salt-treated roots (Supplemental Fig. S2). Exposure to H_2O_2 rapidly induced mRNA accumulation of *APR1* and *APR3*, but not of *APR2* (Fig. 2A). Although an increase in mRNA accumulation occurred after 1 h, the enzyme activity was strongly reduced after 1 h and slowly recovered so that after 5 h it was similar to that in control roots (Fig. 2B). This experiment suggests that the effect of salt on APR may not be primarily caused by ROS.

Regulation of APR by Salt Is ABA Independent

Response to salt stress is commonly regulated via abscisic acid (ABA) signaling (Zhu, 2002). To test whether ABA is involved in regulation of APR by salt, we compared the induction of APR in Arabidopsis mutants deficient in ABA and ABA signaling (see Table II for a description of the mutants) with their corresponding wild types. After 5 h of salt treatment, the mRNA levels for all three APR isoforms and APR activity were increased in all lines compared to untreated controls (Fig. 3, A–D and F). The increase in APR transcripts is evident in the images of gels resolving reverse transcription (RT)-PCR fragments (Fig. 3A). The mRNA level of control salt and ABA-regulated *RD29A* gene was also elevated in all genotypes (Fig. 3E). After quantification, the individual levels of induction differed from 2.5- to 8-fold and were statistically significant at a high confidence level ($P \leq 0.01$) for all genotypes. On the other hand, the mRNA level of cytosolic OASTL was not affected by the salt treatment (Fig. 3A). Thus, it seems that APR is regulated by salt in an ABA-independent manner.

These findings were corroborated by treating Arabidopsis plants with 50 μM ABA. mRNA accumulation of none of the APR isoforms was affected by ABA in the roots after 3 and 5 h, in contrast to treatment with salt (Fig. 4, A–C). In contrast, the *RD29A* gene was clearly induced by both treatments at both time points (Fig. 4D), whereas the transcript of an ABA-insensitive *AtDi19-2* gene (Rodriguez Milla et al., 2006) was induced by salt only (Fig. 4E). Surprisingly, however, ABA treatment resulted in a significant decrease in APR activity at both time points, whereas control salt treatment led to induction of the activity (Fig. 4F). Because the mRNA levels were not affected, it seems that ABA represses APR activity at the posttranscriptional level. These experiments, therefore, indicate that APR is regulated by salt in an ABA-independent manner.

Involvement of Salicylate, Ethylene, Jasmonate, and Nitric Oxide in Regulation of APR by Salt Stress

In the search for components of signaling pathways regulating APR, we tested the known stress-signaling molecules salicylate, ethylene, and jasmonate for their ability to affect APR activity and transcript levels in the roots. Addition of 0.1 mM salicylate to the nutrient solution led to an increase of mRNA levels of all three APR isoforms. On the other hand, 0.2 mM 1-aminocyclopropane carboxylic acid (ACC), which stimulates ethylene production, and 45 μM jasmonate increased accumulation of *APR1* and *APR3* transcripts, but did

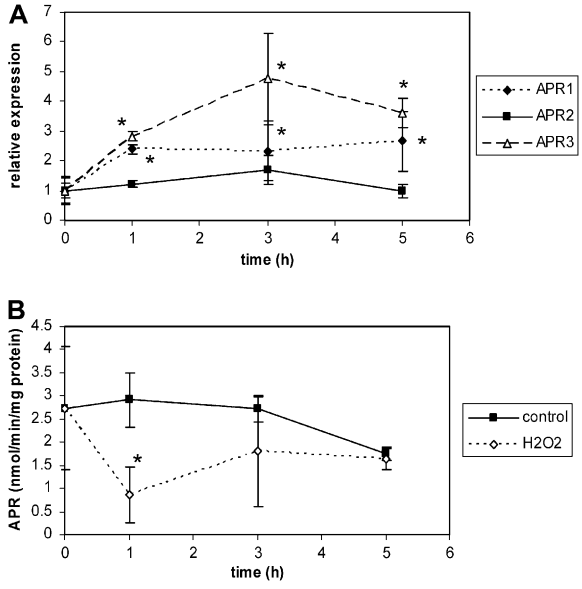

Figure 2. Regulation of APR by H_2O_2. Arabidopsis plants (Col-0) grown in hydroculture were treated with 10 mM H_2O_2. A, Relative mRNA levels of the three APR isoforms in roots were determined by semi-quantitative RT-PCR. Data are presented as ratios of mean transcript levels of four independent RNA preparations from treated versus untreated plants. B, APR activity was measured in crude root extracts. Data are presented as means ± SDs of three independent plants. Values indicated by asterisks are different from control at $P \leq 0.05$.

Table II. *Mutant lines analyzed for regulation of APR by salt stress*

Mutant Allele	Functional Category	Gene	Parental Ecotype	Ref.
aba1	ABA deficient	Zeaxanthin epoxidase	L*er*	Koornneef et al. (1982)
aba2	ABA deficient	Short-chain dehydrogenase/reductase	Col-0	Leon-Kloosterziel et al. (1996)
abi1	ABA insensitive	Protein phosphatase 2C	L*er*	Koornneef et al. (1984)
abi2	ABA insensitive	Protein phosphatase 2C	L*er*	Koornneef et al. (1984)
npr1-2	Blocked salicylate signaling	Transcription activator	Col-0	Cao et al. (1994)
NahG	Salicylate deficient	Overexpressing bacterial salicylate hydroxylase	Col-0	Lawton et al. (1995)
etr1-3	Ethylene insensitive	Ethylene receptor	Col-0	Bleecker et al. (1988)
ein2-1	Ethylene insensitive	NRAMP metal transporter family	Col-0	Guzmán and Ecker (1990)
jar1-1	Jasmonate resistant	Jasmonate:amino acid synthetase	Col-0	Staswick et al. (1992)
CKX	Cytokinin deficient	Overexpressing cytokinin oxidase	Col-0	Werner et al. (2003)
ahk4	Cytokinin insensitive	Cytokinin receptor AHK4	Col-0	Inoue et al. (2001)
axr1-3	Auxin resistant	Ubiquitin-like activating enzyme	Col-0	Estelle and Somerville (1987)
tir1-1	Inhibition of auxin transport	Auxin receptor	Col-0	Ruegger et al. (1998)
gai	GA insensitive	DELLA protein	L*er*	Koornneef et al. (1985)

not affect the *APR2* isoform (Fig. 5, A–C). All three compounds significantly induced APR activity (Fig. 5D). It seems that the increase in mRNA for *APR1* and *APR3* isoforms is sufficient to increase APR activity upon feeding with these phytohormones.

To test whether these hormones are involved in regulation of APR by salt stress, we analyzed plants deficient in the corresponding signaling pathways (Table II). In all these genotypes, mRNA levels of the three APR isoforms and the control *RD29A* gene were induced by the salt treatment (Fig. 6, A–D). Although some variations in the degree of induction or in steady-state transcript levels in control plants were detected in some genotypes, the general up-regulation of APR transcripts by salt was not affected by modulation of the signaling pathways. Surprisingly, in contrast to wild-type Arabidopsis, APR activity was not affected by salt stress in these plants or was even slightly decreased in the genotypes deficient in salicylate signaling (Fig. 6E). Remarkably, the activity in *npr1-2* and *NahG* was consistently higher than in wild-type Arabidopsis. Thus, the induction of APR activity, but not mRNA, seems to be dependent on correctly functioning stress signaling by salicylate, ethylene, and jasmonate. HPLC analysis revealed no increase in total GSH in *ein2* or *jar1* following salt treatment, whereas it was increased in *npr1* plants to a similar degree as in wild-type plants (Table I).

Another molecule associated recently with stress signaling is nitric oxide (NO; Delledonne, 2005). NO signaling can be prevented by the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO). Similar to experiments blocking salicylate, ethylene, and jasmonate signaling, preventing NO signaling by treatment with PTIO only slightly affected the increase in APR mRNA levels by salt treatment, which remained significantly elevated, but prevented the increase in enzyme activity (Fig. 7). NO may thus be another component of the signaling network in regulation of APR by salt stress.

GA Is Required for APR Regulation by Salt

APR has been shown to be regulated by cytokinins (Ohkama et al., 2002); therefore, we tested whether these or two other phytohormones, auxin and GA, are involved in regulation of this enzyme by salt stress. Five further Arabidopsis mutants impaired in phytohormone signaling were used (Table II). These plants were compared to the corresponding wild types for alterations in APR response to salt treatment. In the plants deficient in cytokinin accumulation and signaling and auxin signaling, the mRNA levels of the three APR isoforms were increased upon salt treatment similar to wild type (Fig. 8, A–C), except *APR2* in the CKX plants, which was not induced. Enzyme activity measurements revealed that APR activity was not increased by salt stress in cytokinin and auxin-insensitive genotypes (Fig. 8E). On the other hand, the *APR1* and *APR2* mRNA levels were not significantly affected by salt treatment in the GA-insensitive *gai* plants (Fig. 8, A–C). *APR3* regulation was similar to the corresponding wild type (Landsberg *erecta* [L*er*]) and other genotypes. The induction of *RD29A* mRNA level has been observed in all genotypes (Fig. 8D). APR activity was, however, increased by salt stress in *gai* plants despite no effect of the treatment on transcript levels of *APR1* and *APR2*. Because GAs have not yet been implicated in APR regulation, we treated wild-type plants with 50 μM GA_3 added to the nutrient solution, which resulted in a significant increase in APR mRNA levels and enzyme activity (Supplemental Fig. S3). Total GSH content in *gai* and *tir1* plants was increased by salt stress in a similar way to corresponding wild types.

APR Is Regulated at Translational and Posttranslational Levels

To gain further insight into the mechanism of regulation of APR, crude root extracts were analyzed by western blotting using antiserum against recombinant

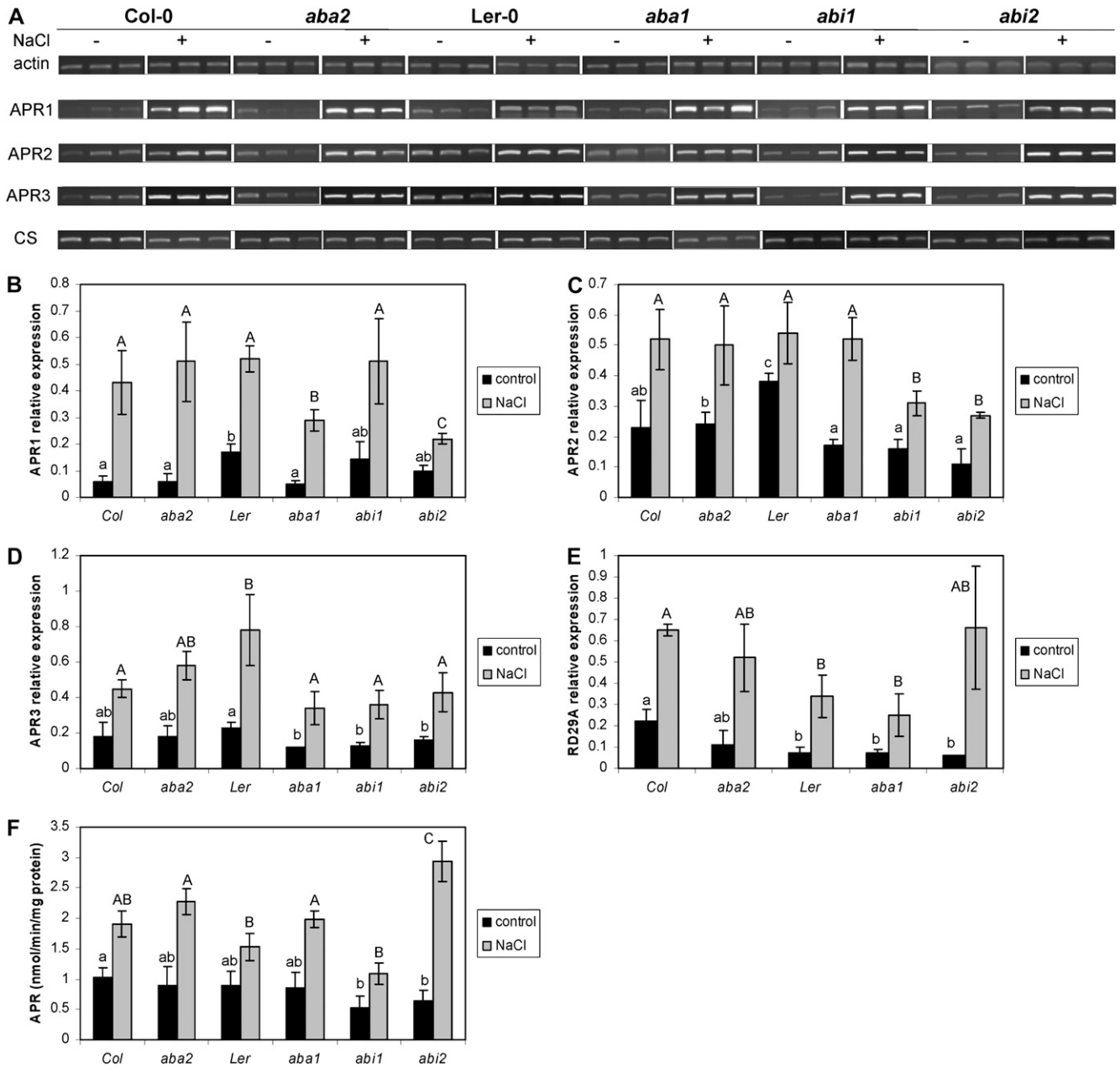

Figure 3. Salt regulation of APR is ABA independent. Arabidopsis mutants deficient in ABA accumulation (*aba1* and *aba2*) or ABA signaling (*abi1* and *abi2*) plus the corresponding wild-type ecotypes Col-0 and L*er* were grown in hydroculture and treated with 150 mM NaCl for 5 h. A, Ethidium bromide-stained gels of RT-PCR fragments of actin, *APR1*, *APR2*, *APR3*, and cytosolic OASTL (CS) from a representative experiment are shown. The relative mRNA levels for *APR1* (B), *APR2* (C), *APR3* (D), and *RD29A* (E) in roots were calculated from the semiquantitative RT-PCR data with the Quantity One software package and standardized with actin 2/7 transcript. F, APR activity was measured in crude root extracts. Data are presented as means ± SDS of three independent plants. All treatments were repeated at least twice with similar results. Different indices indicate values significantly different at $P \leq 0.05$. All values of salt-treated plants are significantly ($P \leq 0.05$) different from nontreated plants.

APR2, which cross-reacts with all three APR isoforms (Kopriva et al., 1999; Koprivova et al., 2000). Several genotypes were used, representing different effects of salt treatment on APR transcript levels and activity. In both wild-type ecotypes, in mutants deficient in or insensitive to ABA, and in the GA-insensitive *gai* plants, APR was more abundant in salt-treated roots (Fig. 9A). Analysis of mutants deficient in salicylate, ethylene, jasmonate, auxin, or cytokinin signaling revealed that, in contrast to wild-type plants, salt treatment of these plants did not induce APR protein levels. The protein accumulation thus correlated well

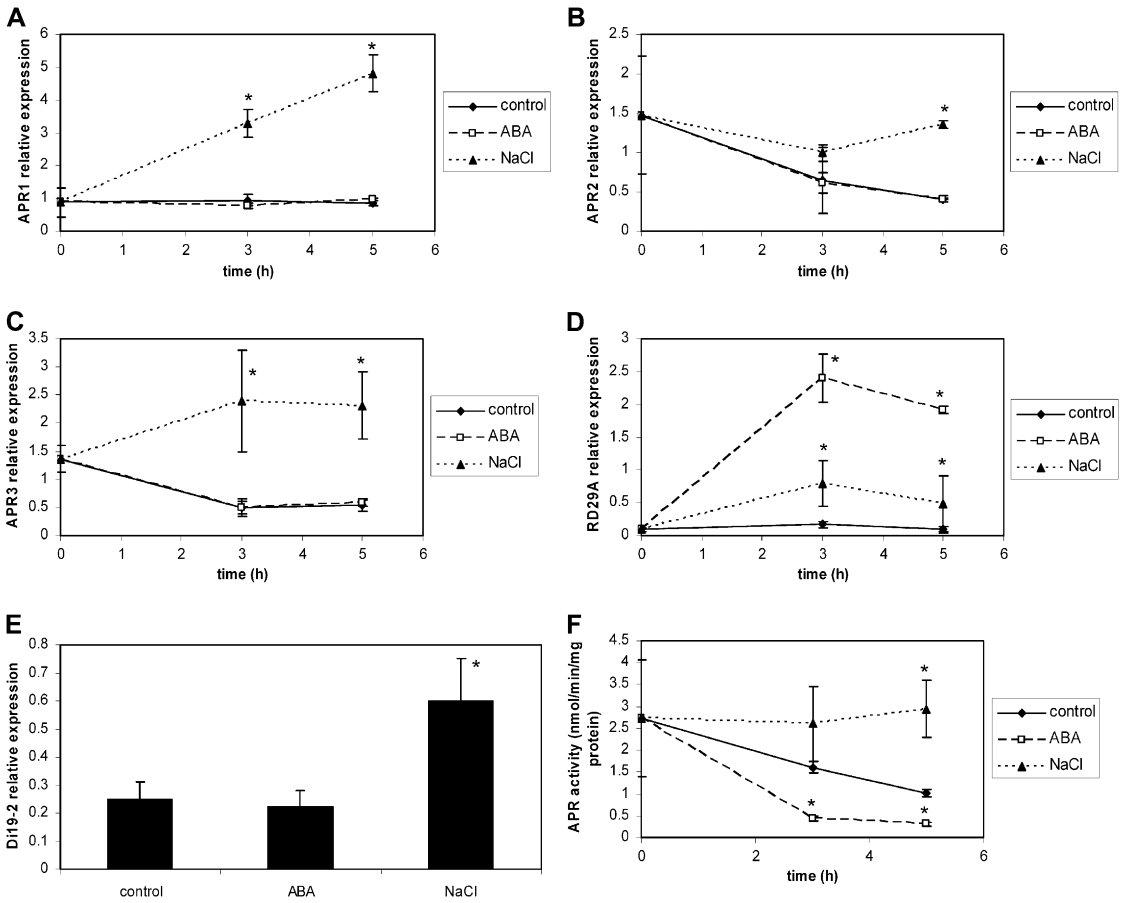

Figure 4. Regulation of APR by ABA. Wild-type Arabidopsis (Col-0) were grown in hydroculture and treated by addition of ABA or NaCl to the nutrient solution to a final concentration of 50 μM or 150 mM, respectively, for 3 or 5 h. Relative mRNA levels for *APR1* (A), *APR2* (B), *APR3* (C), *RD29A* (D), and *AtDi19-2* (E) were determined in roots by semiquantitative RT-PCR and standardized with actin 2/7 transcript. F, APR activity was determined in crude root extracts. Data are presented as means ± SDs of three independent plants. All treatments were repeated at least twice with similar results. Values indicated by asterisks are different from untreated control plants at $P \leq 0.05$.

with the APR activity in these genotypes (Fig. 9A; compare with Figs. 3, 4, 6, and 8). The same strict correlation between APR protein level and activity was observed after feeding various phytohormones (Fig. 9B). Thus, ABA reduced APR protein accumulation, whereas salicylate, ACC, and jasmonate induced APR protein levels to a similar degree as enzyme activity. These results reveal a novel mechanism of APR regulation at the posttranscriptional level. This regulation seems to require the correct functioning of multiple signaling pathways, including those involving salicylate, ethylene, jasmonate, auxin, and cytokinins.

Different results, however, were obtained from analysis of extracts from roots treated with H_2O_2. The signal corresponding to APR at 52 kD was strongly reduced in these extracts, which again correlated with the decrease in enzyme activity. An additional signal also appeared on the blots corresponding to a high molecular mass protein or a protein adduct (Fig. 9C). The same results were obtained using reducing and nonreducing conditions (data not shown). Such a high molecular mass signal was not observed in extracts from salt-treated plants of any genotype.

DISCUSSION

Exposure to high salinity is connected with ionic stress due to accumulation of Na^+ ions, osmotic stress, and ROS production (Hasegawa et al., 2000). Salt stress induces the activities of antioxidative enzymes and accumulation of antioxidants, such as GSH (Ruiz and Blumwald, 2002; Mittova et al., 2003). Therefore, it was not surprising that this appeared to be a suitable treatment for dissecting the regulation of APR by stress. Indeed, in our experimental conditions, GSH content and APR activity were increased by salt treatment (Table I; Fig. 1). The increased APR activity and GSH level in salt-treated roots correlate with previous observations of demand-driven regulation of sulfate assimilation and the key role of APR in control of the pathway (Brunner et al., 1995; Lappartient and Touraine, 1996; Lee and Leustek, 1999; Leustek et al.,

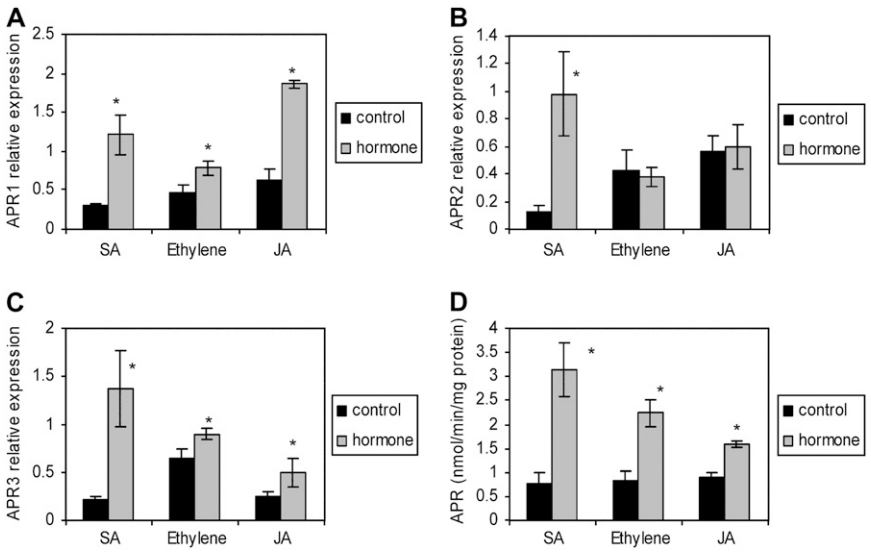

Figure 5. Regulation of APR by phytohormones. Wild-type Arabidopsis (Col-0) were grown in hydroculture and treated with 100 μM salicylate, 200 μM ACC, or 45 μM jasmonate for 5 h. The relative mRNA levels for *APR1* (A), *APR2* (B), and *APR3* (C) in roots were determined by semiquantitative RT-PCR and standardized with actin 2/7 transcript. D, APR activity was measured in crude root extracts. Data are presented as means ± SDs of three independent plants. Values indicated by asterisks are different from untreated plants at $P \leq 0.05$. All treatments were repeated at least twice with similar results.

2000; Westerman et al., 2001; Vauclare et al., 2002; Kocsy et al., 2004; Kopriva, 2006). In fact, other enzymes of sulfate assimilation are also induced by salt, including ATP sulfurylase, Ser acetyltransferase, and a cytosolic isoform of OASTL (Barroso et al., 1999; Ruiz and Blumwald, 2002).

Salt stress signaling is complex and involves numerous pathways with frequent cross-talk. ABA has a pivotal role among these secondary signals; however, both ABA-dependent and ABA-independent signaling pathways have been described (Zhu, 2002). ABA regulates gene expression directly via ABF/AREB transcription factors (Xiong et al., 2002) or indirectly because ABA also induces ROS production (Guan et al., 2000), which in turn activates ROS signaling, affecting cytosolic Ca^{2+}, NO, ethylene, jasmonate, and salicylate. APR regulation by salt is clearly ABA independent, as demonstrated by the same regulation of APR by salt treatment in *aba* and *abi* mutants (Fig. 3). In contrast, the OASTL mRNA was induced after 24 h by salt treatment in an ABA-dependent manner and also by direct ABA treatment (Barroso et al., 1999). In our experiments, the level of cytosolic OASTL mRNA was not increased by salt within the first 5 h of salt exposure (Fig. 3). This suggests that the role of the two genes in the Arabidopsis response to salt stress is different: Whereas OASTL is involved in the acclimation to high salt, APR contributes to the early response. It seems that because of its key role in control of sulfate assimilation, APR has to be increased rapidly to allow a higher rate of Cys synthesis to accommodate the increased demand for GSH.

Because ABA does not seem to be involved in regulation of APR by salt, we addressed the possibility that the regulation is actually triggered by ROS. Indeed, DAB staining revealed that ROS were induced by the salt treatment in our experimental conditions. However, the regulation of APR was different after exposure to salt and to H_2O_2 (Figs. 1 and 2). Because fumigation with ozone resulted in a posttranslational activation of APR (Bick et al., 2001), the reduction in APR activity in H_2O_2-treated roots appears surprising. It is, however, supported by our observation that purified recombinant APR2 from Arabidopsis is inactivated by 0.2% H_2O_2 (S. Kopriva, unpublished data). Presumably, the concentration of H_2O_2 in the root cells after the treatment was higher than after the ozone fumigation and resulted in enzyme inactivation. It also cannot be excluded that the APR regulation differs in roots and leaves or that it is specific to certain types of ROS, such as superoxide. In salt-treated roots, however, the staining revealed even higher ROS concentration and the activity was still increased. The reason for the discrepancy thus might be the subcellular distribution of the ROS between apoplast, cytosol, and plastids. Nevertheless, it seems that ROS were not the primary elicitors of the APR response to salt stress, or perhaps alternative ROS, such as superoxide (O_2^-), participate in this regulation.

In the vast majority of previous experiments, a strict correlation between APR mRNA levels, protein accumulation, and enzyme activity was observed leading to the conclusion that APR is regulated primarily on the transcriptional level (Kopriva et al., 1999; Koprivova et al., 2000; Hesse et al., 2003; Vauclare et al., 2002; Kopriva and Koprivova, 2004). Additional means of affecting APR activity via posttranslational redox regulation has been demonstrated in *Brassica* plants subjected to oxidative stress (Bick et al., 2001) and on recombinant APR2 protein (Kopriva and Koprivova, 2004). In addition, a reduction of APR activity despite an increase in mRNA level was observed after treatment of *Brassica juncea* with cadmium, although this may have been caused by a direct inhibitory effect of cadmium on the enzyme (Lee and Leustek, 1999). Salt treatment of wild-type Arabidopsis resulted in a coordinated increase in APR mRNA, protein, and activity, which suggested a transcriptional regulation in

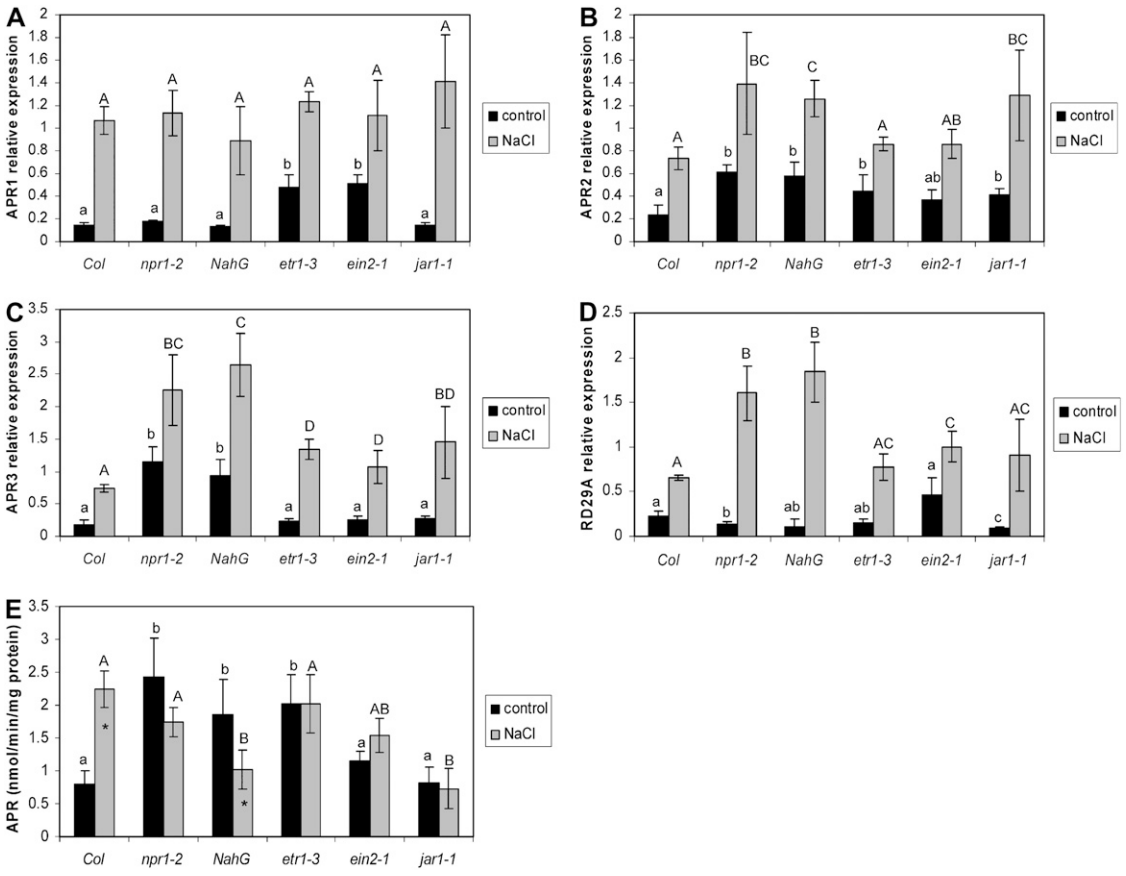

Figure 6. Regulation of APR by salt in mutants deficient in stress signaling. Arabidopsis mutants or transgenic lines deficient in salicylate signaling (*npr1*), salicylate accumulation (*NahG*), ethylene signaling (*etr1* and *ein2*), or jasmonate signaling (*jar1*) plus the corresponding wild type (Col-0) were grown in hydroculture and treated with 150 mM NaCl for 5 h. The relative mRNA levels for *APR1* (A), *APR2* (B), *APR3* (C), and *RD29A* (D) in roots were determined by semiquantitative RT-PCR and standardized with actin 2/7 transcript. E, APR activity was measured in crude root extracts. Data are presented as means ± SDs of three independent plants. All treatments were repeated at least twice with similar results. Different indices indicate values significantly different at $P \leq 0.05$. All values of salt-treated plants in A to D and those marked with asterisks in E are significantly ($P \leq 0.05$) different from nontreated plants.

response to this stimulus as well. From the analysis of mutants disrupted in different signaling pathways, we therefore expected to identify the cascades responsible for the regulation of APR by salt, as described, for example, in Charlton et al. (2005). For most mutants analyzed, the mRNA levels of all three APR isoforms were increased similarly to corresponding wild-type controls. There were small differences in the level of induction in several genotypes; however, the general response was the same. The exception was the *gai* mutant, revealing that the increase of APR mRNA after salt treatment may be dependent on GA signaling. This is quite surprising because GA has not been described previously as a regulator of APR and because disruption of none of the usual stress signaling pathways (jasmonate, salicylate, ethylene, NO) affected the regulation of APR transcripts.

In addition, the analysis revealed that the regulation of the three APR isoforms is not identical. *APR2* mRNA was not induced by H_2O_2, jasmonate, or ACC, in contrast to *APR1* and *APR3* (Fig. 5), and also was not induced by salt stress in the cytokinin-deficient plants (Fig. 8). Only little is known about the biochemical or molecular differences between the three APR isoforms. Based on genomic sequence, *APR1* and *APR3* are more closely related to each other than either of them is to *APR2*. In previous experiments, the isoforms were all regulated in the same way, but with different time and/or strength of the response (Kopriva et al., 1999; Koprivova et al., 2000, Vauclare et al., 2002). These results indicate that the different APR isoforms have specific functions and are regulated differently, which is likely to allow a more precise fine tuning in the plant stress response.

Interestingly, the GSH content in the signaling mutants did not always correlate with APR activity. In *jar1* and *ein2*, APR activity was not induced by salt stress and correspondingly GSH was also not increased. On the other hand, no increase in APR was detected in *tir1* and *npr1*, but the thiols were increased similar to

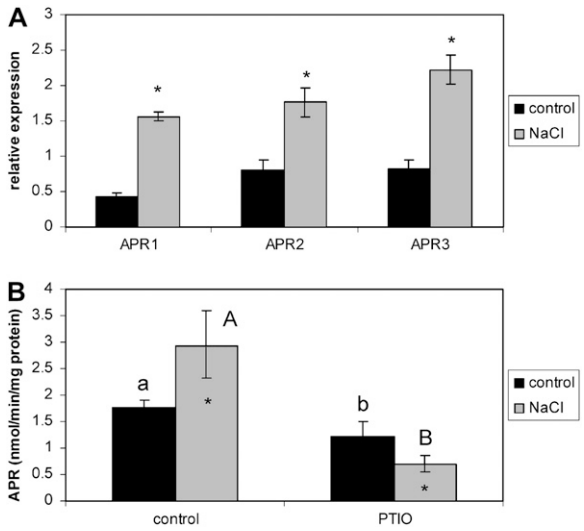

Figure 7. Involvement of NO in regulation of APR by salt stress. Wild-type Arabidopsis (Col-0) were grown in hydroculture and treated with 0.6 mM PTIO for 30 min before exposure to 150 mM NaCl for 5 h. A, The relative mRNA levels for *APR1*, *APR2*, and *APR3* in roots were determined by semiquantitative RT-PCR and standardized with actin 2/7 transcript. B, APR activity was measured in crude root extracts. Data are presented as means ± SDs of three independent plants. All treatments were repeated at least twice with similar results. Values marked with asterisks are significantly ($P \leq 0.01$) different from control values.

wild-type plants. This finding shows that, despite its high control over sulfate assimilation, induction of APR activity is not essential for the increase of GSH synthesis after salt stress and that other components of GSH biosynthesis have to be induced by salt as well to enable its accumulation. The other components are probably under the control of jasmonate and ethylene signaling because disruption of these pathways prevented GSH accumulation. The uncoupling of APR regulation from GSH synthesis has been observed before. Loudet et al. (2007) showed that reduction of total APR activity in *apr2* T-DNA lines to 20% of the wild-type activity did not affect thiol levels.

Had we stopped our analysis at measuring only transcript levels, which is often the case (Charlton et al., 2005; Ma et al., 2006), this would be the end of a very simple story. However, we also measured the APR enzyme activity because this is more relevant for the physiological response of the plant than mRNA level alone. The activity results were very striking. We could rapidly conclude that APR is indeed regulated by salt stress in an ABA-independent manner (Fig. 3). In all other signaling mutants but *gai*, APR activity was not increased upon salt treatment despite the increase in mRNA levels. The uncoupling of responses of mRNA and enzyme activity to salt stress in most of the signaling mutants revealed that the regulation is far more complex than we expected and from what is known from literature (Leustek et al., 2000; Kopriva and Koprivova, 2004; Kopriva, 2006). Therefore, we have to postulate that posttranscriptional regulation of APR occurs, requiring the correct functioning of stress signaling. The finding that APR activity was significantly reduced in H_2O_2-treated plants (Fig. 2) led to a possible explanation for our results. We hypothesized that the disruption of stress signaling pathways resulted in an inefficient detoxification of ROS, elicited by the salt treatment, which inactivated APR. However, because by DAB staining we have not observed any increased ROS production in salt- treated roots of the various signaling mutants (data not shown) and because in extracts from such roots the high molecular mass APR adducts were not detected, this explanation is unlikely. Moreover, in all western-blot experiments the APR protein accumulation strictly correlated with enzyme activity (Fig. 9); therefore, the regulation is likely to be on the level of translation or protein stability.

The lack of induction of APR activity in the signaling mutants could be explained in three ways: the need for a stress-induced activator for the translation of APR; the presence of an inhibitor that is inactivated and/or degraded as a response to stress; or the activation of the APR degradation pathway in the mutants. The results could also possibly be attributed to various pleiotropic effects of the mutations. However, because similar responses were observed in plants where very different signaling pathways have been disrupted and also in plants where the signaling was disrupted by chemical treatment, the pleiotropic effects are probably not the main cause of the observed changes in APR regulation in the mutants. The strict correlation between activity and protein, however, suggests that the posttranslational activation of APR by oxidative stress as described by Bick et al. (2001) does not contribute to the regulation of the enzyme by salt stress.

The increase in APR protein accumulation and activity in *gai* plants was no less surprising because mRNAs of two of the three APR isoforms were not affected in this genotype. It appears that the small increase in *APR3* transcript may be responsible for the increase in enzyme activity. However, the contribution of individual isoforms to total APR activity is not known, and *APR3* has never been considered to be the major isoform. Indeed, in APR2 T-DNA lines, the foliar APR activity was reduced to 20% of wild-type levels, pointing to a more significant role of this isoform than APR3 (Loudet et al., 2007). Therefore, posttranscriptional control of APR involving a GA-modulated mechanism is more likely to explain this result.

Such posttranscriptional regulation has not been described for plant APR before, but is similar to regulation of APR in sulfur starvation response mutants of the green alga *Chlamydomonas reinhardtii*. Specifically, in the *sac2* mutant, APR mRNA was induced by sulfate starvation similar to wild-type *Chlamydomonas*, but a corresponding increase in enzyme activity was prevented by the mutation (Davies et al., 1994; Ravina et al., 2002). This is analogous to our results, and the *SAC2* gene thus seems to be equivalent to our postulated translational regulator. Analysis of the *sac1*

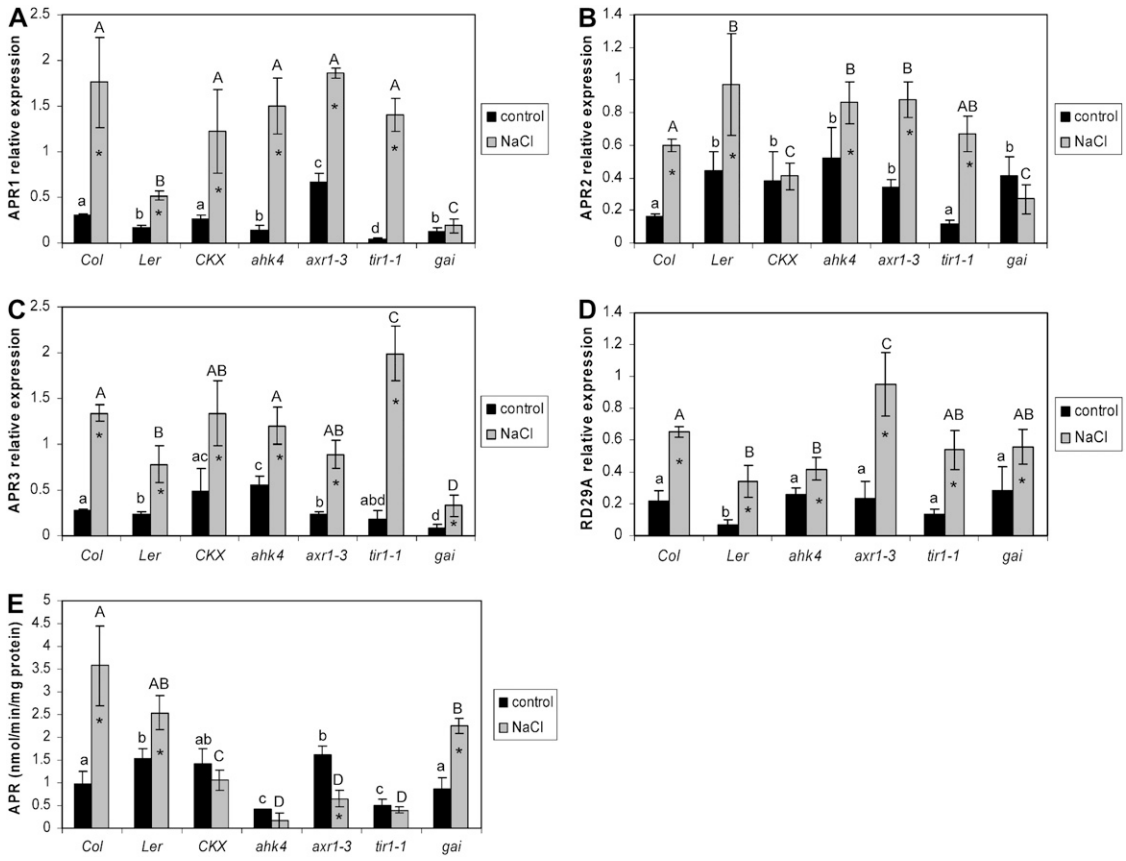

Figure 8. Regulation of APR by salt in mutants deficient in hormone signaling. Arabidopsis mutants or transgenic lines deficient in cytokinin perception (*ahk4*), cytokinin accumulation (*CKX*), auxin signaling (*axr1*), and *tir1* or GA-signaling *gai* plus the corresponding wild types (Col-0 or L*er*) were grown in hydroculture and treated with 150 mM NaCl for 5 h. The relative mRNA levels for *APR1* (A), *APR2* (B), *APR3* (C), and *RD29A* (D) in roots were determined by semiquantitative RT-PCR and standardized with actin 2/7 transcript. E, APR activity was measured in crude root extracts. Data are presented as means ± SDs of three independent plants. All treatments were repeated at least twice with similar results. Different indices indicate values significantly different at $P \leq 0.05$. Values marked with asterisks are significantly ($P \leq 0.05$) different from nontreated plants.

mutant revealed another peculiarity in APR regulation. Transcripts of APR and other genes of sulfate assimilation were not induced by sulfur deficiency in this mutant; nevertheless, the APR activity increased similar to wild type. This was not true for any other enzyme of the pathway; the increases in ATP sulfurylase, Ser acetyltransferase, and OASTL were prevented by the *sac1* mutation (Davies et al., 1994; Ravina et al., 2002). Again, this regulation is remarkably similar to the regulation of APR by salt stress in *gai* plants. The similarity in regulatory mechanisms is very surprising because, until now, discoveries about the regulation of the pathway by sulfur starvation in *Chlamydomonas* could not be confirmed in higher plants despite the great interest in this biological question.

Interestingly, another gene involved in salt stress response is also regulated at the posttranscriptional level (Hua et al., 2001). The mRNA level of *AtP5R* (pyrroline-5-carboxylate reductase), which is involved in Pro synthesis, is increased upon salt treatment, but the protein does not accumulate. The translation of the protein is inhibited during salt stress, via a mechanism dependent on the 5′-untranslated region, which is predicted to form a very strong secondary structure (total free energy -59.7 kcal mol^{-1}; Hua et al., 2001). In silico analysis of 5′-untranslated regions of the three APR genes revealed that they also potentially form strong secondary structures with free energies of -5.8, -18.9, and -22.4 kcal mol^{-1} for *APR1*, *APR2*, and *APR3*, respectively (Supplemental Fig. S4), whereas an average free energy of predicted secondary structures of 5′-untranslated regions is approximately -3 kcal mol^{-1} (Hua et al., 2001). Whether the 5′-untranslated regions of APR genes are indeed involved in the translational regulation awaits further investigation.

In conclusion, we have demonstrated that regulation of APR by salt cannot be wholly attributed to transcriptional regulation. This has important implications for the analysis of plant responses to different stimuli because many studies up to now have concentrated on transcriptome analysis to deduce the in vivo effects of these stimuli. It is clear that we must look at

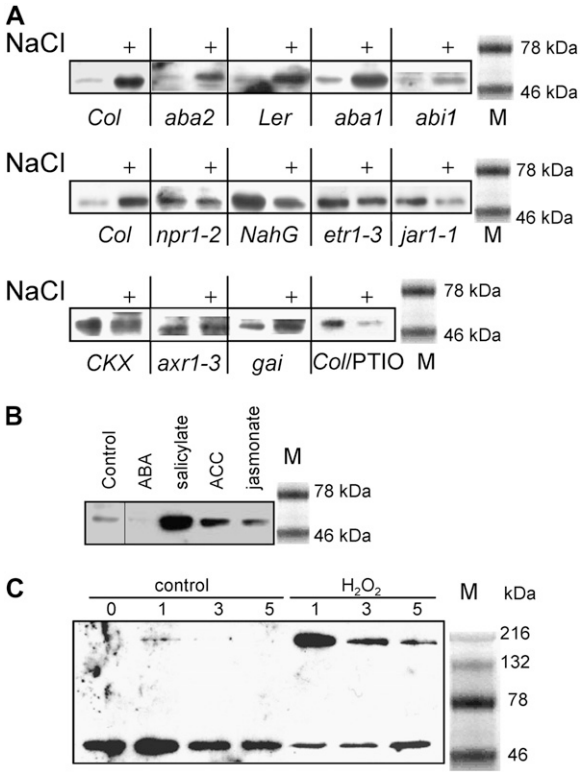

Figure 9. Western-blot analysis. A, APR protein accumulation was compared in various untreated and salt-treated Arabidopsis genotypes by western blotting of the crude root extracts for activity measurement. The analysis was repeated at least twice on independent extracts with similar results. B, Protein extracts from Arabidopsis wild-type (Col-0) roots untreated or treated with ABA, salicylate, ACC, and jasmonate for 5 h were resolved by SDS-PAGE. APR protein accumulation was compared by western blotting with APR2 antiserum. C, Protein extracts from Arabidopsis (Col-0) roots untreated or treated with H_2O_2 for indicated time were resolved by SDS-PAGE. APR protein accumulation was compared by western blotting with APR2 antiserum. M represents molecular mass marker.

enzyme activities together with transcriptional information to gain more physiologically relevant insight into plant responses to their environment.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The seeds of the mutants and transgenic plants were kindly provided by Gary Creissen (*npr1*, *NahG*, *etr1*, *ein2*, *jar1*), Fred Rook (*aba1*, *aba2*, *abi1*), Nick Harberd (*gai*), Robert Sablowski (*axr1*), Jonathan Jones (*tir1*), Geoff Holroyd (*abi1*), and Thomas Schmuelling (CKX), or obtained from The Nottingham Arabidopsis Stock Centre (*ahk4*). The plants were grown in hydroculture in nutrient solution composed of 1.5 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.75 mM KH_2PO_4, 0.75 mM $MgSO_4$, 0.1 mM Fe-EDTA, 10 μM $MnCl_2$, 50 μM H_3BO_3, 1.75 μM $ZnCl_2$, 0.5 μM $CuCl_2$, 0.8 μM Na_2MoO_4, 1 μM KI, and 0.1 μM $CoCl_2$ under a 10-h-light/14-h-dark cycle at constant temperature of 22°C, 60% relative humidity, and light intensity of 160 μmol m^{-2} s^{-1}. The nutrient solution was exchanged weekly. Three weeks after sowing, the plants were transferred into fresh nutrient solution with or without 150 mM NaCl for salt treatment, 10 mM H_2O_2 or other additives, as indicated, and incubated for 5 h under the same conditions. Roots were collected and immediately frozen in liquid N_2.

RNA Extraction and Expression Analysis

Total RNA was isolated from the roots by phenol:chloro-form:isoamylalcohol (25:24:1) extraction and LiCl precipitation. Aliquots of 1 μg were reverse transcribed by SuperScript reverse transcriptase (Invitrogen). For semiquantitative PCR, equivalents of 40 ng of total RNA were amplified by GoTaq Flexi DNA polymerase (Promega) in 20-μL reactions with primers specific for the three APR isoforms APR1 (At4g04610)—APR1f (CTCGTTTCGGTGTTTCATTG) and APR1r (CAATCCCTTGCTCCTAACCA); APR2 (At1g62180)—APR2f (CCACACATCAGCTCCTTCAA) and APR2r (AACGCTGAGTCACATTCACG); and APR3 (At4g21990)—APR3f (TCCAAGCACGTAAACCCTTC) and APR3r (CGGCTTCTCTGAGTTTGTCC). As controls, the cDNA was amplified with primers derived from actin 2/7 (At5g09810)—actf (GGAGCTGAGAGATTCCGTTG) and actr (TGAACAATCGATGGACCTGA); from salt and ABA up-regulated gene *RD29A* (At5g52310)—RD29Af (GGAGCTGAGCTGGAAAAAGAATTTGATCAGAAG) and RD29Ar (CCAATCTGAAGTTTCTCGGCAACCATATCAG); and an ABA-independent salt-inducible gene *AtDi19-2* (At1g02750)—Di19f (ACGCGTCGACATGGAAGACGATATGTGGTGCG) and Di19r (CGCGGATCCGCCTCAGAAGAGTCACATTCATC). The reactions were stopped after 26, 28, and 29 cycles for *APR1*, *APR2*, and *APR3*, respectively, and after 22, 29, and 32 cycles for actin, *RD29A*, and *Di19-2* when the reactions were still in the exponential phase as determined in preliminary experiments (Supplemental Fig. S5). Eighteen microliters of the PCR products were subjected to electrophoresis on ethidium bromide containing 1% agarose gels. The resulting band intensity on a UV transilluminator was calculated with the Quantity One software package (Bio-Rad).

APR Activity Measurement

APR activity was determined as described elsewhere (Kopriva et al., 1999; Koprivova et al., 2000). The roots were homogenized 1:20 (w/v) in 50 mM Na/K phosphate buffer, pH 8, supplemented with 30 mM Na_2SO_3, 0.5 mM 5′-AMP, and 10 mM dithioerythritol (DTE) and the extract was centrifuged for 30 s at 2,000 rpm to remove cell debris. APR activity was measured in the supernatants as the production of [^{35}S]sulfite, assayed as acid volatile radioactivity formed from [^{35}S]APS and DTE (Brunold and Suter, 1990). The protein concentration in the extracts was determined by Bio-Rad protein assay with bovine serum albumin as a standard.

Western Blotting

APR protein accumulation was assessed by western blotting with polyclonal antisera against recombinant APR2 (Kopriva et al., 1999). Aliquots of the crude extracts from APR activity measurements corresponding to 8 μg of protein were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membrane by electroblotting. The blots were developed with SuperSignal West Pico system (Pierce).

GSH Measurements

GSH was extracted from the root tissue by grinding 0.1 g of frozen material in 1 mL of 0.1 M HCl. After centrifugation at 20,000*g* for 10 min, the supernatant was used to measure the content of total GSH after reduction with DTE by HPLC using the monobromobimane derivatization method as described by Creissen et al. (1999).

DAB Staining

H_2O_2 in the roots of plants treated with salt or H_2O_2 was detected by staining with DAB according to Thordal-Christensen et al. (1997).

Statistical Analysis

The data were subjected to ANOVA and multiple range tests (LSD). The results from salt treatments were compared with controls by Student's *t* test at 95% confidence level. Statistical analyses of the data were carried out using SPPS for Windows (release 9.0; SPSS).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. GENEVESTIGATOR microarray data on regulation of APR by salt.

Supplemental Figure S2. Localization of ROS in roots after treatment with NaCl and H_2O_2.

Supplemental Figure S3. Regulation of APR by GA.

Supplemental Figure S4. Predicted hairpin structures of 5′-untranslated regions of APR mRNA.

Supplemental Figure S5. RT-PCR conditions.

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