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An *Arabidopsis* Soil-Salinity–Tolerance Mutation Confers Ethylene-Mediated Enhancement of Sodium/Potassium Homeostasis[W]

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High soil Na concentrations damage plants by increasing cellular Na accumulation and K loss. Excess soil Na stimulates ethylene-induced soil-salinity tolerance, the mechanism of which we here define via characterization of an *Arabidopsis thaliana* mutant displaying transpiration-dependent soil-salinity tolerance. This phenotype is conferred by a loss-of-function allele of *ETHYLENE OVERPRODUCER1* (*ETO1*; mutant alleles of which cause increased production of ethylene). We show that lack of ETO1 function confers soil-salinity tolerance through improved shoot Na/K homeostasis, effected via the ETHYLENE RESISTANT1–CONSTITUTIVE TRIPLE RESPONSE1 ethylene signaling pathway. Under transpiring conditions, lack of ETO1 function reduces root Na influx and both stelar and xylem sap Na concentrations, thereby restricting root-to-shoot delivery of Na. These effects are associated with increased accumulation of RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF)–dependent reactive oxygen species in the root stele. Additionally, lack of ETO1 function leads to significant enhancement of tissue K status by an RBOHF-independent mechanism associated with elevated *HIGH-AFFINITY K^+ TRANSPORTER5* transcript levels. We conclude that ethylene promotes soil-salinity tolerance via improved Na/K homeostasis mediated by RBOHF-dependent regulation of Na accumulation and RBOHF-independent regulation of K accumulation.

INTRODUCTION

Almost all important crop plants are sensitive to high soil salt concentrations (Frommer et al., 1999; Flowers, 2004). High soil salinity is therefore a severe problem in approximately one-third of the world's irrigated agricultural land and is a major constraint on agricultural productivity (Greenway and Munns, 1980; Zhu, 2002; Munns and Tester, 2008). NaCl is the most soluble and widespread soil salt. High soil Na concentrations damage plants by causing increased tissue Na accumulation and K loss (Nassery, 1979; Greenway and Munns, 1980; Lynch and Läuchli, 1984; Zhu, 2002; Munns and Tester, 2008). Accordingly, plants have evolved a variety of salinity tolerance mechanisms, including mechanisms to restrict Na accumulation and K loss (Zhu, 2002; Smith et al., 2010). For example, in *Arabidopsis thaliana*, the product of the *SALT OVERLY SENSITIVE1* (*SOS1*) gene, the plasma membrane Na^+/H^+ antiporter SOS1, transports Na^+ accumulated in outer cell layers of the root back into soil solution, thereby reducing the net influx of Na^+ into inner cell layers of the root and, in turn, root-to-shoot Na^+ delivery (Shi et al., 2000, 2002; Zhu, 2002; Munns and Tester, 2008). Several other components (e.g., SOS2, SOS3, and the calcineurin B-like protein 10 [a putative calcium sensor]) (Liu and Zhu, 1998; J. Liu et al., 2000; Qiu et al., 2002; Quan et al., 2007) are also involved in the signaling pathways by which Na^+ is eventually transported back out of the root, while the *HIGH-AFFINITY K^+ TRANSPORTER1* (*HKT1*) gene product (HKT1) can retrieve Na^+ from the transpiration stream xylem sap before it reaches the shoot (Mäser et al., 2002; Sunarpi et al., 2005; Møller et al., 2009). High salinity also causes strong depolarization of the electrical potential difference across the plasma membrane, leading to tissue K^+ loss via outward-rectifying K^+ channels (Shabala and Cuin, 2008; Jayakannan et al., 2013), in turn impairing metabolic processes. Therefore, the ability of plants to retain tissue K concentration correlates with plant salinity tolerance (Maathuis and Amtmann, 1999; Chen et al., 2005; Cuin et al., 2008; Munns and Tester, 2008). Intriguingly, salinity tolerance can be achieved by manipulating the expression of both Na^+ transporter genes (e.g., *HKT1* and *SOS1*; Shi et al., 2003; Møller et al., 2009; Yang et al., 2009) and K^+ transporter genes (e.g., ARABIDOPSIS K TRANSPORTER1 [AKT1]; Ardie et al., 2010), indicating that differential regulation of Na^+ and K^+ transporters, and thus of ionic homeostasis, can contribute significantly to salinity tolerance.

Soil-salinity stress causes in planta accumulation of reactive oxygen species (ROS), which can result in oxidative stress and cell damage (Miller et al., 2010; Smith et al., 2010). However, in addition to being a toxic agent in salinity stress, ROS also act as an important signaling mediator of plant salinity tolerance (Kaye et al., 2011; Jiang et al., 2012; Ma et al., 2011). For example, *5PTase7* regulates ROS production in saline conditions (likely through activation of NADPH oxidases), and *Arabidopsis* mutants lacking 5PTase7 activity are hypersensitive to salinity (Kaye et al., 2011). Our previous investigations indicated that *RESPIRATORY BURST OXIDASE HOMOLOG F* (*RBOHF*) encodes a specific isoform of NADPH oxidase (RBOHF) that plays

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a key role in soil-salinity tolerance (Jiang et al., 2012). Lack of RBOHF function causes a deficiency in salt-induced accumulation of ROS in the *Arabidopsis* root stele that results in increased Na concentrations both in root stelar cells and xylem sap in saline soil conditions, increased root-to-shoot Na delivery, increased shoot Na accumulation, and consequential soil-salinity hypersensitivity (Jiang et al., 2012). On the other hand, ROS (specifically the hydroxyl radical [$OH^{\bullet}$]) may have adverse effects by activating root Na^+-permeable nonselective cation channels (Demidchik et al., 2003; Foreman et al., 2003) and outward-rectifying K^+ channels (Demidchik et al., 2010). These observations indicate that increased ROS levels play an important role in Na/K homeostasis and salinity tolerance. Since ROS production is influenced by many signals (e.g., biotic attack, abiotic stress, and hormonal signaling; Torres et al., 2002; Kwak et al., 2003; Marino et al., 2012), it is possible that additional factors regulate RBOHF in the mediation of soil-salinity tolerance. Here, we explore the possibility that the stress phytohormone ethylene is such a regulatory factor.

Phytohormones are known to play important roles in regulating ionic homeostasis and plant salt tolerance (e.g., Achard et al., 2006; Wu et al., 2008). For example, salt increases abscisic acid (ABA) levels and activates ABA-dependent signaling pathways (Zhu, 2002), in turn regulating transcriptome-level salt stress responses and physiological adaptation to salt stress (Xiong et al., 2001). Salicylic acid can prevent salinity-induced K loss, thus promoting *Arabidopsis* salinity tolerance (Jayakannan et al., 2013). Kinetin can inhibit K release into the xylem (Collins and Kerrigan, 1974; Hong and Sucoff, 1976), increase K^+ channel-mediated root K uptake (Shabala et al., 2009), and thus alleviate the negative impact of salinity on plants (Tounekti et al., 2011). In addition, polyamines likely play important roles in salinity tolerance (Krishnamurthy and Bhagwat, 1989) and have been implicated in the regulation of ion channel activity (Brüggemann et al., 1998; K. Liu et al., 2000; Shabala et al., 2007; Pandolfi et al., 2010; Zepeda-Jazo et al., 2011). Finally, the stress phytohormone ethylene is also likely involved in the regulation of plant salt tolerance (Zhang et al., 2004; H. Wang et al., 2004; Cao et al., 2007). Ethylene binds to endoplasmic reticulum membrane–localized receptors (e.g., ETHYLENE RESISTANT1 [ETR1]; Chang et al., 1993), thus deactivating CONSTITUTIVE TRIPLE RESPONSE1 (CTR1; a putative mitogen-activated protein kinase kinase kinase; Kieber et al., 1993), in turn inhibiting the activity of a signal transduction cascade that targets the ETHYLENE INSENSITIVE3 (EIN3) transcription factor protein for destruction in the proteasome (Solano et al., 1998; Guo and Ecker, 2003; Potuschak et al., 2003; Yoo et al., 2008; Zhao and Guo, 2011). Accordingly, ethylene promotes the accumulation of EIN3 in the nucleus (Potuschak et al., 2003; Yoo et al., 2008) where EIN3 binds to the promoters of its target genes, thus triggering a diverse array of ethylene responses (Solano et al., 1998; Fujimoto et al., 2000). Amongst EIN3-regulated genes are a number encoding ETHYLENE RESPONSE FACTOR (ERF) transcription factors. Previous reports have shown that overexpression of selected *ERF* genes confers increased salt tolerance (H. Wang et al., 2004; Zhang et al., 2004; Zhang et al., 2012) and that mutants exhibiting constitutive ethylene responses are also relatively salt resistant (Achard et al., 2006). By contrast, mutants deficient for ethylene responses display salt hypersensitivity at different developmental stages (Achard et al., 2006; Lei et al., 2011). These findings suggest that ethylene-regulated signaling pathways play an important role in plant salinity stress adaptation. However, the precise causal molecular and physiological mechanisms underlying this adaptation are largely unknown.

The DELLA proteins, gibberellin-opposable growth-inhibitory components of the gibberellin-GID1-DELLA signaling pathway (Harberd et al., 2009), are also known to play critical roles in plant tolerance to salt (Achard et al., 2006). We previously found that salt-activated ABA and ethylene signaling pathways regulate plant growth and/or development via integration at the level of DELLA function (Achard et al., 2006), suggesting the importance of DELLAs in some aspects of plant growth responses to high salt environments. In this study, we aimed to identify DELLA-independent salinity tolerance mechanisms and hence conducted a genetic screen for the identification of salt-resistant mutants using a multiply mutant progenitor line devoid of all five of the DELLAs encoded by the *Arabidopsis* genome (the global DELLA-deficient line; see Methods). Identified in the absence of DELLAs, such mutants might be expected to define salt-tolerance pathways that operate in a DELLA-independent fashion. In addition, we conducted our genetic screen in soil-grown conditions resembling natural environments rather than in Petri dish conditions (Jiang et al., 2012), anticipating that this would enable identification of salt tolerance mechanisms important in natural and agricultural conditions.

Here, we describe the genetic, molecular, and physiological characterization of an *Arabidopsis* soil-salinity tolerant mutant, *soil salinity tolerant1-1* (*sst1-1*). We first show that *sst1-1* is a loss-of-function allele of the previously described *ETHYLENE OVERPRODUCER1* (*ETO1*) locus, mutations in which cause increased production of ethylene (K.L.C. Wang et al., 2004). Our characterization of *sst1-1* thus reveals an association between in vivo regulation of ethylene synthesis and salt stress tolerance. We next show that lack of ETO1 function promotes soil-salinity tolerance by reducing root-to-shoot Na delivery via an *RBOHF*-dependent mechanism. In addition, we show that ethylene promotion of soil-salinity tolerance is likely in part attributable to an increase in tissue K concentration, possibly via upregulation of the expression of the gene encoding the high-affinity K^+ transporter HIGH-AFFINITY K^+ TRANSPORTER5 (HAK5). Our observations provide a mechanistic understanding of how the stress phytohormone ethylene promotes plant tolerance of soil salinity, which has potential value for the development of salinity-tolerant crop plants.

RESULTS

Isolation and Genetic Characterization of *sst1-1*

We aimed to identify naturally important salt tolerance mechanisms (using soil-based genetic screens; see Jiang et al., 2012) that may have been missed by previous studies using in vitro (Petri dish) conditions (e.g., J. Liu et al., 2000). This is particularly the case because transpirational flow is essentially blocked by

lack of evaporation in enclosed in vitro conditions, and yet the transpiration stream is likely of paramount importance to delivery of Na to shoots in natural and agricultural conditions (Jiang et al., 2012). We therefore screened for mutants tolerant of soil salinity in conditions of active transpiration. Watering soil-grown *Arabidopsis* seedlings once with NaCl solution of increasing concentration (up to 450 mM) caused a progressive decrease in chlorophyll content (Figures 1A and 1B) and progressive increase in plant death (Figure 1C).

We performed a genetic screen for mutants exhibiting pronounced salt tolerance. Approximately 20,000 soil-grown fast-neutron-mutagenized DELLA-deficient (global) M2 seedlings (see Methods; Belfield et al., 2012; Jiang et al., 2012) were watered with 350 mM NaCl. Three weeks later, all surviving plants (as shown in Supplemental Figure 1 online) were selected and allowed to self-pollinate, and the heritability of salinity tolerance was determined in subsequent generations. Among ~10 such mutants, the phenotype of one, *sst1-1*, was shown by backcrossing to be conferred by a single recessive mutant allele (a 3:1 ratio was obtained in the F2 generation of this cross).

sst1-1 conferred a clear soil-salinity tolerance phenotype (Figure 1D). Although there was no detectable difference between the shoots of the DELLA-deficient global progenitor and *sst1-1* (also DELLA-deficient) plants grown in normal conditions (Figure 1D), progenitor plants were yellowing (decreased chlorophyll content) 1 week after being watered with 350 mM NaCl, while *sst1-1* plants remained green after this treatment (Figures 1D and 1E). Whereas the majority of progenitor plants were dead 3 weeks after treatment, the majority of *sst1-1* plants had survived (Figure 1F). By contrast, there were no detectable phenotypic differences between progenitor and *sst1-1* plants following treatment with a sublethal mannitol concentration (see Supplemental Figure 2 online), suggesting that the NaCl tolerance of *sst1-1* plants is distinct from any effect on osmotic tolerance. Notably, although the *sst1-1* mutant was relatively tolerant of soil salinity (Figures 1D to 1F), there were no detectable differences between the salt sensitivities of progenitor and *sst1-1* plants when grown in Petri dish conditions (see Supplemental Figures 3A and 3B online). Thus, *sst1-1* identifies a gene that specifically affects plant sensitivity to soil salinity but not to in vitro salinity.

sst1-1 Is a Mutant *ETO1* Allele

Although there was no detectable phenotypic difference between shoots of global and *sst1-1* grown in normal soil (Figure 1D), we observed that *sst1-1* seedling primary roots were shorter than those of global controls (Figure 2A). In addition, we found that dark-grown *sst1-1* seedlings constitutively exhibited the triple response phenotype (shorter, radially swollen hypocotyl and exaggerated apical hook) characteristic of dark-grown seedlings treated with ethylene (Figure 2B; Guzmán and Ecker, 1990). These observations suggested that *sst1-1* might confer changes in ethylene synthesis, signaling, or response. In further experiments, we found that growth of the *sst1-1* primary root was restored by treatment with silver (Ag^+) ions (Figure 2C). Silver ions block the binding of ethylene to endoplasmic reticulum membrane–localized ETR1-type ethylene receptors (Beyer, 1976), consequently suppressing the phenotype of

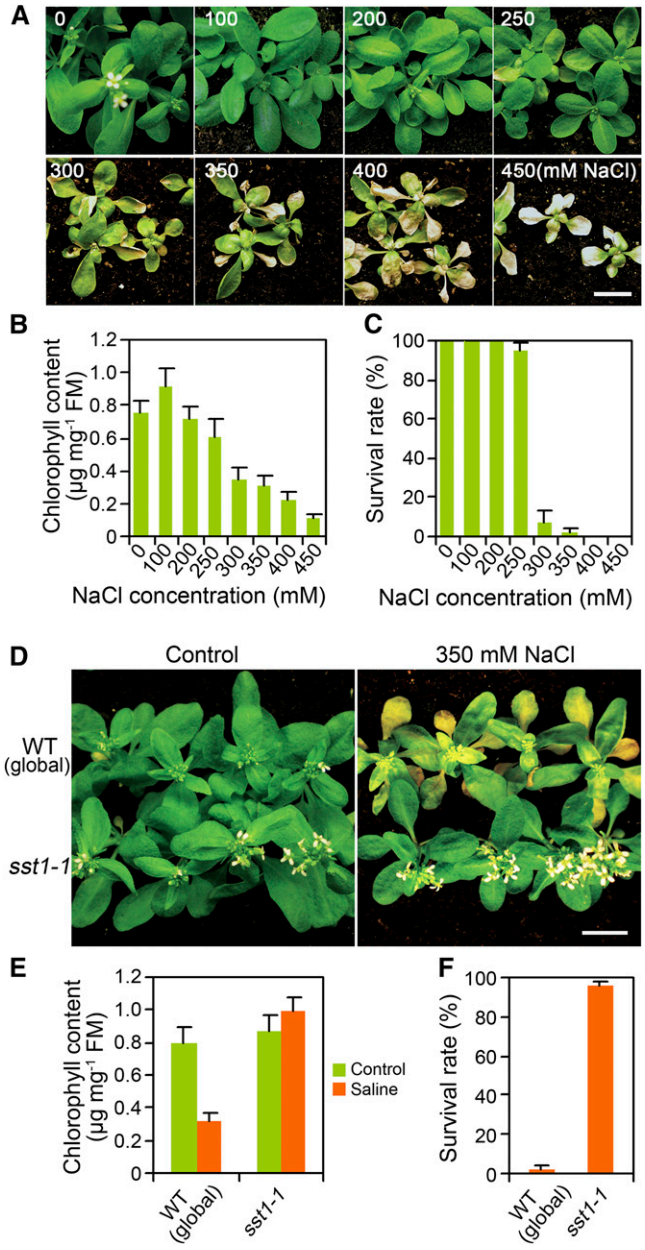


Figure 1. Soil-Salinity Tolerance of *sst1-1*.

(A) Appearance of *Arabidopsis thaliana* wild-type (Landsberg *erecta*) plants following treatment with saline solutions of increasing NaCl concentration. Four-week-old soil-grown plants were watered (concentrations as indicated) once to soil capacity and then subsequently watered as required with nonsaline water. Pictures were taken 10 d after treatment.

(B) Chlorophyll content of plants shown in **(A)**. Chlorophyll content measurements were as described in Methods. FM, fresh mass.

(C) Survival rate (%) of plants 3 weeks following salinity treatments.

(D) to **(F)** Appearance **(D)** and chlorophyll content **(E)** of global progenitor (wild type [WT]) and *sst1-1* mutant plants 1 week following treatment with 350 mM NaCl. Survival rate (%) **(F)** 3 weeks following the salinity treatment. Data shown in **(B)**, **(C)**, **(E)**, and **(F)** are means ± SE of three replicates. Bars = 1 cm in **(A)** and **(D)**.

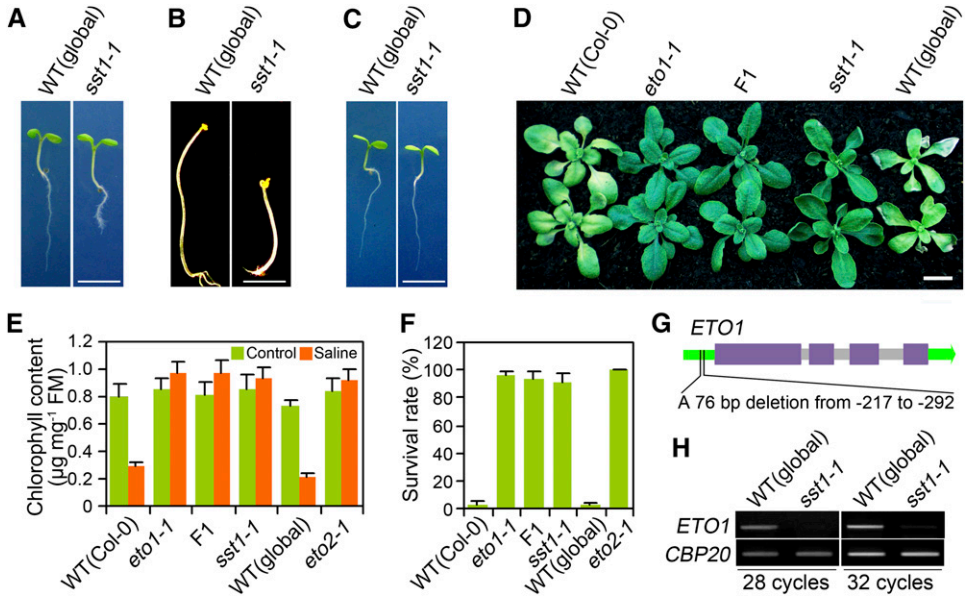


Figure 2. *sst1-1* Is a Mutant *ETO1* Allele.

(A) to **(C)** Appearance of 6-d-old global progenitor (wild type [WT]) and *sst1-1* mutant seedlings grown in light/dark cycles **(A)**, constant darkness **(B)**, or light/dark cycles in the presence of 10 μM Ag^+ **(C)**.

(D) Appearance of plants of various genotypes (as indicated) 10 d following treatment with 350 mM NaCl (plant growth and salinity treatment as described in Figure 1A). F1 is *sst1-1*/*eto1-1* heterozygote. Col-0 is the wild-type control appropriate for *eto1-1*, global for *sst1-1*.

(E) Chlorophyll contents of plants shown in **(D)** (with additional *eto2-1* mutant). Col-0 is the wild-type control appropriate for *eto2-1*. FM, fresh mass.

(F) Survival rate (%) of plants (genotypes as indicated) 3 weeks after treatment with 350 mM NaCl in soil-growth conditions.

(G) Representation of the *ETO1* gene showing the location (promoter) of the deletion in the *sst1-1* allele. Purple boxes represent coding sequences. Narrower pale boxes represent introns. Green boxes represent 5′ and 3′ untranslated regions.

(H) RT-PCR analysis of the abundance of *ETO1* transcripts in wild-type progenitor and *sst1-1* mutant. *CBP20* transcripts provide control.

Bars = 0.5 cm in **(A)** to **(C)** and 1 cm in **(D)**. Data shown in **(E)** and **(F)** are means ± SE of three replicates.

ethylene overproducing mutants (e.g., *eto1-1*; K.L.C. Wang et al., 2004), but not suppressing the phenotype of constitutive ethylene response mutants (e.g., *ctr1-1*; Guzmán and Ecker, 1990). These latter observations suggested the possibility that *sst1-1* might be a mutant *ETO1* allele. *ETO1* encodes a broad-complex/tramtrack/bric-a-brac protein that interacts with type-2 1-aminocyclopropane-1-carboxylate synthase (ACS) ethylene biosynthetic pathway enzymes (e.g., ACS5), thereby causing degradation of ACS proteins and reducing ethylene production (K.L.C. Wang et al., 2004). *ETO1* loss-of-function alleles thus confer increased levels of ACS enzymes and of consequent ethylene production (K.L.C. Wang et al., 2004). Accordingly, we found that F1 heterozygote plants generated by crossing *sst1-1* and *eto1-1* displayed a similar level of salt tolerance to that of *sst1-1* or *eto1-1* homozygotes (Figures 2D to 2F), suggesting that *sst1-1* is indeed a mutant *ETO1* allele. We next determined the DNA sequence of the *ETO1* gene in *sst1-1*, including 5′ and 3′ flanking regions, and identified a 76-bp deletion in the *ETO1* promoter region (Figure 2G; see Supplemental Figure 4 online). Subsequent RT-PCR results indicated that this deletion causes a substantial decrease in the abundance of *ETO1* transcripts (Figure 2H). In addition, the fact that the *sst1-1*/*eto1-1* heterozygote (also heterozygous for the *gai-t6 rga-t2 rgl1-1 rgl2-1* and *rgl3-4* mutations that when homozygous confer the DELLA deficiency of the *sst1-1* progenitor) exhibits tolerance of soil salinity demonstrates that the soil-salinity tolerance conferred by *ETO1* loss-of-function alleles is not dependent on lack of DELLA function. Thus, we conclude that *sst1-1* is a mutant *ETO1* allele (hereafter named *eto1-15*) that results in reduced levels of *ETO1* transcripts.

We next determined if the soil-salinity tolerance of *ETO1* loss-of-function alleles is a property peculiar to *ETO1* or if other mutations conferring increased ethylene levels also lead to increased soil-salinity tolerance. We found that *eto2-1*, a gain-of-function *ACS5* mutant allele of *ETO2* that confers increased ethylene production (K.L.C. Wang et al., 2004), also displayed increased soil-salinity tolerance (see Supplemental Figure 5 online; Figures 2E and 2F). These observations indicated that the increased soil-salinity tolerance conferred by *eto1-1*, *eto1-15*, and *eto2-1* is caused by increased ethylene level.

Lack of ETO1 Function Ameliorates Shoot Na Accumulation and K Depletion in Plants Grown on Saline Soils

Growth in high-salinity environments causes in planta accumulation of Na, depletion of tissue K, and resultant cellular injury (Zhu, 2002; Munns and Tester, 2008). We therefore investigated whether the soil-salinity tolerance conferred by lack of ETO1 function might be due to improved Na/K homeostasis. When analyzed 2 or 5 d after watering with 350 mM NaCl solution, both

the *eto1-15* and *eto1-1* mutants lacking ETO1 function had accumulated much less shoot Na than their respective wild-type controls (Figure 3A). Furthermore, we found that these genotypes also differed in their shoot K concentrations (Figure 3B): the *eto1-15* and *eto1-1* mutants had ~10 to 15% higher ($P < 0.05$) shoot K concentrations (versus the wild type) in control soil conditions, and while shoot K concentrations declined significantly in the wild-type lines following salt treatment (day 0 compared with day 5, $P < 0.05$), the mutants lacking ETO1 function displayed no statistically significant decrease in K content (day 0 compared with day 5, $P > 0.4$; Figure 3B). These observations suggest that lack of ETO1 function confers salt tolerance by ameliorating shoot Na accumulation and salt-induced depletion of shoot K.

Ethylene Regulates Shoot Na and K Content via the ETR1-CTR1–Regulated Ethylene Signaling Pathway

Since lack of ETO1 function causes an increase in ethylene production and ethylene activates downstream signaling pathways

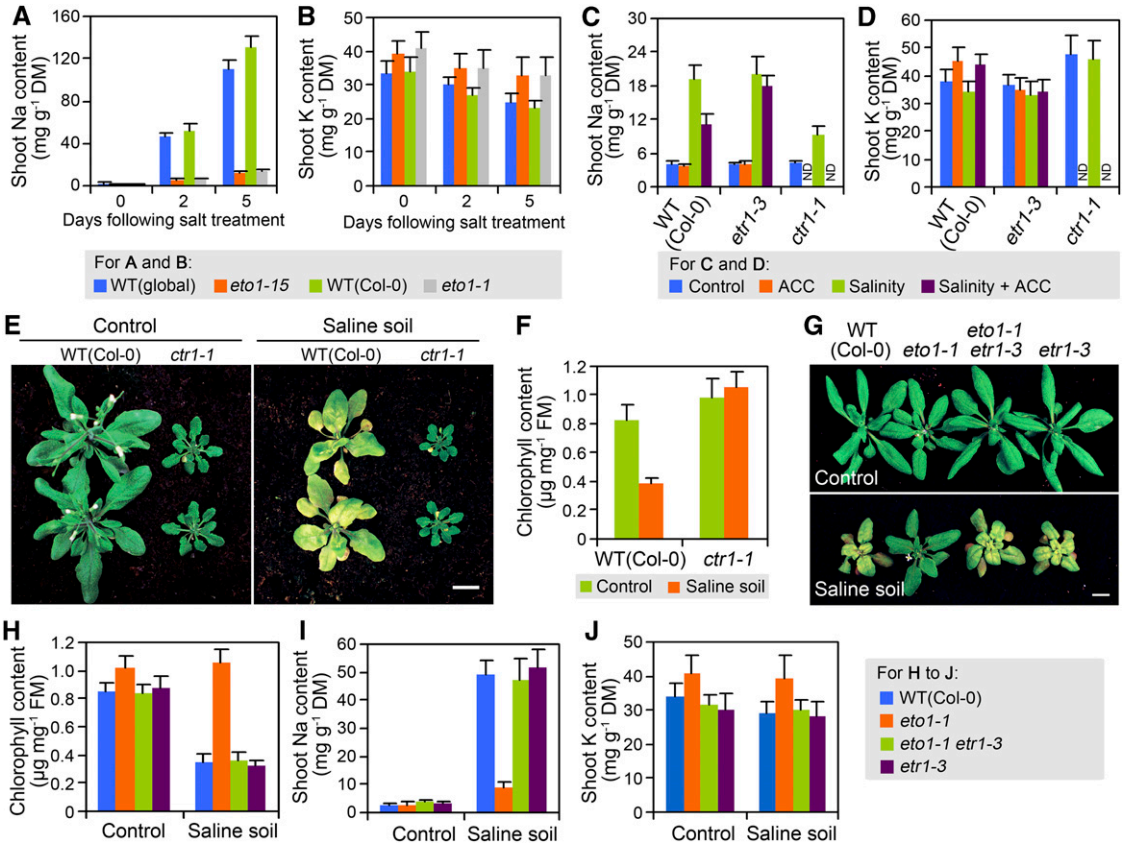


Figure 3. Ethylene Modulates Shoot Na and K Homeostasis via ETR1-CTR1–Regulated Ethylene Signaling.

(A) and **(B)** Na **(A)** and K **(B)** contents of shoots (genotypes as indicated) 2 or 5 d following salt treatment (dry mass [DM]) in soil-grown conditions. Global provides the wild-type (WT) control for *eto1-15* and Col-0 the wild-type control for *eto1-1*. Plant growth and salt treatment were as described in Figure 1A.

(C) and **(D)** Shoot Na **(C)** and K **(D)** contents of ACC-treated versus control plants (genotypes as indicated). ACC treatment was facilitated by use of hydroponically grown plants. Four-week-old plants (genotypes as indicated; see Methods) were treated with quarter-strength MS (control) or quarter-strength MS plus 100 mM NaCl. ACC treatment (50 μM) was applied 2 d prior to salinity treatment. Shoot samples were collected 2 d following onset of salinity treatment. ND, not determined.

(E) Wild-type (Col-0) and *ctr1-1* mutant plants grown in control soil conditions (Control) or for 10 d following treatment with 350 mM NaCl (Saline soil). Plant growth and salinity treatment were as described in Figure 1A.

(F) Chlorophyll contents of plants shown in **(E)**. FM, fresh mass.

(G) Plants (genotypes as indicated) grown in control conditions (Control) or for 10 d following treatment with 350 mM NaCl (Saline soil). Plant growth and salinity treatment were as described in Figure 1A.

(H) Chlorophyll contents of plants shown in **(G)**.

(I) and **(J)** Shoot Na **(I)** and K **(J)** contents of plants (genotypes as indicated) grown in control conditions or for 2 d following salinity treatment (350 mM NaCl; Saline soil). Plant growth and salinity treatment were as described in Figure 1A.

Data shown in **(A)** to **(D)**, **(F)**, and **(H)** to **(J)** are means ± SE of at least three replicates. Bars = 1 cm in **(E)** and **(G)**.

by binding to ethylene receptors (e.g., ETR1; Chang et al., 1993), we next determined if ethylene regulates shoot Na and K concentrations via ETR1-regulated ethylene signaling pathways. 1-Aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, is a widely used chemical ethylene surrogate treatment (Wang et al., 2002). We found that ACC treatment of wild-type plants had no detectable effect on shoot Na concentration under control conditions but caused ~50% reduction in shoot Na concentration 2 d following onset of salinity treatment (Figure 3C). In addition, ACC treatment of wild-type plants led to slightly but significantly ($P < 0.05$) elevated shoot K concentration, both under control conditions and 2 d following the onset of salinity treatment (Figure 3D). By contrast, ACC treatment of the ethylene-insensitive (ethylene receptor) mutant *etr1-3* had no significant effect on shoot Na or K concentration in either control or salinity treatment conditions (Figures 3C and 3D), suggesting that ethylene regulates shoot Na and K concentrations via the ETR1-regulated ethylene signaling pathway. Consistent with this, *ctr1-1* (a loss-of-function allele of *CTR1*, a gene encoding a downstream negative regulator of the ETR1-regulated ethylene signaling pathway; Kieber et al., 1993) displayed a reduced increase in shoot Na concentration compared with wild-type plants following salinity treatment (Figure 3C) and maintained a relatively high shoot K concentration in both control and salinity treatment conditions (Figure 3D). Accordingly, *ctr1-1* displayed tolerance of soil salinity (Figures 3E and 3F). In further experiments with a double mutant *etr1-3 eto1-1* line, we found that *etr1-3* suppressed both the soil-salinity tolerance (Figures 3G and 3H) and the reduced Na and elevated K shoot concentrations (Figures 3I and 3J) characteristic of *eto1-1*. These observations indicate that the ethylene overproduction characteristic of loss of ETO1 function regulates shoot Na and K homeostasis via the ETR1-CTR1–regulated ethylene signaling pathway.

Lack of *ETO1* Function Confers Decreased Xylem Sap Na Concentration and Increased Xylem Sap K Concentration

Growth of plants in high-salinity conditions causes excessive root-to-shoot Na delivery via the xylem in the transpiration stream, but mutants lacking ETO1 function were remarkably effective at restricting accumulation of Na in the shoot when exposed to salinity (Figure 3A). To determine if this effect was related to delivery of Na via the xylem, we compared ion concentrations in xylem sap obtained as root pressure exudate from detopped root systems of mutant plants lacking ETO1 function (*eto1-15*) versus wild-type plants at intervals following the onset of salinity treatment. While xylem sap Na concentrations of both wild-type and *eto1-15* plants increased with a similar time course following the start of salinity treatment (Figure 4A), that of *eto1-15* increased much less and averaged ~60% of the wild type (Figure 4A). In addition, *eto1-15* plants showed consistently higher xylem sap K concentrations (versus the wild type) in both control and salinity-treated plants (Figure 4B). The lower Na concentration and higher K concentration in the xylem sap of *eto1-15* plants therefore contribute directly to the reduced shoot Na accumulation and enhanced shoot K accumulation characteristic of mutants lacking ETO1 function (Figures 3A and 3B).

Lack of *ETO1* Function Reduces Root Na Influx and Xylem Na Loading and Promotes Retention of Relatively High Root K

To determine whether ETO1 modulates xylem sap Na and K concentrations by regulating root Na and K uptake or xylem sap loading, or through a combination of both of these processes, we also examined the time course of changes in bulk root Na and K concentrations in wild-type and *eto1-15* plants following exposure to salinity. We found that bulk root Na concentration was consistently lower in *eto1-15* than in wild-type plants over the first 2 d of salinity treatment (Figure 4C), indicating that lack of ETO1 function reduced (net) Na influx into the root during this time. Estimates of the slopes of the curves at the start of salinity treatment indicated that the initial rate of Na uptake into roots of *eto1-15* plants was ~70% of that into wild-type plants. By the third day of exposure to salinity and thereafter, the bulk root Na concentrations of *eto1-15* and wild-type plants were indistinguishable (Figure 4C), whereas *eto1-15* plants maintained consistently lower xylem sap Na concentrations (versus the wild type) for at least 4 d following salinity treatment (Figure 4A), indicating that the rate of xylem loading of Na was lower in *eto1-15* plants even at comparable bulk root Na content. Thus, it appears that reduced accumulation of shoot Na in *eto1-15* plants is attributable both to lower influx of Na into the root and to reduced xylem Na loading. In addition, we found that *eto1-15* bulk root K concentrations were consistently higher ($P < 0.05$) than those of the wild type in both salinity-treated and control plants (Figure 4D), an observation consistent with the high shoot and xylem sap K concentrations characteristic of *eto1-15* (Figures 3B and 4B).

Enhanced Na Tolerance and Shoot Na Homeostasis of Mutants Lacking ETO1 Function Is Dependent upon RBOHF Function

An earlier study suggested a linkage between ethylene and RBOHF in the induction of stomatal closure (Desikan et al., 2006). In addition, we previously showed that lack of RBOHF function causes overaccumulation of shoot Na and hypersensitivity to soil salinity (Jiang et al., 2012). We therefore next determined if the salinity tolerance conferred by lack of ETO1 function is dependent upon RBOHF function by making an *eto1-15 rbohf-4* double mutant (*rbohf-4* is an *RBOHF* loss-of-function allele [the same allele as *sss1-1* described by Jiang et al., 2012]). *eto1-15 rbohf-4* plants displayed the salinity hypersensitivity characteristic of *rbohf-4* (Figures 5A to 5C), and, while *eto1-15* survived treatment with 350 mM NaCl (Figures 1D to 1F, 5B, and 5C), *eto1-15 rbohf-4* plants were bleached and killed by NaCl treatments at 150 mM and above (Figures 5A to 5C; see Supplemental Figure 6 online). These observations indicate that ethylene confers soil-salinity tolerance via mechanisms dependent upon the function of RBOHF.

Analyzing the elemental content of plants treated with either 150 or 350 mM NaCl, we next found that, while *eto1-15* as expected accumulated markedly less shoot Na than the wild type, the *eto1-15 rbohf-4* double mutant exhibited shoot Na concentrations markedly higher than in wild-type plants and similar

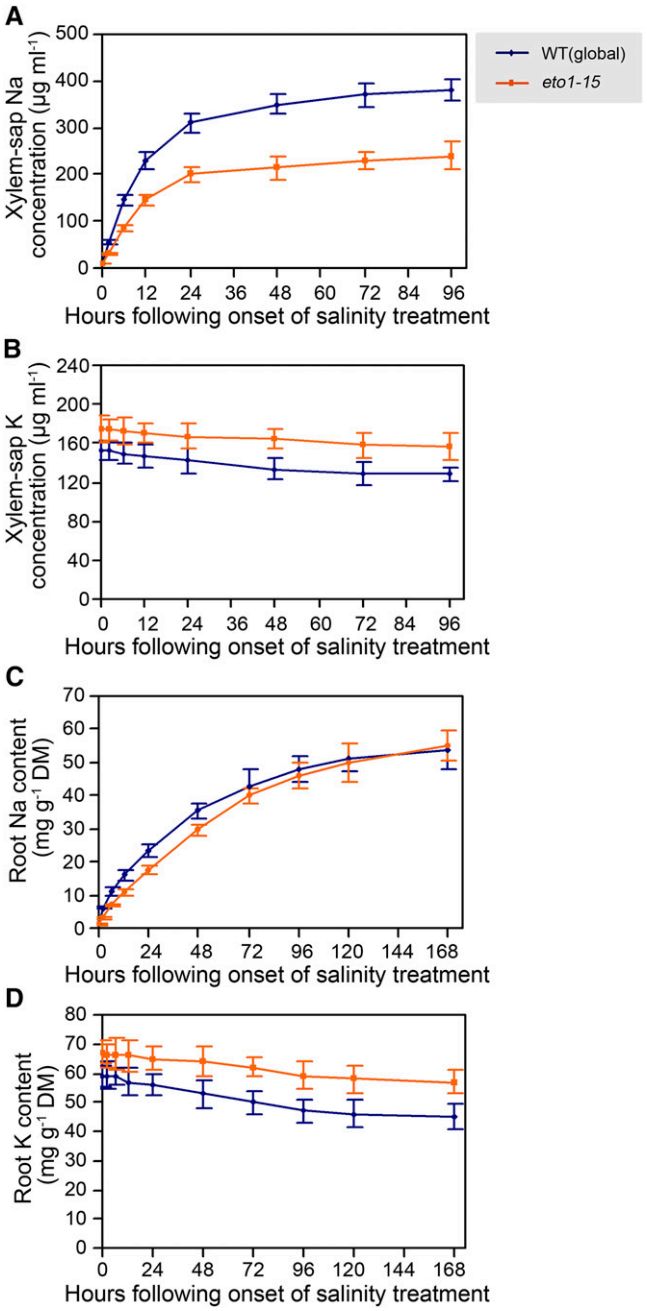


Figure 4. Lack of ETO1 Function Alters Root and Xylem Sap Na and K Homeostasis.

(A) and **(B)** Xylem sap Na **(A)** and K **(B)** concentrations at intervals following onset of salinity treatment. WT, the wild type.

(C) and **(D)** Root Na **(C)** and K **(D)** content at intervals following onset of salinity treatment. DM, dry mass.

To facilitate the collection of root samples and to compare the dynamics of changes in Na and K concentrations in xylem sap and root, hydroponically grown plants were used in these experiments. Four-week-old hydroponically grown wild-type (global) and *eto1-15* plants were treated with quarter-strength MS (control) or quarter-strength MS plus 100 mM NaCl. Xylem sap (exudation rates comparable between the two genotypes) and root samples were collected as described in Methods. Data shown are means ± SE of three replicates.

to those accumulated in the *rbohf-4* single mutant (Figure 5D). These observations indicate that ethylene regulates shoot Na accumulation predominantly via an RBOHF-dependent mechanism. In contrast with the ethylene regulation of shoot Na concentration, we found that *eto1-15* and *eto1-15 rbohf-4* displayed similar shoot K concentrations under both control and salinity-treated conditions (Figure 5E), indicating that RBOHF function does not contribute to the ethylene regulation of shoot K concentration.

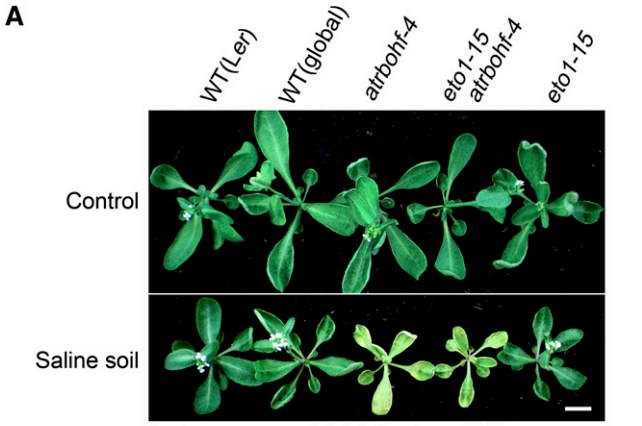
Lack of ETO1 Function Increases RBOHF-Dependent ROS Production in the Root Stele

Our previous work showed that RBOHF-dependent production of ROS in root stelar cells regulates root-to-shoot Na delivery (Jiang et al., 2012), while the observations above indicate that the effect of ethylene in ameliorating shoot Na accumulation is RBOHF dependent (Figure 5D). We therefore next determined if lack of ETO1 function reduces shoot Na concentrations by increasing RBOHF-regulated ROS production in the root stele. Intriguingly, we first found that *eto1-15* displayed a constitutively elevated level of stelar cell ROS (as detected by nitroblue tetrazolium [NBT] staining; Figure 6A; see Supplemental Figure 7 online). Second, the *eto1-15 rbohf-4* double mutant displayed an intensity of NBT staining similar to that of the *rbohf-4* single mutant, indicating that the ethylene-induced stele ROS accumulation characteristic of *eto1-15* is RBOHF dependent (Figure 6A). Thirdly, while salinity treatment increased the NBT signal in stelar cells of wild-type plants, it also further increased the already elevated NBT signal in *eto1-15* plants (Figures 6A and 6B). The constitutively elevated levels of root stele ROS, together with the further increase induced by salinity treatment, could therefore explain the reduced root-to-shoot Na delivery conferred by *eto1-15* (Figure 3A).

We next determined if ethylene regulates stele ROS production through the ETR1-regulated ethylene signaling pathway and found that *etr1-3* suppressed the high root stele NBT staining of *eto1-1* in control conditions, while in these same conditions, *ctr1-1* roots displayed constitutively high levels of NBT staining (Figure 6C). These observations suggest that stele ROS accumulation due to lack of ETO1 function is dependent on the ETR1-regulated ethylene signaling pathway. Interestingly, while *etr1-3* suppressed the increased NBT staining of *eto1-1*, the *etr1-3* mutant nevertheless displayed a salinity-induced increase in NBT staining, albeit of smaller magnitude than that of the wild-type control ($P < 0.05$; Figure 6C), thus suggesting that salinity-induced root stele ROS accumulation is partially but not fully dependent upon ETR1-regulated ethylene signaling.

Ethylene Increases Root Stele *RBOHF* Transcript Levels

Our previous study indicated that salinity causes root stele ROS accumulation at least partially via increased *RBOHF* transcript levels (Jiang et al., 2012). We next found that *eto1-1*, *eto1-15*,



B

150 mM NaCl

Lines	Total plants	Surviving plants	Rate (%)
WT(L*er*)	33	33	100
WT(global)	27	27	100
atrbohf-4	31	0	0
eto1-15 atrbohf-4	27	0	0
eto1-15	26	26	100

350 mM NaCl

Lines	Total plants	Surviving plants	Rate (%)
WT(L*er*)	33	3	9
WT(global)	27	1	4
atrbohf-4	31	0	0
eto1-15 atrbohf-4	27	0	0
eto1-15	26	25	96

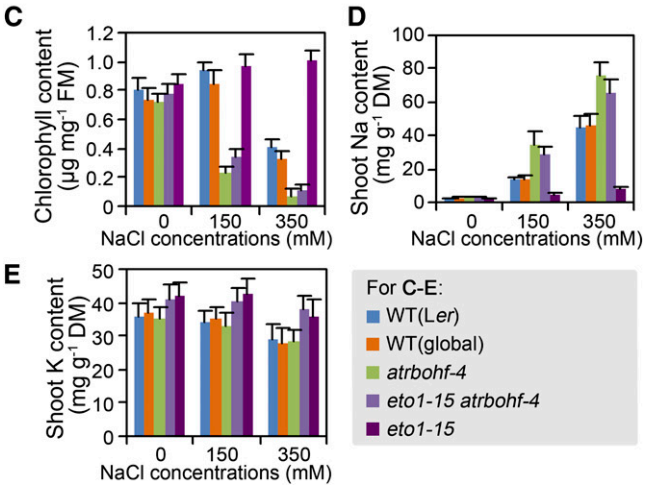


Figure 5. Ethylene Regulates Shoot Na Homeostasis via an RBOHF-Dependent Mechanism.

(A) Plants (genotypes as indicated) grown in control conditions (Control) or 7 d following treatment with 350 mM NaCl (Saline soil). Plant growth and salinity treatments were as described in Figure 1A. WT (*Ler*), wild-type Landsberg *erecta*. Bar = 1 cm.

(B) Plants (genotypes as indicated) surviving treatment with 150 or 350 mM NaCl (expressed as number of surviving plants and rate of survival [%] 3 weeks after treatment).

(C) Chlorophyll content (expressed as percentage of content of plants grown in control conditions) of shoots (genotypes as indicated) 7 d following treatment with 150 or 350 mM NaCl solutions. FM, fresh mass.

(D) and **(E)** Shoot Na **(D)** and shoot K **(E)** contents of plants (genotypes as indicated) 2 d following NaCl treatments (NaCl concentrations as indicated) in soil-growth conditions. DM, dry mass.

Results shown in **(C)** to **(E)** are means ± SE of three independent replicates.

and *ctr1-1* root tissues displayed elevated *RBOHF* transcript levels in control conditions (Figures 6D and 6E). Furthermore, while *eto1-1* displayed elevated levels of *RBOHF* transcript, in the *eto1-1 etr1-3* double mutant they were indistinguishable from wild-type levels (Figure 6E), suggesting that lack of ETO1 function increases *RBOHF* transcript levels via a mechanism dependent upon the ETR1-regulated ethylene signaling pathway. ACC treatment of wild-type plants also caused an increase in *RBOHF* transcript levels (Figure 6F), and ACC treatment of *pRBOHF*:*GUS* (for β-glucuronidase) plants caused an increase in root GUS staining, predominantly in stelar tissue (Figure 6G). These observations suggest that ethylene elevates ROS production in root stele at least partially by increasing *RBOHF* transcript levels.

Salinity treatment resulted in increased *RBOHF* transcript levels in wild-type plants and further increased the already elevated *RBOHF* transcript levels in *eto1-1* and *ctr1-1* plants (Figure 6E). The additive effects of salinity and ethylene/ethylene signaling on *RBOHF* transcript levels probably explains their additive effects on root stele ROS levels (Figures 6A to 6C). Salinity treatment also caused an increase in *RBOHF* transcript levels in the *etr1-3* mutant, although the magnitude of this increase was smaller than in wild-type plants ($P < 0.05$; Figure 6E), suggesting that factors additional to the ETR1-regulated ethylene signaling pathway mediate salinity-induced increases in root stele *RBOHF* transcript levels.

Ethylene Regulation of Root Na Influx Is Dependent upon RBOHF Function

Our previous study indicated that RBOHF regulates root Na influx at relatively early stages of salinity treatments (Jiang et al., 2012). Furthermore, the observations described above show that lack of ETO1 function reduces net influx of Na into the root, at least during the first 2 d of salinity treatment (Figure 4C). We next determined if this effect of lack of ETO1 function is RBOHF dependent by comparing the temporal dynamics of root Na accumulation in *eto1-15*, *rbohf-4*, *eto1-15 rbohf-4*, and wild-type roots in response to salinity treatment. We observed that the *rbohf-4* mutation conferred a significantly greater increase in bulk root Na content during the first 2 d of salinity treatment not only in the wild-type background but also in the *eto1-15* mutant background (Figure 7A), indicating that ethylene regulation of root Na influx is largely RBOHF dependent. However, we also found that root Na concentration in *eto1-15 rbohf-4* remained lower than that of *rbohf-4* (at least for the first 2 d of salt treatment), suggesting that RBOHF-independent mechanisms also play subsidiary roles in ethylene regulation of root Na influx. After prolonged salinity treatment (≥4 d), all tested genotypes had accumulated similar bulk root Na concentrations (Figure 7A).

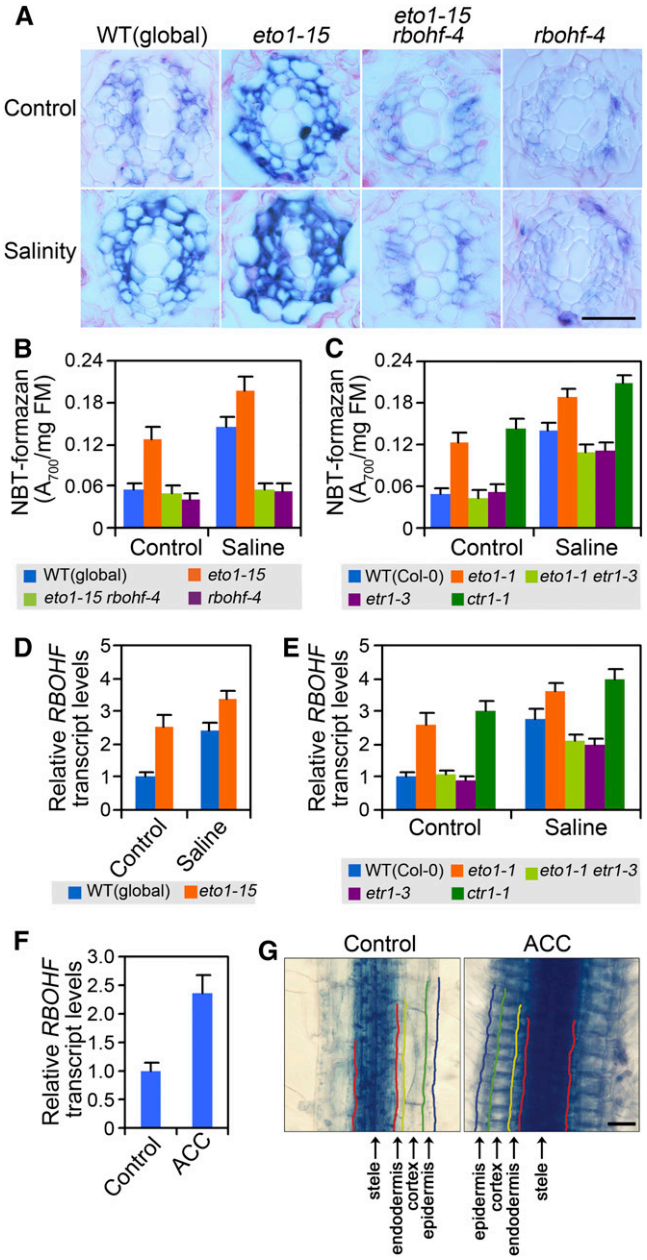


Figure 6. Ethylene Regulates RBOHF-Mediated ROS Production in Root Vascular Tissue.

(A) The effect of salinity treatment on NBT-visualized ROS levels in the stele of wild-type (WT; global), *eto1-15*, *eto1-15 rbohf-4*, and *rbohf-4* roots. Four-week-old hydroponically grown plants (genotypes as indicated) were treated with quarter-strength MS (control) or quarter-strength MS plus 100 mM NaCl. Root samples were collected for NBT staining (see Methods) 3 d following the treatments. Bar = 50 μm.

(B) and **(C)** Colorimetric quantification of NBT-formazan deposition in root tissues of plants (genotypes as indicated) under control conditions or 3 d following onset of salinity treatments (see Methods). FM, fresh mass. Data are means ± SE of three replicates.

(D) to **(G)** Ethylene increases *RBOHF* transcript levels in root vasculature tissue. Real-time PCR analysis of *RBOHF* transcript levels in root tissues (genotypes and treatments as indicated) (**[D]** to **[F]**). Plant growth and treatments were as described in Figures 3C and 3D. Root tissues were collected 3 d following the onset of the salinity (**[D]** and **[E]**) or ACC **(F)** treatment. Data shown in **(D)** to **(F)** were expressed as fold increase of *RBOHF* transcript levels in experimental samples over that in untreated wild-type controls and are means ± SE of three replicates. Effect of ACC treatment on GUS staining of mature *pRBOHF*:*GUS* roots **(G)**. Colored lines have been added to demarcate the different tissues for ease of visualization. Bar = 50 μm.

Ethylene Regulation of Xylem Sap and Root Stele Na Concentrations Are Dependent upon RBOHF Function

We next compared the temporal dynamics of xylem sap Na concentration in *eto1-15*, *rbohf-4*, *eto1-15 rbohf-4*, and wild-type plants in response to the onset of salinity treatment. We found that the *rbohf-4* mutation conferred a significant increase in xylem sap Na concentration in both the wild-type background and the *eto1-15* mutant background (Figure 7B), indicating that ethylene regulation of xylem Na loading is largely RBOHF dependent. However, the *eto1-15 rbohf-4* xylem sap Na concentrations were consistently lower than those of *rbohf-4*, suggesting that RBOHF-independent mechanisms also play subsidiary roles in the ethylene regulation of xylem Na loading. Consistent with our previous observations that ethylene regulation of shoot K concentration is not dependent on RBOHF function (Figure 5E), ethylene regulation of bulk root and xylem sap K concentrations were unaffected by *rbohf-4* (see Supplemental Figure 8 online).

Our previous studies indicated that RBOHF-catalyzed stelar ROS production decreases xylem sap Na concentrations mainly by reducing stelar Na concentrations (Jiang et al., 2012). In addition, our observations described above indicate that lack of ETO1 function causes an increase in RBOHF-dependent ROS accumulation in the root stele (Figures 6A and 6B) and a decrease in xylem sap Na concentration (Figure 4A). We next determined if the decrease in xylem sap Na concentration conferred by lack of ETO1 function is also attributable to decrease in root stelar Na concentration. We visualized Na accumulation in the stele of salinity-exposed roots using CoroNa Green dye (a green fluorescent indicator of Na^+; Oh et al., 2009). Fluorescence was barely detectable in root cells of all tested genotypes under control conditions but was prominent in epidermal cells of roots following exposure to NaCl (Figure 7C). Fluorescence was faintly but clearly detectable in the stelar cells of salinity-treated wild-type roots (Figures 7C and 7D) but was not detected in equivalent cells of salinity-treated *eto1-15* roots (Figures 7C and 7D). Thus, less Na^+ accumulated in the root vasculature of salinity-treated *eto1-15* versus wild-type plants. By contrast, fluorescence became clearly detectable in the vasculature tissue of salinity-treated *eto1-15 rbohf-4* roots (Figures 7C and 7D), indicating that the reduced root vascular Na^+ accumulation characteristic of *eto1-15* is dependent upon RBOHF function.

Salinity and Ethylene Increase the Abundance of Gene Transcripts Encoding the HAK5 K^+ Transporter

The observations described above indicate that ETO1 confers tolerance of soil salinity by regulating RBOHF-dependent Na

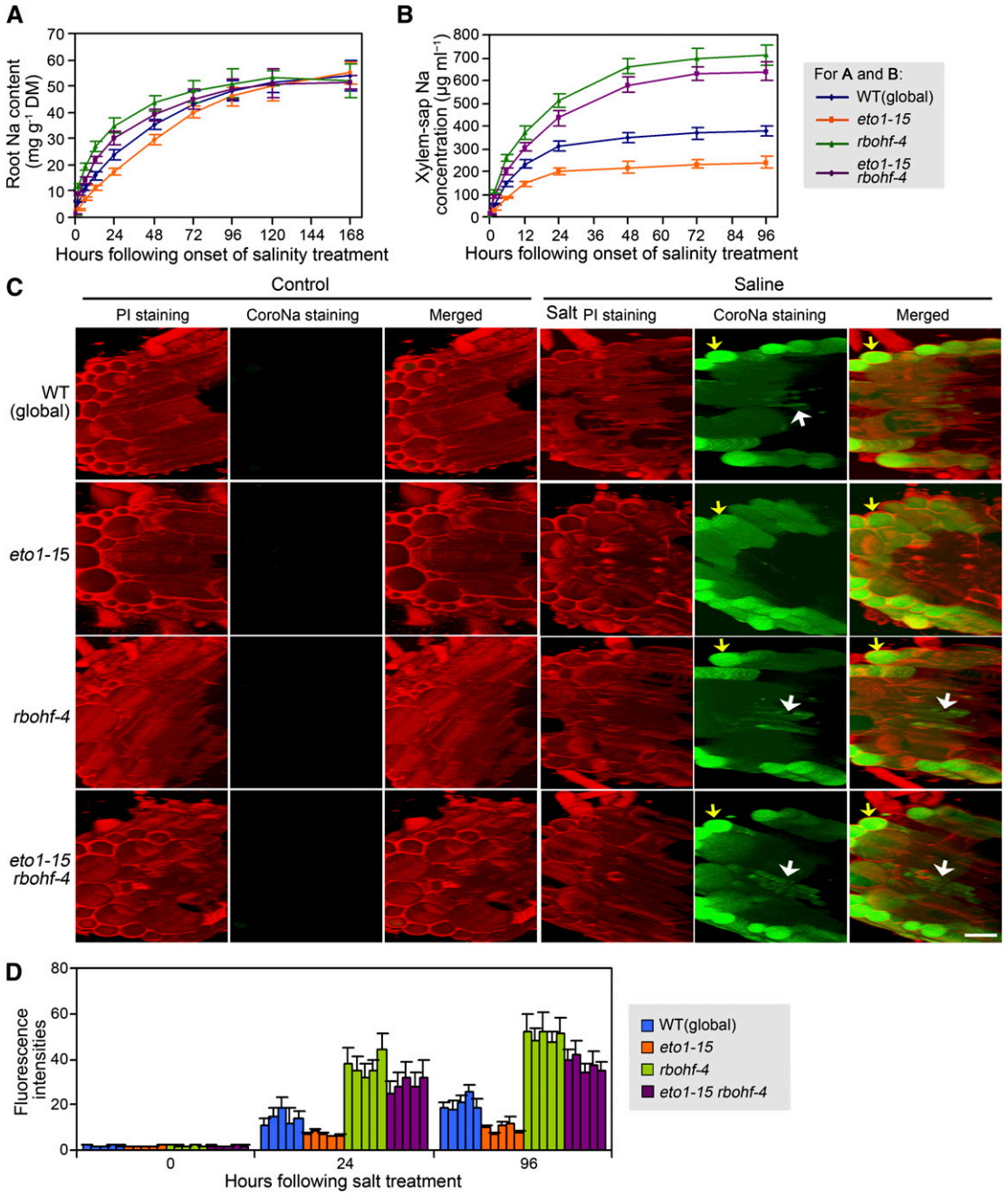


Figure 7. ETO1 Regulates Root Na, Xylem Sap Na, and Root Vascular Tissue Na Concentration via an RBOHF-Dependent Mechanism.

(A) and **(B)** Root **(A)** and xylem sap **(B)** Na concentrations in control conditions or following salinity treatment (genotypes as indicated). Plant growth, salinity treatments, and sample collections were as described in Figure 4. Data are means ± SE of three replicates. Data for global and *eto1-15* are reproduced from Figure 4 to allow direct comparison of the effect of *rbohf-4*. DM, dry mass.

(C) Cellular localization of root Na^+ (genotypes as indicated) in control conditions or following salinity treatment (Saline). Confocal images of roots stained with CoroNa Green (green) and propidium iodide (PI; red) are shown. Plant growth and salinity treatment was as described in Figure 4. CoroNa Green staining was as described in Methods. Arrows highlight the CoroNa Green signal in epidermal (yellow) or vascular (white) cells. Bar = 50 µm.

(D) Quantitative comparison of CoroNa Green fluorescence intensities in root pericycle cells 24 and 96 h following onset of salinity treatment. Five individual plants were measured for each genotype. Values are means ± SE for 10 pericycle cells from each individual plant.

homeostasis and RBOHF-independent K homeostasis (Figures 3A, 3B, and 7; see Supplemental Figure 8 online). Previous studies identified possible roles for a number of different ion transporters in the regulation of Na and K homeostasis (reviewed in Demidchik and Maathuis, 2007; Munns and Tester, 2008; Shabala and Cuin, 2008; Kronzucker and Britto, 2011). Among the genes that have been functionally characterized, loss of function of genes encoding HKT1 or SOS1 Na^+ transporters has been found to confer Na hypersensitivity (Rus et al., 2001; Shi et al., 2002), while ectopic overexpression of *SOS1* or stele-specific expression of *HKT1* increases salinity tolerance (Shi et al., 2003; Møller et al., 2009). In addition, AKT1 (a Shaker-type K^+ channel) and HAK5 (a high-affinity K^+ transporter belonging to the KUP/HAK/KT family) are the two K^+ transporters that play a major role in K acquisition by the roots, particularly in the low concentration range (Hirsch et al., 1998; Gierth et al., 2005; Pyo et al., 2010). We therefore next determined if ethylene signaling modulates Na and K homeostasis by regulating the levels of gene transcripts encoding these transporters. We found that *eto1-15*, *eto1-1*, *ctr1-1*, and wild-type plants all displayed similar levels of expression of *SOS1*, *HKT1*, and *AKT1* under control conditions and that transcript levels were generally unresponsive to salt treatment (except for a slight increase in *SOS1* expression in all genotypes in response to salt; see Supplemental Figure 9 online). This suggests that ethylene does not regulate Na and K homeostasis via transcript-level regulation of the abundance of SOS1, HKT1, or AKT1. By contrast, we found that the levels of *HAK5* transcripts in *eto1-1*, *eto1-15*, and *ctr1-1* were consistently approximately twofold higher than in wild-type plants in control conditions (Figure 8A) and that *HAK5* transcript levels displayed a transient three- to fivefold increase in response to salinity in all genotypes (peaking at 6 h following onset; Figure 8A). In addition, we found that *etr1-3* suppressed the elevated *HAK5* expression level conferred by *eto1-1* (Figure 8B), indicating that lack of ETO1 function promotes the increase in *HAK5* transcript level via the ETR1-CTR1–regulated ethylene signaling pathway. The elevated *HAK5* transcript level in mutants lacking ETO1 function and in *ctr1-1* potentially explains the relatively high K concentration characteristic of these mutants (Figures 3B, 3D, 4B, and 4D). Finally, consistent with the observation that lack of ETO1 function confers retention of high K concentrations in the shoot, xylem sap, and root via an RBOHF-independent mechanism (Figure 5E; see Supplemental Figure 8 online), we found that ethylene regulation of *HAK5* transcript levels was RBOHF independent (Figure 8C).

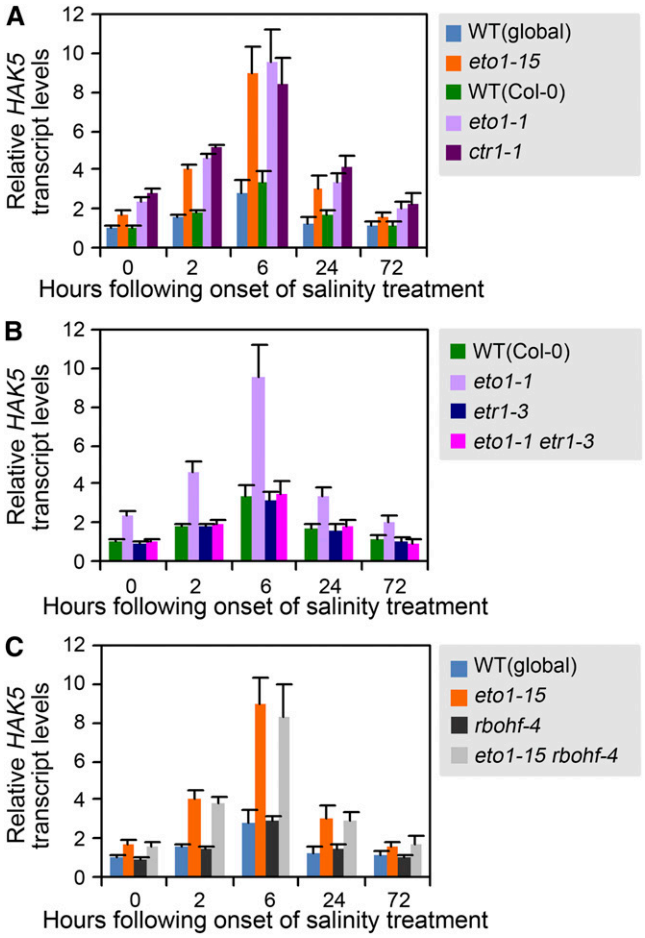


Figure 8. Salinity and Ethylene Regulate *HAK5* Transcript Levels.

Effect of increasing duration of salinity treatment on *HAK5* transcripts in root tissues of plants (genotypes as indicated). Plant growth and salinity treatments were as described in Figure 4A. Samples were collected at the time points indicated following onset of salinity treatments. Real-time PCR analysis was as described in Methods. Data were expressed as fold change of *HAK5* transcript levels in experimental samples over that in untreated wild-type controls at the onset of salinity treatment. Values are means ± SE of three biological replicates. Data for Col-0, *eto1-1*, global, and *eto1-15* in **(B)** and **(C)** are reproduced from **(A)**. WT, the wild type.

DISCUSSION

Increased in Vivo Ethylene Production Confers Soil-Salinity Tolerance

Ethylene synthesis is induced in plants by various biotic and abiotic environmental stresses, including pathogen attack, wounding, drought, flooding, salinity, low phosphorus, and low potassium (Borch et al., 1999; Wang et al., 2002; Achard et al., 2006; Broekaert, et al., 2006; Jung et al., 2009; Shi et al., 2012), and ethylene-mediated responses are often part of the crucial adaptive strategies that enable survival of those stresses. For example, the ethylene-inducible *Sub1A* gene (encoding an ERF-like protein) confers submergence tolerance in rice (*Oryza sativa*; Xu et al., 2006). In addition, ethylene signaling negatively regulates freezing tolerance (Shi et al., 2012) and positively regulates low potassium tolerance in *Arabidopsis* (Jung et al., 2009). Here, by employing a mutant screen using soil-grown plants in transpiring conditions, we discovered that a mutation causing enhanced ethylene production via loss of ETO1 function confers soil-salinity tolerance (Figures 1D to 1F). Our further demonstration that the *eto2-1* mutant allele (which also causes enhanced ethylene production; K.L.C. Wang et al., 2004) similarly confers soil-salinity tolerance (Figures 2E and 2F; see Supplemental Figure 5 online) provides further genetic evidence

that an increase in in vivo ethylene production confers plant soil-salinity tolerance.

While previous studies have indicated that enhanced ethylene signaling (as conferred by the *ctr1-1* mutation or by overexpression of selected ERF factors) results in salinity tolerance in various conditions (H. Wang et al., 2004; Achard et al., 2006; Zhang et al., 2012), our discovery that increased ethylene production promotes soil-salinity tolerance is particularly notable since *eto1-15* displays a survival rate comparable with that of wild-type controls under in vitro salinity conditions (see also Divi et al., 2010; see Supplemental Figure 3 online). This apparent discrepancy is likely to be due to the large differences in transpiration rate characteristic of the two experimental systems, which in turn may provide insight into the mechanisms underlying this conditional salinity tolerance phenotype. Since salinity stress is well known to increase the levels of transcripts specified by genes regulating ethylene biosynthesis (e.g., *ACS2* and *ACS7*), and, hence, ethylene emission (Achard et al., 2006), our observations thus indicate that salinity-induced ethylene is an especially potent promoter of salt tolerance in conditions in which transpiration is active.

RBOHF Is an Important Mediator of Ethylene-Regulated Soil-Salinity Tolerance and Na Homeostasis

As mentioned in the Introduction, ROS production is both a consequence of the damage that stress causes and a signaling component that mediates adaptive stress responses. The results here indicate that ethylene promotes soil-salinity tolerance by increasing RBOHF-dependent ROS production (at least partially via increasing RBOHF transcript levels [Figure 6]) but also possibly via posttranslational activation of RBOHF (Sirichandra et al., 2009; Yun et al., 2011; Drerup et al., 2013). ROS are versatile molecules mediating a variety of biological responses in plants, including programmed cell death, development, hormone signaling, and environmental responses (Mittler et al., 2004, 2011; Torres et al., 2005; Kwak et al., 2006; Miller et al., 2009, 2010). The levels of in planta ROS are regulated by the interplay of ROS-generating and ROS-scavenging mechanisms (Mittler et al., 2004), and the NADPH oxidases, or respiratory burst oxidase homologs (RBOHs), are the most thoroughly studied plant ROS-generating enzymes (Marino et al., 2012). The *Arabidopsis* genome encodes 10 RBOH enzymes (encoded by genes *RBOHA* to *RBOHJ*; Torres et al., 2002), and expression of individual *RBOH* genes is commonly restricted to particular cells/tissues and regulates unique biological functions (e.g., *RBOHC* regulates the growth of root hair cells; Foreman et al., 2003). Our previous study indicated that ROS produced by RBOHF in the root stele confer soil-salinity tolerance (Jiang et al., 2012). Here, we show that the soil-salinity tolerance of *eto1-15* is substantially dependent upon ethylene regulation of RBOHF-mediated ROS production in the root stele (Figures 5 to 7), indicating that RBOHF-produced ROS is an important mediator of ethylene signaling in soil-salinity tolerance. Our findings also need to be considered in light of a previous study indicating that ethylene causes stomatal closure via RBOHF-mediated ROS production (Desikan et al., 2006). Taken together, these observations indicate that RBOHF is likely to be an important mediator of ethylene signaling cascades and highlight the potential value of determining the function of the ethylene RBOHF regulon in response to other stresses and developmental cues.

As a key ROS-generating enzyme, RBOHF is an essential component of various biological processes, including induction of the phytohormone salicylic acid during the incompatible interaction (Chaouch et al., 2012), localized deposition of lignin (Lee et al., 2013), ABA and salicylic acid regulation of stomatal closing (Kwak et al., 2003; Khokon et al., 2011), jasmonic acid regulation of gene expression (Maruta et al., 2011), and regulation of sodium homeostasis in saline soil conditions (Jiang et al., 2012). Previous studies have demonstrated that the activity of RBOHF is regulated by many factors and at various levels. For example, ABA treatment and high salinity stress increase *RBOHF* transcript levels, thus increasing RBOHF-mediated ROS production (Kwak et al., 2003; Ma et al., 2011; Jiang et al., 2012). In addition, the activity of RBOHF may be positively regulated by phosphorylation (e.g., by OPEN STOMATA1; Sirichandra et al., 2009), by CBL1/9-CIPK26 complexes (Drerup et al., 2013), or by phosphatidic acid binding (Zhang et al., 2009), and alternatively may be negatively regulated by direct binding of CIPK26 (Kimura et al., 2013). Intriguingly, some of these actual or potential RBOHF regulatory factors are also involved in the regulation of salinity tolerance (e.g., ABA and phosphatidic acid; Yu et al., 2010).

When plants are grown in conditions of high soil salinity, a damaging excess of Na is delivered to the shoot via the xylem in the transpiration stream (Zhu, 2002; Munns and Tester, 2008). Our previous study indicated that RBOHF-mediated stele-specific ROS production plays an important role in regulation of root-to-shoot Na delivery and Na tolerance in transpiring plants exposed to soil salinity (Jiang et al., 2012). Here, we show that ethylene promotes soil-salinity tolerance by reducing root-to-shoot Na delivery via regulation of RBOHF-dependent ROS accumulation in root stele (Figures 5 to 7). Our observations indicate that, under high-salinity conditions, loss of ETO1 function increases RBOHF-dependent ROS accumulation in root stele (Figures 6A and 6B) and that this reduces stelar Na concentration (Figures 7A, 7C, and 7D), which in turn lowers the xylem sap Na concentration (Figure 7B), thus decreasing delivery of Na to the shoot, resulting in reduced shoot Na accumulation (Figure 3A) and enhanced salinity tolerance (Figure 1D). Lack of RBOHF function substantially suppresses the reduced shoot Na accumulation and soil-salinity tolerance characteristic of *eto1-15* (Figure 5), indicating that ethylene regulation of salinity tolerance is largely dependent upon RBOHF-mediated regulation of tissue Na homeostasis. By contrast, a recent study has indicated that ethylene regulation of salt tolerance in *Arabidopsis* is not dependent upon a decrease in tissue Na content (Yang et al., 2013). This discrepancy is likely the consequence of the experimental system employed by Yang et al. (2013) being distinct from ours: under their experimental conditions, wild-type (Columbia-0 [Col-0]) plants treated with 200 mM NaCl exhibited a higher yield biomass than plants treated with 50 mM NaCl (Yang et al., 2013). However, our results are in accordance with this in showing that maintenance of high shoot K concentration is associated with ethylene-mediated salinity tolerance, although

our genetic evidence indicates that this is independent of RBOHF function (Figures 3J and 5E; see Supplemental Figure 8 online).

Delivery of Na^+ from the soil solution to shoot is a complex process that most probably involves the concerted action of several different transporters, but the key steps are believed to be (1) Na^+ uptake into the root across the epidermis, (2) Na^+ transport across the endodermis into cells of the central vascular cylinder (stele), (3) Na^+ loading from stelar parenchyma cells into tracheary elements of the xylem, and (4) Na^+ delivery to the shoot by mass flow in the transpiration stream (Amtmann and Sanders, 1999; Tester and Davenport, 2003; Munns and Tester, 2008; Kronzucker and Britto, 2011). Here, we show that, under saline conditions, the considerable reduction in shoot Na content caused by lack of ETO1 function (Figures 3A and 5D) is associated with reduced xylem sap Na concentration (Figure 4A). Interestingly, although the initial rate of root Na^+ influx was lower in the *eto1-15* mutant following onset of salinity treatment, xylem sap Na^+ concentration was maintained at reduced levels compared with the wild type in the steady state even when bulkroot Na concentrations had reached identical values in the two lines (Figures 4A and 4C). The simplest explanation for this observation is that the reduced Na^+ concentration in the root stelar tissue of the *eto1-15* mutant (Figures 7C and 7D) leads directly to lower rates of xylem loading and hence to a lower xylem sap Na^+ concentration. Other approaches, such as quantitative cryoanalytical electron microscopy, have also indicated the close relationship between Na^+ concentration in stelar cells and xylem loading of Na^+ for delivery to the shoot in the transpiration stream (Läuchli et al., 2008). In addition, our observations indicate that ethylene-regulated Na homeostasis is largely dependent on RBOHF-mediated ROS production in root stele (Figures 5 to 7), although the exact components of the signaling process that link salinity-induced ethylene production and enhanced levels of ROS remain to be defined.

Much interest surrounds the role of specific transport systems in the uptake and accumulation of mineral ions from the soil, so an important goal for future work will be identification of specific downstream targets of the signaling cascade initiated by salinity-induced ethylene production. Manipulation of expression of the Na^+ transporters *SOS1* and *HKT1* has revealed their important roles in regulating root Na acquisition and xylem sap Na concentration (Shi et al., 2003; Møller et al., 2009), but our results show that RBOHF-produced ROS regulates Na homeostasis by a mechanism that is not dependent on changes in *SOS1* or *HKT1* transcript levels (see Supplemental Figure 9 online). However, the activity of such transporters can also be regulated by posttranscriptional mechanisms, as has been established for SOS1 (e.g., Qiu et al., 2002), so it remains possible that RBOHF-produced ROS could be involved in such posttranscriptional regulation. This is particularly possible because SOS1 interacts with the ROS signal regulators MAPK6 (Yu et al., 2010) and RCD1 (Katiyar-Agarwal et al., 2006) and because of indications that ROS may play a role in regulating Na^+/K^+ antiporter activity (Zhou et al., 2012). Root Na^+ influx, and possibly a component of xylem Na^+ loading, is also likely attributable to nonselective cation channels (Demidchik et al., 2002; Tester and Davenport, 2003; Demidchik and Maathuis, 2007; Munns and Tester, 2008). It will be interesting to determine if RBOHF-produced ROS is involved in regulation of the activity of these channels when the specific genes encoding them have been identified.

Ethylene Regulates *HAK5* Transcript Levels and Tissue K Homeostasis

Plant salinity tolerance is achieved by some degree of Na/K homeostasis, which involves restricting tissue Na accumulation and maintaining adequate K concentrations, for example, by increasing the capacity for tissue K accumulation (Shi et al., 2003; Shabala and Cuin, 2008; Horie et al., 2009; Møller et al., 2009; Ardie et al., 2010; Kronzucker and Britto, 2011). We found that, compared with wild-type plants, mutants lacking ETO1 function show constitutively elevated tissue K concentrations under control conditions and less K depletion following salinity treatment (Figures 3B, 4B, and 4D). A link between the soil-salinity tolerance conferred by lack of ETO1 function and retention of higher tissue K concentrations may be provided by the constitutively elevated levels of transcripts encoding the high-affinity K^+ transporter HAK5 observed in the *eto1-1*, *eto1-15*, and *ctr1-1* mutants (Figure 8A). *HAK5* is the only gene amongst those encoding KUP/HAK/KT family transporters whose transcript levels have consistently been found to be upregulated by K^+ deficiency (Ahn et al., 2004; Armengaud et al., 2004; Gierth et al., 2005; Rubio et al., 2008), but there have been contradictory reports of the response of *HAK5* transcript abundance to salinity. Long-term exposure of wild-type *Arabidopsis* to NaCl for 6 d (Ahn et al., 2004) or 16 d (Nieves-Cordones et al., 2010) was found to cause either no change or a reduction, respectively, in *HAK5* transcript level. However, *HAK5* transcript expression is less sensitive to downregulation by salt in the halophyte *Thellungiella halophila* than in *Arabidopsis* (Alemán et al., 2009), while transcript levels for four *HAK* genes actually increase in response to salt treatment in the halophilic plant *Mesembryanthemum crystallinum* (Su et al., 2002). Our results reveal that *HAK5* expression increases in *Arabidopsis* in response to NaCl, but only transiently, with transcript levels peaking at 6 h and returning to close to basal levels within 24 h (Figure 8). Thus, both the elevated basal levels of *HAK5* transcripts as well as the greater amplitude of the short-term response to salt treatment may contribute to the maintenance of tissue K concentrations and enhanced salinity tolerance conferred by lack of ETO1 function.

The ethylene-dependent increase in *HAK5* transcripts in response to low K^+ has been shown to be mediated by RAP2.11, an ethylene-inducible ERF transcription factor that binds to the *HAK5* promoter (Kim et al., 2012). Although ethylene regulation of *HAK5* expression is mediated via ROS production (Shin and Schachtman, 2004; Jung et al., 2009), our results show that this is independent of RBOHF function (Figure 8C). Interestingly, *HAK5* is expressed mainly in the root hairs and epidermis (Ahn et al., 2004), in which Na accumulation is particularly pronounced (Figure 7C). The response of *HAK5* transcripts to low K^+ was found to be dependent upon the function of another member of the NADPH oxidase gene family, *RBOHC* (also known as *ROOT HAIR DEFECTIVE2*; Foreman et al., 2003), as *HAK5* was not upregulated in response to K^+ deprivation in *rhd2* mutant plants (Shin and Schachtman, 2004). Thus, the elevated

K status of roots (Figure 4) and improved salinity tolerance (Figures 1 to 3) associated with lack of ETO1 function may be attributable in part to enhanced uptake of K^+ into the roots via the high-affinity transporter HAK5. ROSs have also been implicated in direct modulation of ion channel activity and could, for example, play a role in activating the K^+ outward channels responsible for K^+ loading from the stelar tissue into the xylem (Gaymard et al., 1998; Demidchik et al., 2010; Garcia-Mata et al., 2010). However, the increased xylem sap K^+ concentration conferred by lack of ETO1 function is also independent of RBOHF-mediated ROS production (see Supplemental Figure 8B online), so the higher rate of xylem loading of K^+ is probably a simple function of the elevated bulk root K^+ concentration observed in the *eto1-15* mutant plants (see Supplemental Figure 8A online).

The Mechanism of Ethylene Regulation of Soil-Salinity Tolerance

In summary, we have shown that ethylene regulates *Arabidopsis* soil-salinity tolerance via the canonical ETR1-CTR1 ethylene signaling pathway and that this regulation targets distinct aspects of organismal Na and K homeostasis. Ethylene production is increased in saline conditions, and here we show that increased ethylene reduces shoot exposure to soil Na by reducing bulk root Na influx and predominantly by reducing the Na content of the xylem sap, hence reducing delivery of Na to the shoot via the transpiration stream. The ethylene effect on xylem sap Na is dependent on the function of a specific respiratory burst oxidase, RBOHF, a function that we previously demonstrated to be necessary for soil-salinity tolerance and to be likely due to RBOHF-catalyzed ROS production (Jiang et al., 2012). In addition, we have shown that ethylene regulates K homeostasis via an RBOHF-independent mechanism that possibly involves upregulation of gene transcripts encoding the K^+ transporter HAK5.

While our observations demonstrate that ethylene modulates root-to-shoot Na delivery via regulation of RBOHF-dependent ROS accumulation in root stelar tissues (Figures 5 to 7), a previous study indicated that engineering enhanced expression of a gene (*HKT1;1*) encoding the HKT1 Na^+ transporter in root stelar cells of *Arabidopsis* can reduce root-to-shoot Na delivery, thus increasing salinity tolerance (Møller et al., 2009). These observations support the notion that regulation of Na concentration in root vasculature and xylem sap is crucial for regulating root-to-shoot Na delivery in saline conditions, suggesting the possibility that cell type–specific engineering of ethylene signaling or ROS production could increase the soil-salinity tolerance of plants. Since genes encoding components of the ethylene signaling pathway and RBOHF-related enzymes exist in the genomes of many crops (Xu et al., 2006; Wong et al., 2007; Lightfoot et al., 2008), it is possible that cell type–specific engineering of ethylene signaling or ROS production might provide a route towards increase in the soil-salinity tolerance of a wide range of crop species.

METHODS

Plant Materials

The global-*DELLA* (*gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-4*; global) *Arabidopsis thaliana* line and fast-neutron-mutagenized M2 seeds (global background) were as previously described (Belfield et al., 2012). *eto1-1*, *eto2-1*, and *etr1-3* are in the Col-0 genetic background (Guzmán and Ecker, 1990; K.L.C. Wang et al., 2004). The *pRBOHF-GUS* transgenic line and *rbohf-4* mutant (global background) were also as previously described (Jiang et al., 2012).

Soil and Hydroponic Plant Culture

Arabidopsis plants were grown on soil and in hydroponic conditions as previously described (Jiang et al., 2012).

Mutant Screen

To screen for soil-salinity–tolerant mutants, M2 seeds (global background, mutagenized via fast-neutron bombardment; Belfield et al., 2012) were first sown on soil (Erin multipurpose compost). Four-week-old seedlings were subsequently watered with 350 mM NaCl solution to soil capacity. After 3 weeks, all surviving plants were transferred to normal soil conditions and allowed to set seed. Heritability of the salt tolerance of candidate mutants was validated in subsequent generations.

Transcript Analyses

Total RNA was extracted using a Qiagen RNeasy kit. cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen). For quantitative RT-PCR, SYBR green PCR master mix (ABI) was used on an Applied Biosystems 7300 real-time PCR system. Expression levels of genes were determined from three independent biological replicates. For each biological replicate, we ran at least two technical replicates. The *CBP20* (At5g44200) gene was used as an internal positive control. Primer pairs are as described in Supplemental Table 1 online.

Xylem Sap Collection

We developed an efficient method for collection of xylem sap from hydroponically grown *Arabidopsis* plants modified from Roosens et al. (2003). In brief, rosette leaves and inflorescence stems were excised at the top of the hypocotyl. The hypocotyls of detopped plants were then gently inserted into flexible plastic tubing (Tygon Laboratory Tubing) of suitable diameter to form a tight seal round the stem (see Supplemental Figure 10 online). Sap exuding from the detopped plants by root pressure was collected for up to 6 h (see Supplemental Figure 10 online). This method enabled the collection of up to 100 μL of xylem sap from a single detopped plant.

Determination of Elemental Concentrations

For determination of Na and K concentration in root or shoot tissue, samples were harvested, oven-dried for at least 24 h at 80°C, weighed, and then digested in concentrated (69%, v/v) HNO_3 for at least 12 h for elemental extraction. Concentrations of Na and K were determined in appropriately diluted samples in an air-acetylene flame by atomic absorption spectrophotometry using a double-beam optical system with deuterium arc background correction (AAnalyst 100; Perkin-Elmer). Measurement of xylem sap Na and K concentration was performed as previously described (Jiang et al., 2012).

Determination of Chlorophyll Concentrations

Leaves from control and salt-treated plants were collected, weighed fresh, washed in distilled water, and extracted in 80% (v/v) acetone at room temperature in darkness (overnight), and the concentration of chlorophyll was determined according to Lichtenthaler (1987).

Determination of ROS Accumulation

We used NBT (Sigma-Aldrich) to detect ROS production. Four-week-old hydroponically grown plants were treated with quarter-strength Murashige and Skoog (MS; control) or quarter-strength MS plus 100 mM NaCl. Whole root systems were collected 3 d following treatment onset and stained with freshly made NBT solution (1.0 mg mL^{-1} NBT in 10 mM NaN_3 and 10 mM phosphate buffer, pH 7.5) for 1 h. NBT-stained samples were then used to quantify the generation of formazan (Myouga et al., 2008) and to analyze the intensity of NBT staining in root vasculature. For histological sectioning of the NBT-stained samples, the samples were fixed in 4% paraformaldehyde in PBS at pH 7.0. After 24 h, samples were rinsed in PBS and dehydrated in an ethanol series of 30, 50, 70, and 85% (60 min each), then in 95% ethanol (0.1% eosin; overnight), and finally in 100% ethanol (0.1% eosin; 2 × 60 min). The samples were then processed in a Histoclear series of 25, 50, 75, and 100% (60 min each) and subsequently in Histoclear containing increasing volumes of wax chips (0.25, 0.5, and 0.75 volume) at 60°C for 2 h each. The samples were then placed in freshly melted wax overnight at 60°C. Finally, samples were placed in molds and sectioned with a Leica RM 2135 rotary microtome (10 μm). Sections were examined using a Leica DMRB light microscope, and photographs were obtained with a charge-coupled device digital camera (Micropublisher 5.0 RTV).

Visualization of Cellular Na^+ Concentration

Four-week-old hydroponically grown seedlings were treated with 100 mM NaCl in liquid media, following which whole root systems were incubated for 8 h in a Petri dish in the same media supplemented with 2.5 μM CoroNa Green-AM (Invitrogen). Propidium iodide (2.5 μg mL^{-1}; Invitrogen) was added as a counterstain for the plant cell wall prior to confocal microscopy. Z stacks of fluorescent images were recorded using a Zeiss LSM 510 confocal system with fixed settings of laser power, pinhole, and photomultiplier gain for all samples. Excitation and detection wavelengths were as previously described (Oh et al., 2010). The fluorescence intensity was calculated using Zeiss LSM 510 software.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: *ETO1*, At3g51770; *ETR1*, At1g66340; *RBOHF*, At1g64060; *SOS1*, At2g01980; *HKT1*, At4g10310; and *HAK5*, At4g13420.

Supplemental Data

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

Supplemental Figure 1. Identification of a Soil-Salinity–Tolerant Mutant.

Supplemental Figure 2. The *sst1-1* Mutation Has No Detectable Effect on Osmotic Stress Tolerance.

Supplemental Figure 3. *sst1-1* Does Not Confer in vitro Salinity Tolerance.

Supplemental Figure 4. The *sst1-1* Mutant Allele Carries a Mutant *ETO1* Promoter.

Supplemental Figure 5. The *eto2-1* Mutant Allele Confers Soil-salinity Tolerance.

Supplemental Figure 6. Lack of RBOHF Function Suppresses the Soil-Salinity Tolerance Conferred by *eto1-15*.

Supplemental Figure 7. The Effect of Salinity Treatment on NBT-Visualized ROS Levels in Mature Root Stele (Genotypes as Indicated).

Supplemental Figure 8. ETO1 Regulates Root and Xylem Sap K Concentration via an RBOHF-Independent Mechanism.

Supplemental Figure 9. The Effect of Salinity Treatment on At *SOS1*, At *HKT1*, and At *AKT1* Transcript Levels.

Supplemental Figure 10. Xylem Sap Collection from a Detopped Hydroponically Grown *Arabidopsis* Plant.

Supplemental Table 1. List of Primers Used in RT-PCR Experiments (Figures 2H, 6D and 6E and 8; Supplemental Figure 9).

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AUTHOR CONTRIBUTIONS

C.J., J.A.C.S., and N.P.H. designed the research. C.J., E.J.B., and J.A.C.S. performed the experiments. C.J., E.J.B., Y.C., J.A.C.S., and N.P.H. contributed to writing the article.

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