Effect of salt and osmotic stress upon expression of the ethylene receptor ETR1 in *Arabidopsis thaliana*

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Abstract In hormone perception, varying the concentrations of hormone, receptor, or downstream signaling elements can modulate signal transduction. Previous research has demonstrated that ethylene biosynthesis in plants is regulated by abiotic factors. Here we report that exposure of *Arabidopsis* plants to NaCl reduced expression of the ethylene receptor ETR1. The change in gene expression was reflected at the protein level based on immunoblot analysis. Further analysis supports a general effect of osmotic stress upon the expression level of ETR1. The reduction in ETR1 levels should cause increased sensitivity of the plant to ethylene. These results suggest that plant responses to abiotic stress are modulated by changes in the expression level of ethylene receptors.

Key words: Ethylene signal transduction; Receptor; ETR1; Salt stress; Osmotic stress; *Arabidopsis thaliana*

1. Introduction

The gaseous hormone ethylene is a key regulator of plant growth and development [1,2]. Ethylene is also involved in plant responses to biotic stresses such as pathogen attack and abiotic stresses such as freezing, wounding, and drought [1–5]. Extensive research has demonstrated that ethylene biosynthesis is regulated by numerous factors including biotic and abiotic stresses [1,2]. For example, both hypoosmotic and hyperosmotic shocks induce 1-aminocyclopropane-1-carboxylate synthase, the key enzyme regulating ethylene biosynthesis [6].

In *Arabidopsis*, ethylene is perceived by a five-member family of receptors: ETR1, ETR2, ERS1, ERS2, and EIN4 [7,8]. The receptors contain N-terminal transmembrane domains that encompass the ethylene-binding site, ethylene binding having been demonstrated for ETR1 and ERS1 [9–11]. The ethylene receptor ETR1 localizes to the endoplasmic reticulum of *Arabidopsis* [12]. In the C-terminal half, the receptors contain regions with homology to histidine kinases and in some cases the receiver domains of response regulators [7,8]. These are signaling elements originally identified as parts of bacterial two-component systems that are now known to also exist in plants, fungi, and slime molds [13–15].

Signal transduction can be regulated by changes in the levels of hormone, receptor, or downstream signaling elements. Although various factors have been demonstrated to regulate ethylene levels in the plant [1,2], only limited information is available on the regulation of ethylene receptor levels. Interestingly, one factor that affects the expression of ethylene receptors is ethylene itself, which induces the expression of *ETR2*, *ERS1*, and *ERS2*, but not of *ETR1* and *EIN4* [16]. We show here that expression of the ethylene receptor *ETR1* is downregulated by salt and osmotic stress at the transcript and protein levels. This decrease in receptor levels should cause increased sensitivity of the plant to ethylene. Thus, abiotic stresses, in addition to regulating ethylene signal transduction by modulating hormone levels, may also do so by modulating receptor levels.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana (ecotype Columbia) seeds were stratified for 2 days at 4°C. Seeds were then placed on sterile filter paper on top of 1% (w/v) agar plates of half-strength Murashige and Skoog (MS) basal medium (pH 5.65–5.85) with Gamborg's vitamins (Sigma). Seedlings were grown for 7 days under continuous illumination with 60 μE/m^2/s fluorescent light at 22°C. The filter paper with seedlings was then transferred to 100×20 mm plates containing 10 ml liquid MS medium with NaCl, $NaNO_3$, KCl, mannitol, or sorbitol at concentrations and for incubation times as indicated.

2.2. RNA isolation and Northern blot analysis

Total RNA was extracted from 5 g *Arabidopsis* seedlings treated with 200 mM NaCl for 0, 2, 24, 36, 48 and 72 h using Invitrogen Plant RNA Purification Reagent according to manufacturer's instructions. mRNA was isolated using the Promega PolyATract mRNA isolation system. For Northern blot analysis, RNA was separated on 1% (w/v) agarose gels containing glyoxal and dimethylsulfoxide using the Ambion NorthernMax-Gly kit. RNA was transferred to nylon membrane by the capillary method and fixed by ultraviolet (UV) crosslinking. Hybridizations were performed using buffers supplied with the NorthernMax-Gly kit. Single-stranded DNA antisense probes were made using primers designed to anneal at the 3′ end of the selected genes. Radiolabeled probes were made and the blot stripped between hybridizations by using the Ambion Strip-EZ polymerase chain reaction (PCR) kit. Radioactivity was phosphor-imaged and quantitated with a Bio-Rad Molecular Imager FX using the accompanying Quantity One software.

2.3. Protein isolation and immunoblot analysis

For isolation of *Arabidopsis* membranes, plant material was homogenized at 4°C in extraction buffer (30 mM Tris, pH 8.5, 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 20% (v/v) glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin as protease inhibitors. The homogenate was strained through Miracloth (Calbio-

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chem) and centrifuged at 8000×*g* for 15 min. The supernatant was centrifuged at 100 000×*g* for 30 min, and the resulting membrane pellet resuspended in 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol with protease inhibitors. Protein concentration was determined by a modification of the Lowry assay [17] in which samples were treated with 0.4% (w/v) sodium deoxycholate [18]. Bovine serum albumin was used as a standard.

For immunoblot analysis, membranes were mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer and incubated at 37°C for 1 h. Proteins were fractionated by SDS–PAGE using 8% (w/v) polyacrylamide gels [19]. After electrophoresis, proteins were either stained with Coomassie blue or electrotransferred to Immobilon nylon membrane (Millipore). Immunoblotting was performed using anti-ETR1 (401–738) or anti-(H^+-adenosine triphosphatase (ATPase)) polyclonal antibodies. Anti-ETR1 (401–738) was prepared against a glutathione S-transferase (GST) fusion protein with amino acids 401–738 of ETR1 [20]. The anti-(H^+-ATPase) antibody [21] was provided by Dr. M. Sussman (University of Wisconsin, Madison, WI, USA). Immunodecorated proteins were visualized and quantified as described [22].

3. Results

3.1. *mRNA expression of* ETR1 *is repressed by NaCl treatment*

To examine the expression of the ethylene receptor *ETR1* in response to abiotic stress, 7-day-old green seedlings were exposed to medium containing 200 mM NaCl, a level of salt that has been demonstrated to lead to stress responses and changes in gene expression in *Arabidopsis* [23]. The mRNA level of *ETR1* was analyzed in seedlings following treatment with NaCl for 2, 24, 36, 48, and 72 h (Fig. 1). No change in *ETR1* expression was observed following the 2-h NaCl treatment. With the 24-h treatment, however, the expression level of *ETR1* was reduced to 30% of the control level. *ETR1* expression remained at this reduced level following the longer term treatments with NaCl.

3.2. *Repression of ETR1 mRNA expression by NaCl treatment is reflected at the protein level*

To examine changes at the protein level following NaCl treatment, membranes were isolated from green seedlings exposed to 200 mM NaCl for various lengths of time and prepared for immunoblot analysis (Fig. 2A). The protein levels of ETR1 did not show any appreciable change through 36 h of NaCl treatment. However, after 48-h treatment, the level of ETR1 was reduced to approximately half of its original level. After 72-h treatment, the level of ETR1 was reduced still further. The loss of membrane proteins was not a general feature of the salt stress response in *Arabidopsis*. When analyzed by SDS–PAGE, many Coomassie blue-stained membrane proteins showed little change in abundance over the entire 72 h of salt treatment (Fig. 2A). In addition, immunoblot analysis revealed that the plasma membrane H^+-ATPase showed no significant change in abundance after 72 h of salt treatment (Fig. 2A). Examination of the sensitivity of ETR1 expression to varying levels of NaCl (Fig. 2B) indicated that the effects of NaCl could be distinguished after 72-h treatment with 100–150 mM NaCl.

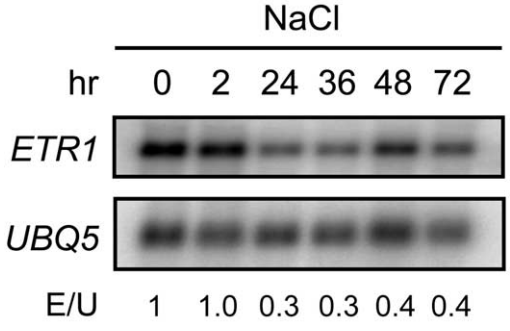

Fig. 1. Expression of *ETR1* in response to NaCl treatment. mRNA was isolated from 7-day-old *Arabidopsis* seedlings following 2, 24, 36, 48, or 72 h of treatment with MS medium containing 200 mM NaCl. Control plants (0 h NaCl) were grown on MS medium lacking NaCl for 72 h. The Northern blot was probed with an *ETR1* probe and with an *UBQ5* probe as an internal control. The numbers below each lane represent the level of *ETR1* expression after normalization to the expression level of *UBQ5*.

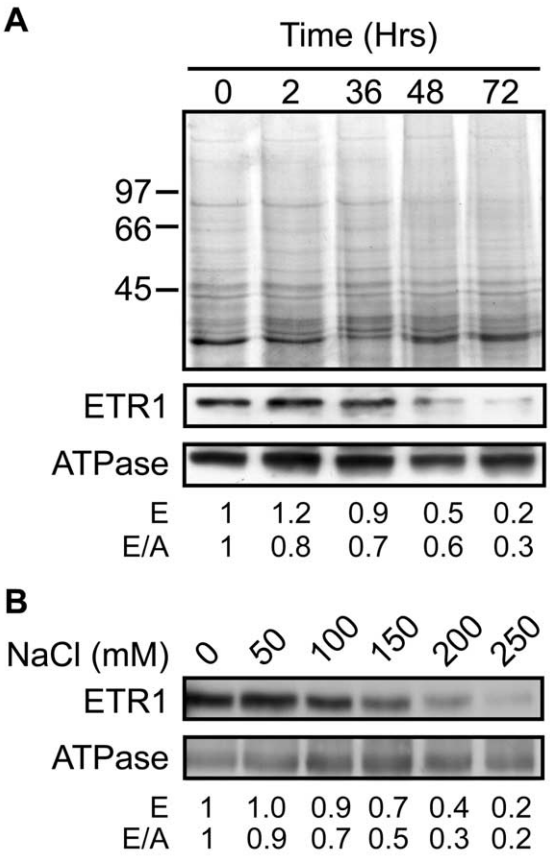

Fig. 2. Sensitivity of ETR1 expression to treatment with NaCl. A: Time course for the effect of salt stress upon protein levels of ETR1. Membrane proteins were isolated from 7-day-old seedlings following 0, 2, 36, 48, or 72 h of treatment with 200 mM NaCl. SDS–PAGE gel profiles of 50 μg membrane proteins are shown from each time point. Migration positions of molecular weight markers are indicated in kDa. Specific membrane proteins were visualized by immunoblot using antibodies against ETR1 and H^+-ATPase. The expression level of ETR1 was quantified densitometrically (*E*) and also normalized against immunologically determined levels of the H^+-ATPase (*E*/*A*) as an internal control. B: Dose dependence of ETR1 expression of plants treated for 72 h with different concentrations of NaCl.

3.3. *Expression of ETR1 is repressed by treatment with osmotica*

To determine if the effects of NaCl treatment were due to ion toxicity and/or osmotic stress [24,25], the effects of additional salts as well as of mannitol and sorbitol upon expres-

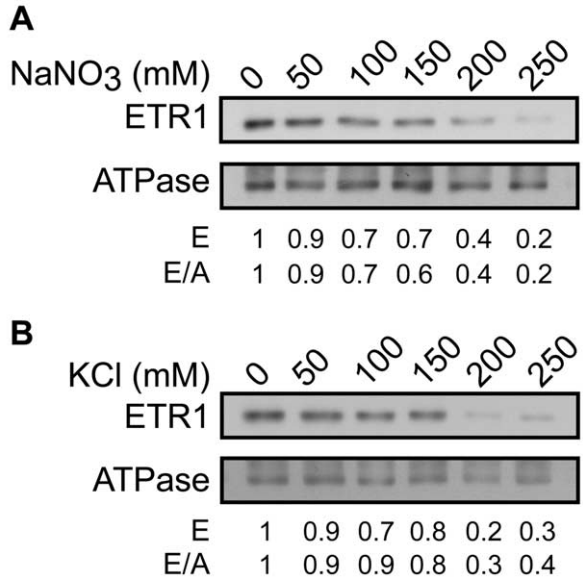

Fig. 3. Sensitivity of ETR1 expression to treatment with various salts. 7-day-old seedlings were treated with different concentrations of $NaNO_3$ (A), or KCl (B) for 72 h. Expression levels of ETR1 were quantified densitometrically (*E*) and also normalized against levels of the H^+-ATPase (*E*/*A*) as an internal control.

sion of ETR1 were examined. Wild-type seedlings were treated with two ionic osmotica, $NaNO_3$ and KCl, over the same concentration range previously examined with NaCl. Immunoblot analysis demonstrated that the protein level of ETR1 exhibited a similar trend of decrease after treatment with $NaNO_3$ or KCl for 72 h (Fig. 3). In both cases, the level of ETR1 exhibited an obvious decrease when the salt concentration reached 100–150 mM, similar to the results observed with NaCl treatment.

To investigate the expression of ETR1 following osmotic stress, seedlings were treated with mannitol and sorbitol for 72 h, and then examined for their level of ETR1 expression (Fig. 4). Mannitol and sorbitol are non-ionic osmotica that are commonly used to examine the effects of osmotic stress on plants [24,26]. The concentrations of mannitol and sorbitol covered a similar range of osmotic pressures to those analyzed with the salt treatments (e.g. growth media with 250 mM NaCl had an osmotic pressure of 650 mOsm; growth media with 600 mM mannitol had an osmotic pressure of 800 mOsm). The ETR1 protein level decreased as the concentration of the osmoticum increased, consistent with an effect of osmotic stress upon the expression of ETR1. However, the reduction in ETR1 protein levels was not as pronounced as that observed with the ionic osmotica. These results suggest that a reduction in ETR1 protein levels may result from both ionic and osmotic stresses upon the plant.

4. Discussion

Ethylene receptors serve as negative regulators of the ethylene signal transduction pathway [27]. Thus a decrease in receptor levels is predicted to sensitize the plant such that it responds to lower levels of ethylene than usual. If the receptors are reduced sufficiently in number, then ethylene responses are predicted to be induced even without an ethylene stimulus. These predictions have been confirmed experimentally. Loss-of-function mutations have been isolated in members of the ethylene receptor family in *Arabidopsis* [22,27,28]. A single loss-of-function mutation in the ethylene receptor *ETR1* results in a plant with increased sensitivity to ethylene, such that it demonstrates a partial ethylene growth response to the basal level of endogenous ethylene [29]. Plants containing a combination of ethylene receptor mutations display a constitutive ethylene response phenotype in the absence of ethylene [22,27,28]. Similar results have been also obtained from tomato by taking an antisense-based approach to decrease the levels of ethylene receptors [30]. Reduced expression levels of a single ethylene receptor, *LeETR4*, resulted in increased ethylene sensitivity and the induction of such ethylene responses as fruit ripening, flower senescence, and leaf epinasty.

As reported here, the ethylene receptor *ETR1* showed reduced expression levels when *Arabidopsis* seedlings were subjected to salt and osmotic stress. Based on Northern blot analysis, the level of reduction was about 3-fold following a 24-h NaCl treatment, with transcript remaining at this level following treatment for longer periods of time. An examination of ETR1 protein levels indicated that the reduction at the message level was reflected at the protein level but with a 24-h lag. This lag could arise from a requirement for the ETR1 message to fall below a threshold level before protein levels are affected or be due to a slow rate of turnover for the ETR1 protein.

The reduction in ETR1 levels following salt and osmotic stress should make the plant more sensitive to ethylene. If other receptors are similarly affected, these abiotic stresses could lead to activation of ethylene responses independent of the presence of ethylene. The discovery that expression of ETR1 decreases during salt stress is therefore consistent with the activation of ethylene responses constituting a component of the plant stress response. Given the lag observed between the initial exposure of plants to salt stress and the reduction in

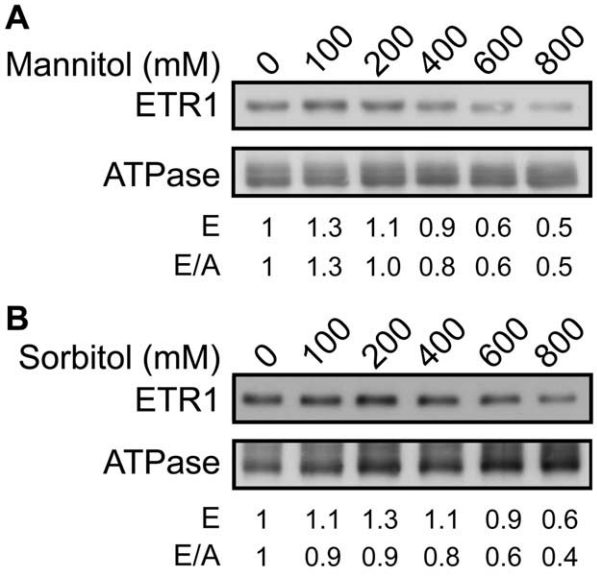

Fig. 4. Sensitivity of ETR1 expression to treatment with non-ionic osmotica. 7-day-old seedlings were treated with different concentrations of mannitol (A), or sorbitol (B) for 72 h. Expression levels of ETR1 were quantified densitometrically (*E*) and also normalized against levels of the H^+-ATPase (*E*/*A*) as an internal control.

ETR1 at both the transcript and protein levels, activation of ethylene signal transduction by this mechanism could be a secondary but potentially important response. According to this hypothesis, the rapid response of plants to abiotic stress would involve increased ethylene production [6], but maintenance of an extended stress response would be facilitated by a reduction in ethylene receptor levels.

Extensive research has demonstrated that ethylene biosynthesis is regulated by environmental stresses and other plant hormones [2,31], and that levels of ethylene vary in different plant tissues and at different developmental stages [2]. More recently, the expression of several ethylene receptors has been demonstrated to increase in response to ethylene and pathogen infection, although ETR1 is not one of the receptors whose expression is affected by these stimuli [16,32]. The results reported here demonstrate decreased expression of the ethylene receptor ETR1 in response to abiotic stress. Taken together, these results indicate that levels of the ethylene receptors are dynamically regulated thereby providing another means by which ethylene signal transduction can be activated and repressed.

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