An AP2 Domain-Containing Gene, ESE1, Targeted by the Ethylene Signaling Component EIN3 Is Important for the Salt Response in Arabidopsis

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Salt Response in Arabidopsis

Ethylene Signaling Component EIN3 Is Important for the

Plants usually experience many biotic and abiotic stresses, including pathogen attack, salt, drought, cold, and wounding. High salinity is a major abiotic stress responsible for substantial losses of crop production (Xiong et al., 2002b; Zhu, 2003; Chinnsamy et al., 2006). Through decades of studies, several signaling pathways have been proved to be associated with the salt response, such as the salt-overly-sensitive pathway that regulates ion homeostasis, the inducer of CBF expression/dehydration-responsive element-binding (ICE-DREB) pathway that controls the expression of DRE/CRT (for dehydration response element/C-repeat)-containing genes, and the mitogen-activated protein kinase cascade that regulates the generation of osmolytes and antioxidants (Xiong et al., 2002a, 2002b; Seki et al., 2003; Zhu, 2003; Mehlmer et al., 2010). Moreover, other pathways, such as abscisic acid and ethylene signaling pathways, have been revealed to be involved in the response to salt stress (Achard et al., 2006; Kempa et al., 2006; Yang et al., 2010).

Ethylene is a simple and very important gaseous hydrocarbon plant hormone, and its signaling pathway has been proposed to include the ethylene receptors CTR1, EIN2, and ETHYLENE INSENSITIVE3 (EIN3) and other components in Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa), and other species (Bleecker and Kende, 2000; Wang et al., 2002; Chang and Bleecker, 2004; Guo and Ecker, 2004; Chen et al., 2005; Yin et al., 2010). It is reported that ethylene participates in modulating the pathogen defense response, photomorphogenesis, and seedling growth in the etiolated condition (Penmetsa and Cook, 1997; Solano and Ecker, 1998; Zhong et al., 2009). In such modulations, transcriptional control conferred by the transcription factor EIN3 is pivotal. For example, EIN3 transcriptionally activates the expression of Ethylene Response Factor1 (ERF1), SID2, EBF2, FLS2, PORA, and PORB, some of which have the core sequence 5’-ATGTA-3’ within their promoters (Solano et al., 1998; Konishi and Yanagisawa, 2008; Chen et al., 2009; Zhong et al., 2009; Boutrot et al., 2010). The negative
regulation of EBF1 and EBF2 in EIN3 accumulation (Guo and Ecker, 2003; Potuschak et al., 2003) is transcriptionally modulated by EIN3 (Konishi and Yanagisawa, 2008). The transcriptional activation of EIN3 on ERF1 confers the regulation of ethylene in the pathogen defense response (Solano and Ecker, 1998; Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). In addition, EIN3-like (EIL) and ERF proteins as a transcriptional complex coordinately regulate the expression of ripening-related genes in the ripening process of kiwifruit (Actinidia deliciosa; Yin et al., 2010).

Moreover, ethylene has also been regarded as an abiotic stress hormone. This regulation is also mediated by ethylene signaling (Yamaguchi-Shinozaki and Shinozaki, 2006; Zhang and Huang, 2010; Zhu et al., 2010). From 122 putative ERF genes, we screened 31 putative ethylene- and salt-responsive ERF genes, we determined that three ERF genes (At3g23220, At2g25820, and At5g25190) were simultaneously induced by 1-aminocyclopropane-1-carboxylic acid (ACC) and NaCl. Thus, we named these genes as ethylene- and salt-inducible ERF genes (ESE1–ESE3, respectively). One ESE gene, ESE1 (At3g23220), functioning downstream of EIN3 as a transcriptional complex in the salt response, is further investigated in this report. We first found that ESE1 is a downstream target of the ethylene signaling component EIN3. Moreover, ESE1 is an important regulator for the salt response during seed germination and seedling development through the modulation of the EIN3–ESE1 transcriptional complex.

RESULTS

Genome-Wide Analyses Reveal Ethylene- and Salt-Inducible ERF Genes

Increasing evidence indicates that ERF proteins regulate a variety of biotic and abiotic stress responses and also plant development (Gilmour et al., 2000; Park et al., 2001; Fowler and Thomashow, 2002; Agarwal et al., 2006; Chinnusamy et al., 2007; Jung et al., 2007; Zhang et al., 2011). Although many genes have been reported as involved in the salt response, a genome-wide analysis of ERF genes in the salt response has not been reported. To address the potential functions of ERF genes in such processes, we consulted a large number of microarray data (Genvestigator). From 122 putative ERF genes, we screened 31 candidate genes that were induced by salt, ACC, or ethylene but reduced by inhibitors of ethylene biosynthesis, aminoethoxyvinylglycine (AVG), or ethylene action, AgNO₃ (Supplemental Table S1). After confirmation using real-time quantitative PCR (qPCR) amplification in wild-type Col-0 without (as a control) or with 10 μM ACC and 100 mM NaCl, we found that the transcripts of At5g25190, At1g21910, At1g44830, At2g25820, At5g44210, and At3g23220 increased more than 2-fold after ACC induction (Fig. 1A), while those of At5g25190, At2g25820, At3g61630, At5g47230, and At3g23220 were induced more than 2-fold after salt treatment (Fig. 1B), indicating that At5g23220, At2g25820, and At5g25190 were simultaneously induced by ACC and NaCl. These three ethylene- and salt-inducible ERF genes are named ESE1 (At3g23220), ESE2 (At2g25820), and ESE3 (At5g25190).

Expression of the ERF Gene ESE1 Is Transcriptionally Modulated by EIN3/EIL1

Increasing amounts of research indicate that EIN3 and ERF proteins play pivotal roles during salt stress (Achard et al., 2006; Cao et al., 2007; Wang et al., 2007; Wu et al., 2008; Yoo et al., 2008; Zhu et al., 2010), so identification of the connection between the two transcription factors in this process is important. After
confirmation using qPCR amplification, we found that the transcripts of \textit{ERF1} (as a positive control), \textit{ESE1}, \textit{ESE2}, and \textit{ESE3} were suppressed in \textit{ein3-1} and/or \textit{eil1-3} mutants compared with those in wild-type Col-0 under normal growth conditions (Supplemental Fig. S1A), suggesting that the ERF genes might be transcriptionally regulated by EIN3. For the identification of EIN3 with sequence $5'\text{ATGTA-3'}$ (Solano et al., 1998; Chen et al., 2009), we then searched the core sequence in the promoters of \textit{ERF1}, \textit{ESE1}, \textit{ESE2}, and \textit{ESE3} (Supplemental Fig. S1B), showing that all the putative target genes of EIN3 contain two to six core sequences in their promoters. To confirm whether the induction of \textit{ESE1} by salt was dependent on the regulation of EIN3, we examined the \textit{ESE1} transcripts in Col-0, \textit{ein2}, \textit{ein3-1}, \textit{eil1-3}, \textit{ein3 eil1}, and transgenic lines overexpressing \textit{EIN3} (EIN3ox). Under normal growth conditions, the expression of \textit{ESE1} significantly decreased in \textit{ein3-1} and \textit{eil1-3} and was almost suppressed in \textit{ein2} and the \textit{ein3 eil1} double mutant, but it increased more than 6- to 8-fold in EIN3ox lines, compared with that in Col-0. Under 100 mM NaCl treatment, the expression of \textit{ESE1} was greatly repressed in the \textit{ein3 eil1} double mutant. Although salt induction greatly increased the transcripts of \textit{ESE1} in different genotypes, statistical analysis showed that the expression of \textit{ESE1} was significantly repressed in \textit{ein2}, \textit{ein3-1}, and \textit{eil1-3} but enhanced in EIN3ox lines, compared with Col-0 under salt treatment (Fig. 2A). Moreover, treatment with inhibitors of AVG and AgNO$_3$ reduced the expression of \textit{ESE1}, while ethylene overproduction mutants \textit{eto1}, \textit{eto2}, and \textit{eto3} enhanced the expression of \textit{ESE1} (Fig. 2B), indicating that \textit{ESE1} is a salt-inducible and ethylene-modulated gene downstream of EIN3/EIL1.

\textit{ESE1} encodes a member of the ERF proteins that contains a single conserved AP2/ERF domain, extending from amino acid 13 to 71. In addition, MEGA 4.0 analyses of \textit{ESE1} to \textit{ESE3}, \textit{ERF1}, and 12 salt-related AP2/ERF proteins from different plant species indicated that the full-length amino acid sequence of \textit{ESE1} is very close to \textit{ERF1} but shares less than 23% identity with other reported ERF proteins (Supplemental Figure 1).

Figure 1. Identification of the putative ethylene- and salt-inducible ERF genes. A, Expression of ERF genes in Arabidopsis Col-0 with or without $10 \mu M$ ACC treatment. B, Expression of ERF genes in Arabidopsis Col-0 with or without 100 mM NaCl treatment. Transcript levels of the ERF genes are indicated relative to the level of the control in Col-0 taken as 1, referring to the transcripts of \textit{TUB4} in the same sample. Error bars (so) are based on three independent experiments.

Figure 2. The expression of \textit{ESE1} is modulated by ethylene signaling components. A, Expression of \textit{ESE1} in Arabidopsis Col-0, \textit{ein2}, \textit{ein3-1}, \textit{eil1-3}, \textit{ein3 eil1-3}, and EIN3ox lines with or without 100 mM NaCl treatment. \textit{P} values (each genotype versus Col-0 under salt induction) were determined by two-tailed Student’s \textit{t} test assuming equal variance (* \textit{P} < 0.05). B, Expression of \textit{ESE1} in Col-0, \textit{eto1}, \textit{eto2}, and \textit{eto3}, and treatment with 10 \mu M ACC and inhibitors (5 \mu M each) of ethylene perception and biosynthesis in Col-0, respectively. Transcripts of the \textit{ESE1} gene are indicated relative to the level of the control in Col-0 (taken as 1), referring to the transcripts of \textit{TUB4} in the same sample. Error bars (so) are based on three independent experiments.
Fig. S2A), which contain a conserved AP2/ERF domain (Supplemental Fig. S2B), suggesting that ESE1 is a novel functionally unknown ERF protein. In addition, ESE1 activated the transcription of HIS3 and LacZ reporter genes in a yeast system (Supplemental Fig. S2C), indicating that ESE1 is a transcriptional activator.

The Promoter of ESE1 Is Directly Targeted by EIN3

To investigate whether and how the expression of ESE1 is regulated by EIN3, we first performed a yeast one-hybrid assay. The generation of reporters with ESE1 promoters of different length, pD1, pD2, pD3, and pD4, and effectors of full length (pEIN3-F), the N-terminal area (pEIN3-N), and the C-terminal area (pEIN3-C) of EIN3 were described using a yeast one-hybrid assay (Fig. 3A). After the effector pEIN3-F was transformed into the yeast competent cells carrying the pD1/D2/D3/D4-lacZ reporter vectors, we found that EIN3 significantly activated the activity of β-galactosidase in the pD4 reporter but not in the other reporters (Fig. 3A). Similarly, we verified that
the effector of pEIN3-N activated the expression of \( \beta \)-galactosidase in the pD4 reporter (Fig. 3B), indicating that the N-terminal area of the EIN3 protein interacts with the D4 region (−452 to +24 bp) of the ESE1 promoter in yeast.

We then detected the binding of EIN3 to the ESE1 promoter in vivo using a chromatin immunoprecipitation (ChIP) assay. Because EIN3 protein exists in plants at an extremely low level (Chen et al., 2009), our attempts to test endogenous EIN3-ESE1 promoter binding were unsuccessful, although ACC was used to stabilize the protein. We then used EIN3ox lines for our ChIP assay to demonstrate the transcriptional cascade in plants. We used the ethylene precursor ACC (Potuschak et al., 2003) to treat seedlings of wild-type Col-0, EIN3ox-1, and EIN3ox-4 that contained a 6×MYC tag. Using DNAs immunoprecipitated by myc antibody as a template, combined with the use of a promoter of TUB4 as the loading control, qPCR amplification revealed the increased signals of the ESE1 promoter D4P2 region (−344 to −194 bp) and EBF2 (as a positive control; −296 to −122 bp) but not in the ESE1 promoter D4P1 and D4P3 regions in the two EIN3ox lines. Our analysis showed that both EIN3ox lines showed similar results; only the qPCR data from EIN3ox-4 are presented (Fig. 3C). Because the fragment D4P2 contains the core sequence (Supplemental Fig. S1B), it is possible that EIN3 interacts with a fragment of D4P2 to activate the expression of the salt-inducible gene ESE1.

The tobacco (Nicotiana tabacum) transient cotransformational system was used to further support EIN3 binding to the promoter of ESE1 in vivo. Under the activation of EIN3, the GUS activity significantly increased in the reporters in which the EIN3-binding core sequence was present (e.g. ESE1-p1, ESE1-p2, ESE1-p3, ESE1-p4, ESE1-p7, and EBF2-p), while GUS had low activity in reporters that did not contain an EIN3-binding core sequence (e.g. ESE1-p5 and ESE1-p6). Moreover, the GUS activity was greatly reduced in the reporters, so that the fragment between −336 and −194 bp was deleted in ESE1-p8 and ESE1-p9 (Fig. 4), supporting the ChIP result that the D4P2 fragment in the ESE1 promoter was required for interaction with EIN3.

Next, we applied an electrophoretic mobility shift assay (EMSA) to further delimit the EIN3 target site in the D4P2 subfragment. D4P2 was fragmented into five segments: D4P2-1, D4P2-2, D4P2-3, D4P2-4, and D4P2-5 (Fig. 5A). Using these segments as probes, we revealed that EIN3-N but not EIN3-C and glutathione S-transferase (GST) only (as negative controls) retarded the movement of EBF2 (as a positive control; −254 to −220 bp; Konishi and Yanagisawa, 2008) and D4P2-3 probes (Fig. 5A). In the region pD4P2-3, there is a core sequence (−266 to −262 bp) of EIN3-binding sequence as for the ERF1 and SID2 promoters, 5′-ATGTA-3′ (Solano et al., 1998; Chen et al., 2009). Competition and mutation assays were performed to further examine the specific binding. Our assay showed that D4P2-3M almost inhibited the binding activity, indicating that the core sequence played a crucial role (Fig. 5B). The binding activity of EIN3-N was completely inhibited with an excess of unlabeled D4P2-3 (Fig. 5C) but not with an excess of unlabeled pD4P2-3M (data not shown), indicating that the N-terminal area of EIN3 physically interacted with the D4P2-3 region (−288 to −246 bp) of the ESE1 promoter. These results suggest...
that the 5′-ATGTA-3′ core sequence is essential for EIN3 binding to the promoter of ESE1.

Either Overexpression of EIN3 or ESE1 Enhances the Expression of Salt-Related Genes and the Salt Response

Although the above assays revealed the interaction of EIN3 with the promoter of a functionally unknown gene, we are interested in whether this novel ESE1 gene functions in the salt response. To test this hypothesis, we first tried to identify T-DNA insertion mutants from the Arabidopsis Biological Resource Center (ABRC). From the available mutants, we confirmed that a T-DNA insertion (SALK_128736) in the ESE1 promoter region resulted in very low expression of ESE1 in the ese1 mutant compared with wild-type Col-0 (Fig. 6, A and B). A complementation assay with the 35S promoter-driven ESE1 expression in this mutant rescued the tolerance to salt during seed germination and seedling growth in response to salt stress (data not shown). We also tried to generate RNA interference ESE1 plants; however, we failed to get specific RNA interference transgenic lines because ESE1 belongs to the ERF family that contains a conserved ERF domain (Supplemental Fig. S2, A and B). Then, the overexpression of ESE1 in Col-0 (ESE1ox) and in the ein3-1 background (ESE1ox in ein3-1, EIN3ox lines, ein3-1, ese1, and overexpressor ERF1ox (CS6142) was used in the following salt response. Under normal growth conditions, the germination in mutants (ein3-1 and ese1) and transgenic plants (EIN3ox, ESE1ox, and ESE1ox in ein3-1) showed no obvious differences from the wild-type Col-0. But after 100 mM NaCl treatment, seed germination in the EIN3ox and ESE1ox lines was evidently higher than in Col-0. Importantly, seed germination in ESE1ox in ein3-1 was significantly higher than in ein3-1, indicating that ESE1 genetically functions downstream of EIN3. Statistical analysis revealed that the germination in ese1 and ein3-1 was significantly more sensitive to salt than for Col-0 (Fig. 6C).

We then examined the seedling response under NaCl treatment. We did not observe obvious differences among mutants, transgenic seedlings, and wild-type Col-0 under normal growth conditions. However, seedling growth in ein3-1, ein3-1 eil1-3, and ese1 was obviously retarded compared with that in Col-0 after salt stress of 100 mM NaCl for 7 d, showing shorter root, smaller leaf with shorter petiole (i.e. backward growth of the leaf blade), and slower leaf growth phenotypes, while seedlings of the transgenic plants EIN3ox, ESE1ox, and ESE1ox in ein3-1 only displayed the slower leaf growth phenotype (Fig. 6D). Further analyses showed that the shoot weight in mutants ein3-1, ein3-1 eil1-3, and ese1 was significantly less than in Col-0, whereas EIN3ox, ESE1ox, and ESE1ox in ein3 retained the fresh weight of shoots after salt treatment (Fig. 6E), demonstrating that EIN3 and ESE1 as a transcriptional cascade is important for the salt response. Moreover, the double mutant ein3-1 eil1-3

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**Figure 5.** The ESE1 promoter physically interacts with the EIN3 protein in vitro. A, Interaction of EIN3 with the ESE1 promoter. The top panel shows the positions of the probes used in EMSA. Numbers indicate the positions of the nucleotides at the 5′ and 3′ ends of each probe relative to the translation start site. The bottom panel displays the detection of probes after reaction with the N-terminal or C-terminal area of EIN3 and the GST protein (as a negative control). B, Interaction confirmation using mutated probe D4P2-3M after reaction with the N-terminal area of EIN3 or the GST protein (as a negative control). The sequences of D4P2-3 and D4-P2-3M are described in “Materials and Methods.” C, Competition assay of unlabeled probe D4P2-3 after reaction with the N-terminal area of EIN3 in the presence of labeled probe D4P2-3.
displayed more severe development retardation after the salt treatment (Fig. 6, D and E), supporting that EIN3/EIL1 genes function in the salt response, consistent with the regulation of EIN3/EIL1 expression in response to salt (Fig. 2A). Interestingly, ERF1ox did not show obvious differences from wild-type Col-0 under 100 mM NaCl treatment during seed germination and seedling development (Fig. 6, C–E), indicating that ERF1 as a downstream regulator of EIN3 in ethylene signaling (Solano and Ecker, 1998) is not involved in the salt response.

To explain how the EIN3-ESE1 transcriptional complex affects the salt response, we compared the Na⁺/K⁺ ratio in wild-type and transgenic seedlings with or without salt treatment. Our results showed no obvious differences in Na⁺/K⁺ ratio for roots and shoots (data not shown). Because Pro content and electrolyte leakage are indicators of the salt response, we then measured changes in these parameters. EIN3ox and ESE1ox transgenic lines had significantly higher Pro contents and lower relative electrolyte leakage under 100 mM NaCl treatment than did the wild-type Col-0, ein3-1, and ese1d (Supplemental Fig. S3, A and B). Importantly, overexpressing ESE1 in ein3-1 transgenic plants led to higher Pro content levels and lower relative electrolyte leakage under treatment with 100 mM NaCl than ein3-1, indicating that ESE1 transcriptionally downstream of EIN3 could retain Pro accumulation and electrolyte leakage. However, the data that overexpression of ERF1 showed no evident difference in Pro accumulation and electrolyte leakage with and without NaCl treatment (Supplemental Fig. S3, A and B) further revealed that ESE1 but not ERF1 is involved in the salt response.

To determine whether EIN3 and ESE1 coactivated the downstream salt-related genes, we first checked the expression of salt-related genes in these mutants. Due to the functional redundancy of ERF genes, and ese1 being a knockdown mutant, the transcript levels of salt-related genes such as RD29A and COR15A in ese1 did not show obvious differences from those in wild-type Col-0 (data not shown). Alternatively, we detected the gene expression with qPCR amplification using 7-d-old seedlings of EIN3ox and ESE1ox lines. As expected, under normal growth conditions, EIN3ox had more than 9-fold more transcripts of COR15A and more than 2-fold the expression of RD29A, P5CS2, and HLS1. Coincidentally, ESE1ox also had more than 6-fold expression of COR15A and more than 2-fold expression of RD29A, P5CS2, and HLS1. Moreover, the expression levels of COR15A, RD29A, P5CS2, and HLS1 were obviously increased in EIN3ox and ESE1ox lines after 100 mM NaCl treatment (Fig. 7). In addition, to distinguish whether the induction of the genes was salt dependent or EIN3-ESE1 dependent, we further detected the expression of ERF5 (At5g47230) and AtERF9 (At5g44210), which were observed not to be salt inducible (Fig. 1B). Our data showed that the expression of these genes was not obviously changed before and after salt treatment (Fig. 7). Thus, it is highly likely that EIN3 and ESE1 coactivated the expression of salt-related genes.

ESE1 Physically Binds to the Promoters of Salt-Related Genes

Accumulating evidence shows that ERF proteins interact not only with the GCC box but also with the DRE/CRT element (Park et al., 2001; Hao et al., 2002; Zhang et al., 2004). As ESE1 is a novel ERF protein (Supplemental Fig. S2), we then further demonstrated the direct activation of ESE1 in the expression of salt-related genes in vivo and in vitro. Our research showed that the GST-ESE1 fusion protein bound to DRE. With an increase of unlabeled DRE, the binding of ESE1 to DRE significantly decreased, and 200-fold excess of unlabeled DRE resulted in complete loss of binding activity (Fig. 8A). Similar results were ob-
tained with the GCC box: an excess of unlabeled probe clearly decreased the binding activity of ESE1 to the GCC box (Fig. 8B). Use of the ChIP assay, combined with qPCR detection, showed coimmunoprecipitation of ESE1 and the promoter fragments of salt-related genes RD29A, COR15A, P5CS2, and HLS1 (Fig. 8C), suggesting the direct binding activity of ESE1 to these genes in vivo. The promoters of RD29A (Kasuga et al., 1999) and COR15A (Jaglo-Ottosen et al., 1998) contain DRE and the promoters of P5CS2 and HLS1 (Lehman et al., 1996) contain the GCC box in their respective promoters; therefore, the fact that EIN3ox and ESE1ox simultaneously triggered the expression of salt stress-responsive genes implied that the transcriptional complex of EIN3 and ESE1 played an important role in the expression of salt-related genes.

DISCUSSION

Ethylene and ethylene signaling components play important roles in plants under salt stress (Achard et al., 2006; Cao et al., 2007; Wang et al., 2007). For example, the knockout mutant of EIN3 is more sensitive to salt stress than in Col-0 during germination and postgermination development (Achard et al., 2006; Cao et al., 2007; Wang et al., 2007; Yoo et al., 2008). However, overaccumulation of EIN3 in the ebf1 ebf2 mutant enhances tolerance to salt stress (Achard et al., 2006), indicating that EIN3 is an important regulator in the salt response. Interestingly, overexpression of a number of ERF genes can enhance stress tolerance, including to salt (Agarwal et al., 2006; Nakano et al., 2006; Wu et al., 2007, 2008; Zhang and Huang, 2010; Zhu et al., 2010; Fukao et al., 2011), indicating that ERF proteins also play crucial roles in response to salt stress. In our research here, through mining the available microarray data and combined transcriptional confirmation, we identified three ERF genes (ESE1–ESE3) that were induced by both ethylene and high salt. Further analyses focused on one ESE gene addressed the idea that the EIN3-ESE1 transcriptional cascade is associated with the salt response. Our evidence showed that EIN3 bound to the promoter of ESE1 in vitro and in vivo, with both ACC and EIN3ox inducing ESE1 expression, while ein2 suppressed, ein3 partially suppressed, but ein3 eil1 almost suppressed the expression of ESE1, indicating that EIN3 transcriptionally modulates the transcription of ESE1. In turn, ESE1 bound to promoters of salt-related genes. Moreover, either EIN3ox or ESE1ox is sufficient to enhance transcript levels of salt-related genes and salt tolerance. In addition, ESE1ox in ein3 enhanced the salt response during seed germination and seedling growth. Therefore, this study revealed that ESE1 is a downstream component in the salt response, elucidating a crucial event of the EIN3-ESE1 transcriptional complex in the salt response, thereby connecting the regulation of EIN3 and the downstream ERF protein ESE1 in the salt response.

Ethylene signaling is considered to be a key regulator in the pathogen response, cooperating with salicylic acid and/or jasmonic acid, resulting in the expression of a large array of defense genes (Beckers and Spoel, 2006; Mur et al., 2006; Bari and Jones, 2009; Zander et al., 2010). In Arabidopsis, both ethylene and salicylic acid signaling pathways are necessary to mount an effective defense response (Schenk et al., 2000; Berrocal-Lobo et al., 2002). Furthermore, the ethylene and jasmonic acid signal pathways also interact with each other in regulating the expression of defense genes (Xu et al., 1994). Downstream of the multiple interactions between different defense pathways, transcriptional factors display very important roles in regulating the expression of functional genes, including pathogenesis-related proteins. For example,
the ERF1 protein activates the expression of PDF1.2, conferring resistance to Botrytis cinerea (Berrocal-Lobo et al., 2002), and this regulation is transcriptionally controlled by the ethylene signaling component EIN3 (Solano and Ecker, 1998), demonstrating that transcriptional modulation is very important for the pathogen response. In this report, we further showed that ESE1 is one downstream target of EIN3 in the salt response. ESE1 encodes a member of the ERF proteins, belonging to a group of 17 proteins including ERF1, AtERF1, AtERF2, and AtERF14 (Sakuma et al., 2002; Nakano et al., 2006); ERF1 and ESE1 are classified in the same group, and these two ERF proteins have very high similarities in amino acid sequences. Although our data and results from Solano and Ecker (1998) demonstrated that ESE1 and ERF1 are downstream targets of EIN3, the modulation of the two ERF genes is different. ERF1 was not induced by salt stress (data not shown), and ERF1ox did not show obvious different phenotypes from wild-type Col-0 during seed germination and seedling development, indicating that ERF1 was not involved in salt stress. Distinct from ERF1, the expression of ESE1 was activated by EIN3ox, salt, and ethylene, implying the functional coordination of EIN3-ESE1 under salt stress. In fact, ethylene, by affecting F-box proteins EBF1/2, stabilizes the EIN3 protein (Guo and Ecker, 2003; Potuschak et al., 2003), resulting in the ethylene response. Thus, our data here further reveal the regulatory targets of the ethylene signaling component EIN3 in the salt response, deepening our understanding of ethylene in the stress response.

EIN3 is a member of a small family of DNA-binding proteins, including EIL1 to EIL5, among which EIL1 has high identity to EIN3 (Chao et al., 1997). The coregulation of EILs was shown in seedling greening (Zhong et al., 2009). In our study, we also observed that single mutants ein3-1 and eil1-3 retained the expression of ESE1, while the ein3 eil1 double mutant and an upstream component mutation of EIN2 almost lost expression of ESE1, indicating the coordination of EIN3-EIL1 in the expression of downstream genes. To show sufficient regulation of EIN3, we first demonstrated that the N-terminal region of EIN3 identifies the ESE1 promoter through physical interaction with the core sequence (5'-ATGTA-3'), which was also present in the ERF1 and SID2 promoters (Solano et al., 1998; Chen et al., 2009). Next, in order to prove the transcriptional regulation of EIN3 on ESE1 in plants, using EIN3ox lines, combined with the approach of the tobacco transient cotransformational system, we further demonstrated the EIN3 binding to the promoter of ESE1 in vivo. In addition, by comparing the expression of salt- and non-salt-related genes in EIN3ox and ESE1ox lines, with or without NaCl treatment, we observed changes in salt-related but not in non-salt-related genes. Thus, this study revealed that the transcriptional regulation of ESE1 was at least in part dependent on EIN3.

The interaction of ERF proteins with the GCC box and/or DRE/CRT is considered to activate the expression of these genes, resulting in responses to abiotic and biotic stresses (Liu et al., 1998; van der Fits and Memelink, 2001; Xue and Loveridge, 2004; Nakano et al., 2006; Wu et al., 2007, 2008). Although ERF proteins are involved in the salt response by controlling the expression of downstream genes, the regulation...
tion of ESE1 in the salt response was first identified, which binds to DRE and the GCC box, subsequently activating the expression of salt-related genes and the salt response. More importantly, the modulation of EIN3 in the expression of the salt-related ESE1 reveals, to our knowledge for the first time, that the transcriptional complex of EIN3-ESE1 is important for the activation of salt-related genes in such a process.

Plant salt tolerance is a complex trait that involves multiple physiological and biochemical mechanisms. In such a process, the transcriptional event is a key step for plant adaptation to salt stress. In this study, we focused on how EIN3 transcriptionally regulated ESE1 expression to increase salt tolerance in Arabidopsis. Based on our study, we propose a regulatory model for EIN3 regulation in the expression of ESE1 under salt stress in Arabidopsis (Fig. 9). In this regulation, ESE1 is one of the target genes of EIN3 that binds to the ESE1 promoter. Consequently, ESE1 identifies DRE and the GCC box as transcriptionally activating the expression of salt-related genes, resulting in enhanced adaptation to salt stress.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) mutants (ein2, ein3-1, eil1-3, eto1, eto2, and eto3) were obtained from the ABRC, and the ein3 eto1 double mutant was provided by Prof. H.W. Guo at Peking University. The background of these mutants is Col-0. Plants overexpressing EIN3 or ESE1 (EIN3ox or ESE1ox, respectively) were generated by Agrobacterium tumefaciens strain LBA4404 transformation into Arabidopsis Col-0 by floral dip, using a construct that contained the full-length coding region of EIN3 (At3g20770) or ESE1 (At3g23220) in a modified pCAMBIA1300 vector that contains a 6×MYC tag. Plants overexpressing ESE1 in ein3-1 (ESE1ox in ein3-1) were made by Agrobacterium transformation into the ein3-1 mutant. Seeds were germinated on Murashige and Skoog (MS) medium and selected with hygromycin resistance. Then, 2-week-old hygromycin-resistant plants were transferred to soil and grown in a greenhouse under normal growth conditions. Transgenic plants were verified by qPCR amplification. We obtained 12, 15, and 20 lines of EIN3ox, ESE1ox, and ESE1ox in ein3-1, respectively. After confirmation of the insertion copy with hygromycin selection in T2, the T3 and T4 generation transgenic seeds were used for our assays. During the generation of transgenic plants, we observed that constitutive ethylene responses were displayed in T2 of EIN3ox, such as dark green leaves and shorter petioles, but were not observable in T3 and T4 of EIN3ox. The T-DNA insertion mutant escl (SAIKL_128736) was obtained from the ABRC. Transgenic plant ERFIox (CS6142) obtained from the ABRC constitutively overexpresses ERF1 under the control of the cauliflower mosaic virus 35S promoter. All seeds were first surface sterilized, placed on plates of MS medium containing 0.5% phytagel, and kept at 4°C for 3 d to break dormancy. Then they were transferred to growth chambers with 70% humidity under a 16/8-h light/dark regime at 21°C.

Seed Germination and Seedling Development under Salt Stress

For seed germination assay, surface-sterilized seeds were plated on MS medium with or without 100 mM NaCl and kept at 21°C with a 16/8-h light/dark regime. Germinated seeds were counted daily. The percentage of seed germination was calculated from about 100 seeds per treatment. For seedling development, 5-d-old seedlings from MS medium plates were transferred to MS plates containing 0 or 100 mM NaCl and incubated at 21°C with a 16/8-h light/dark regime for 7 d to record the phenotypic changes.

Gene Expression Analyses

From the MS medium plates, 7-d-old seedlings were carefully transferred onto Whatman filter paper with treatments of 100 mM NaCl, 10 μM ACC, 5 μM AgNO3, 5 μM AVG, or water (as a control) for 3 h. The treated seedlings were then sampled to extract total RNA by Trizol Reagent (Tiangen). 5 μg of RNA was synthesized into cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer’s instructions, then the expression of genes was followed by qPCR analysis (SYBR Premix; Takara). PCR amplifications were performed on 96-well optical reaction plates with 45 cycles of denaturing for 15 s at 95°C, annealing for 20 s at 56°C, and extension for 45 s at 72°C. Expression levels were normalized to that of TUB4. All gene-specific primers used in the qPCR experiments are given in Supplemental Table S2.

Transcriptional Activation Assay

The effector plasmid was constructed by inserting the full-length coding region of EIN3 downstream of the yeast GAL4 DNA-binding domain in pGBK7T (Clontech). The fusion plasmid EIN3-pGBK7T and the vectors (pGBK7T-53/pGADT-Rec T and pGBK7T-lam/pGADT-Rec T as positive and negative controls) were transformed into yeast strain Y190 (Clontech). The transformants were selected by growth on synthetic dextrose/Leu-Trp His plates to test the expression of the HIS3 reporter gene; subsequently, β-galactosidase activity was used to test the expression of the LacZ reporter gene.

Yeast One-Hybrid Assay

Yeast one-hybrid screening was carried out according to the manufacturer’s protocol (Matchmaker One-Hybrid System; Clontech). Fragments of ESE1 promoter (~1,965 to +24 bp) divided into D1 (~1,965 to ~1,406 bp), D2 (~1,475 to ~976 bp), D3 (~985 to ~472 bp), and D4 (~452 to ~242 bp) were amplified by PCR amplification and cloned into the pLaCZ reporter, yielding pD1, pD2, pD3, and pD4, respectively. Then the reporter vectors were separately integrated into the genome of the yeast strain YM4271 after linearizing the vectors at the Ncol site.

The full length EIN3 (1–628 amino acids) as well as the N-terminal (1–314 amino acids) and C-terminal (315–628 amino acids) regions were cloned downstream of the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene in the yeast expression vector pYEpGAP, yielding effectors pEIN3-F, pEIN3-N, and pEIN3-C. After confirmation of the integration of the reporters, the yeast one-hybrid assay was conducted to transform effectors into the yeast competent cells carrying the reporter pD1, pD2, pD3, or pD4 following the manufacturer’s instructions (Clontech). The transformants were selected on synthetic dextrose/-Ura-Trp medium. The β-galactosidase filter-lifted assay was performed according to the manufacturer’s protocol (Clontech).

ChIP Assay

The ChIP assay was performed as described previously (Gendrel et al., 2005; Saleh et al., 2008). Seven-day-old seedlings (approximately 2 g) of Col-0, EIN3ox-1, EIN3ox-4 (with ACC treatment for 3 h), and ESE1ox-9 were first fixed in 1% formaldehyde for 10 min in a vacuum and neutralized with 0.125 mM glycine in a vacuum for an additional 5 min for cross-linking. After washes with 40 mL of ice-cold sterile deionized water, the samples were ground in liquid nitrogen to extract the chromatin. The DNA was sheared into approximately 500-bp fragments by sonication. After centrifuging, 300 μL of the supernatant was diluted to 3 mL, and 60 μL of protein G agarose/salmon sperm DNA (Upstate) was added for preclearing at 4°C for 1 h. The chromatin was then divided into two 1.5-mL aliquots. Then, 30 μL of monoclonal a-myc 9E10 immobilized onto Sepharose fast-flow beads (9E10 affinity matrix; Covance) was added to one tube, and the other was treated as an input control. After incubating at 4°C overnight, beads were washed with wash buffer and then eluted with elution buffer. After the eluates were subsequently digested with Proteinase K (Merck) and RNase, extraction with phenol/ chloroform, and precipitation with ethanol plus sodium acetate, the purified DNA was resuspended in 100 μL of deionized water. The DNA fragments were determined by qPCR amplification using gene-specific primers (Supplemental Table S2). An unrelated DNA sequence from the promoter of the TUB84 gene was used as an internal control (Zhang et al., 2010). Quantification
is involved in the normalization of each immune precipitation (IP) or control threshold cycle (Ct) to the input DNA sample Ct to obtain a ΔCt (ΔCt IP or ΔCt control), and then the relative enrichment of each fragment was calculated using the following equation: 2–\(\Delta\Delta C_t\) IP = 2–\(\Delta\Delta C_t\) control.

**Agrobacterium-Mediated Transient Cotransformation**

*Agrobacterium* strain LBA4404 harboring individual constructs was inoculated in 20 ml of induction medium containing Luria-Bertani medium, 2 mM phosphate, 1% Glc, 20 mM MES (pH 5.5), and 100 μM acetylsorbose as well as rifampicin and kanamycin (Yang et al., 2000). After overnight culture at 28°C, *Agrobacterium* cells were collected to filtrate tobacco (*Nicotiana tabacum*) leaves for GUS activity assay (Jefferson et al., 1987).

**EMS**

To construct plasmids for the expression of N-terminial (1–314 amino acids) and C-terminal (315–628 amino acids) regions of EIN3 protein in *Escherichia coli* BL21, DNA fragments corresponding to the two regions were obtained by PCR amplification and inserted into the multicloning sites of the pGEX-6p-1 (Amersham). Purification of the fusion protein was conducted according to the Glutathione Sepharose 4B (GE) handbook.

EMS was performed as described for the digoxigenin (DIG) Gel Shift Kit (Roche). The probes, such as D4P2-3 (5′-TCTTCATATTATAATGACAGCAGCCACGCAA-3′, with ATGTA highlighted with a single line) and D4P2-3M (5′-TCTTCATATTATATCCCCCAGAGCAGCCACGCAA-3′), with the mutations marked with double lines), were achieved by synthesis in oligonucleotides (Supplemental Table S2). The 3′-DIG-labeled probes were then independently prepared by annealing of the synthesis oligonucleotides. Each binding reaction (20 μl), containing 100 ng of recombinant protein, 1 ng of labeled DNA probe, 5 ng of poly-d-Lys, 50 ng of poly[d(A-T)], 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol, 2% (w/v) Tween 20, and 30 mM KCl, was incubated at room temperature for 30 min. The reaction mixtures were then loaded onto 5% polyacrylamide gels to separate free and bound DNA. The DNA on the gel was then transferred onto nylon membranes (GE). After UV cross-linking, the DNA on the membrane was detected using anti-digoxigenin-conjugated alkaline phosphatase (DIG Gel Shift Kit; Roche).

**Measurement of Pro Content and Electrolyte Leakage**

Five-day-old seedlings were transferred onto MS medium containing 0 and 100 mM NaCl. After culturing for 10 d, 0.1 g of fresh leaves was harvested for measurement of the Pro content and electrolyte leakage, following the methods described previously (Bates et al., 1973; Cao et al., 2007). Percentage electrolyte leakage was calculated as the ratio of the conductivity before autoclaving to that after autoclaving.

**Supplemental Data**

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

**Supplemental Figure S1.** Confirmation of putative target genes of EIN3.

**Supplemental Figure S2.** The ERF protein ESE1 displays transactivation activity.

**Supplemental Figure S3.** ESE1 improves Pro accumulation but reduces electrolyte leakage.

**Supplemental Table S1.** Genome-wide analyses of ethylene- and salt-inducible ERF genes from microarray data.

**Supplemental Table S2.** Oligonucleotides and primers used in this study.

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**LITERATURE CITED**

Achard P, Cheng H, De Grauwe L, Decat J, Schouteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 311: 91–94

Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25: 1263–1274

Bari R, Jones JD (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69: 473–488

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205–207

Beckers GJ, Spoel SH (2006) Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant Biol (Stuttg) 8: 1–10

Berrocal-Lobo M, Molina A (2004) Ethylene response factor 1 mediates Arabidopsis resistance to the soilborne fungus *Fusarium oxysporum*. Mol Plant Microbe Interact 17: 763–770

Berrocal-Lobo M, Molina A, Solano R (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J 29: 23–32

Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18

Boutrot F, Segonzac C, Chang KN, Qiao H, Ecker JR, Zipfel C, Rathjen JP (2010) Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. Proc Natl Acad Sci USA 107: 14502–14507

Cao WH, Liu J, He XJ, Mu RH, Zhou HL, Chen SY, Zhang JS (2007) Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol 143: 707–719

Cao WH, Liu J, Zhou QY, Cao YR, Zheng SF, Du BX, Zhang JS, Chen SY (2006) Expression of tobacco ethylene receptor NTHK1 alters plant responses to salt stress. Plant Cell Environ 29: 1210–1219

Chang C, Bleecker AB (2004) Ethylene biology: more than a gas. Plant Physiol 136: 2895–2899

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 89: 1133–1144

Chen H, Xue I, Chintamanani S, Germain H, Lin H, Cui H, Cai R, Zuo J, Tang X, Li X, et al (2009) ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFI-CIENT2 expression to negatively regulate plant innate immunity in Arabidopsis. Plant Cell 21: 2527–2540

Chen YE, Etheridge N, Schaller GE (2005) Ethylene signal transduction. Ann Bot (Lond) 95: 901–915

Chinnusamy V, Zhu J, Zhu JK (2006) Salt stress signaling and mechanisms of plant salt tolerance. Genet Eng (N Y) 27: 141–177

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12: 444–451

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14: 1675–1690

Fukao T, Yeung E, Bailey-Serres J (2011) The submergence tolerance regulator SUB1A mediates crosstalk between submergence and drought tolerance in rice. Plant Cell 23: 412–427

Gendrel AV, Lippman Z, Martiisen R, Colot V (2005) Profiling histone modification patterns in plants using genomic tiling microarrays. Nat Methods 2: 213–218

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124: 1854–1865

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by SCAF(D/D:ERF)-dependent proteolyis of EIN3 transcription factor. Cell 115: 667–677

Guo H, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol 7: 40–49

Hao D, Yamasaki K, Sarai A, Ohme-Takagi M (2002) Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC motifs. Biochemistry 41: 4202–4208

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