Seed germination is regulated through elaborately interacting signaling networks that integrate diverse environmental cues into hormonal signaling pathways. Roles of gibberellic acid and abscisic acid in germination have been studied extensively using Arabidopsis (Arabidopsis thaliana) mutants having alterations in seed germination. Auxin has also been implicated in seed germination. However, how auxin influences germination is largely unknown. Here, we demonstrate that auxin is linked via the IAA30 gene with a salt signaling cascade mediated by the NAM-ATAF1/2-CUC2 transcription factor NTM2/Arabidopsis NAC domain-containing protein 69 (for NAC with Transmembrane Motif1) during seed germination. Germination of the NTM2-deficient ntm2-1 mutant seeds exhibited enhanced resistance to high salinity. However, the salt resistance disappeared in the ntm2-1 mutant overexpressing the IAA30 gene, which was induced by salt in a NTM2-dependent manner. Auxin exhibited no discernible effects on germination under normal growth conditions. Under high salinity, however, whereas exogenous application of auxin further suppressed the germination of control seeds, the auxin effects were reduced in the ntm2-1 mutant. Consistent with the inhibitory effects of auxin on germination, germination of YUCCA 3-overexpressing plants containing elevated levels of active auxin was more severely influenced by salt. These observations indicate that auxin delays seed germination under high salinity through cross talk with the NTM2-mediated salt signaling in Arabidopsis.
and Jürgens, 2004; Quint and Gray, 2006; Abel, 2007). It also plays a role in plant responses to biotic and abiotic stresses (Navarro et al., 2006; Park et al., 2007). Notably, recent studies in several plant species support the role of auxin in seed germination (Birgit et al., 2005), although the underlying signaling schemes have not been explored at the molecular level.

Global gene expression studies in Arabidopsis support the role of auxin in seed germination. It has been observed that genes encoding auxin flux carriers and biosynthetic enzymes are up-regulated by GA in germinating seeds, suggesting that GA activity influences auxin level and transport during seed germination (Ogawa et al., 2003; Carrera et al., 2007). Investigation of auxin homeostasis and measurements of endogenous auxin contents of free indole-3-acetic acid (IAA) have been explored at the molecular level.

Assays using the DR5-GUS reporter system have shown that GUS activity is elevated in the radicle after cold imbibition (Ni et al., 2001; Liu et al., 2007). In addition, it has been found that whereas germination sensitivity is mimicked in the germinating seeds of Scots pine (Pinus sylvestris; Ljung et al., 2001). A similar result has been obtained from germination assays of bean (Phaseolus vulgaris) seeds (Bialek and Cohen, 1989).

More direct evidence sustaining the involvement of auxin in seed germination has been inferred from germination assays using transgenic plants overexpressing miR160 or its target Auxin Response Factor10 (ARF10; Liu et al., 2007). Seed germination of the miR160-overproducing transgenic plants is hypo-sensitive to ABA. In contrast, that of transgenic plants overexpressing a miR160-resistant ARF10 gene is hypersensitive to ABA. Interestingly, this ABA hypersensitivity is mimicked in the germinating seeds of wild-type plants in the presence of exogenous auxin application, supporting signaling cross talk between auxin and ABA.

The NAC (for NAM-ATAF1/2-CUC2) transcription factors constitute one of the largest transcription factor families in plant genomes (Ooka et al., 2003; Olsen et al., 2005b). The Arabidopsis genome contains more than 100 NAC members. Roles of many NAC transcription factors have been demonstrated in diverse developmental processes and plant responses to biotic and abiotic stresses, such as floral development (Sablowski and Meyerowitz, 1998), apical meristem formation (Hibara et al., 2003), stress responses and signaling (Balazadeh et al., 2010; Jensen et al., 2010; Seo et al., 2010), cell cycle control (Kim et al., 2006), and germination under high salinity (Kim et al., 2008). A few NAC transcription factors, such as AtNAC2 functioning in root development (He et al., 2005), have been suggested to mediate auxin signaling in the salt stress response. However, no NAC protein has been shown to play a role in auxin-salt stress signaling cross talk during seed germination. Notably, some NAC proteins are membrane associated, and controlled proteolytic activation of the membrane-bound NAC transcription factors has been proposed to serve as an adaptive strategy that ensures rapid transcriptional responses to abrupt environmental changes (Seo et al., 2008).

In this work, we demonstrate that the plasma membrane-bound NAC transcription factor NTM2 integrates auxin and salt signals in regulating Arabidopsis seed germination. In this signaling scheme, auxin signals are incorporated via the IAA30 gene into the NTM2-mediated salt signaling pathway. Therefore, we propose that NTM2 serves as a molecular link that interconnects a developmental feedback loop of auxin signaling with a salt signal transduction pathway during seed germination.

RESULTS

NTM2 Is a Plasma Membrane-Bound NAC Transcription Factor

We have recently reported that a membrane-bound NAC transcription factor, NTM1 (for NAC with Transmembrane Motif1; At4G01540) regulates cell division by modulating cytokinin signaling (Kim et al., 2006). Notably, an adjacent locus (At4G01550) also encodes a NAC protein, designated NTM2, having a similar structural organization and a high sequence homology to the NTM1 protein (Supplemental Fig. S1). The NTM2 protein was designated Arabidopsis NAC domain-containing protein 69 (ANAC069) in the previous report (Ooka et al., 2003). Therefore, we decided to examine whether NTM2/ANAC069 is functionally similar to NTM1.

The NTM2 protein consists of 457 residues (Fig. 1A). Like the NTM1 protein, a NAC DNA-binding domain resides in the N-terminal region, and a transmembrane (TM) motif is predicted in the far C-terminal region. To investigate the physiological role of NTM2, we produced a few truncated NTM2 forms (Fig. 1A). The ΔTM construct included residues 1 to 420 and lacked the TM motif. The ΔC construct consisted of residues 1 to 287 and was similar in size to the transcriptionally active nuclear forms of NTM1 (Kim et al., 2006) and NTL8 (Kim et al., 2008) as well as to the known nuclear NAC proteins (Kim et al., 2006; Ruiming et al., 2007). The NTM2 gene constructs were transformed into Landsberg erecta (Ler) plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

As previously reported with NTM1, NTL6, and NTL8 (Kim et al., 2006, 2008; Seo et al., 2010), transgenic plants overexpressing the full-size NTM2 form (35S:NTM2) and those overexpressing the ΔTM form (35S:ΔTM) were phenotypically indistinguishable from control plants (Fig. 1B). In contrast, those overexpressing the ΔC form, which is presumably analogous to a transcriptionally active NTM2 form, exhibited a dwarfed appearance with small, curled leaves, suggesting that membrane release of the NTM2 protein is essential for its activity. However, no leaf serration was observed, unlike the transgenic plants overexpressing a ΔC form.
of the NTM1 protein (Kim et al., 2006), suggesting that the physiological roles of the NTM1 and NTM2 proteins are at least partly distinct from each other.

To determine the subcellular localization of the NTM2 protein, a GFP-coding sequence was fused in-frame to the 5’ ends of the NTM2 gene sequences and the GFP-NTM2 gene fusions were transiently expressed in Arabidopsis protoplasts. The results showed that whereas the full-size NTM2 protein was localized at the plasma membranes, the ΔC form was detected predominantly in the nucleus (Fig. 1C).

We employed a GAL4 transient expression system in Arabidopsis protoplasts (Miura et al., 2007) to investigate the transcriptional activation activities of NTM2 protein. The NTM2 gene sequences were fused in-frame to the 3’ end of the GAL4 DNA-binding domain-coding sequence in the effector vector (Fig. 1D, top panel). The effector vectors, the reporter vector having the GUS reporter gene, and the vector containing the Renilla luciferase gene, which was included to normalize the measurements, were cotransformed into Arabidopsis protoplasts. The assays revealed that the NTM2 and ΔTM proteins possess discernible levels of transcriptional activation activities, showing that the NTM2 protein is a transcriptional activator (Fig. 1D, bottom panel). The ΔC protein exhibited the highest activity, which was much higher than that of the ΔTM protein, suggesting that membrane release itself is not adequate for the transcriptional activation activity. It is also envisioned that the C-terminal sequence of ΔTM, which is missing in ΔC, may confer an inhibitory effect on the transcriptional activation activity of NTM2, as suggested with NTL6 (Seo et al., 2010). Together, these observations indicate that the NTM2 protein is a plasma membrane-localized transcriptional activator.

The NTM2 Gene Is Induced by High Salinity

Many NAC proteins are involved in plant responses to various environmental stresses, such as high salinity, cold, and dark-induced leaf senescence (Olsen et al., 2005b; Kim et al., 2008; Seo et al., 2010). We found that the phenotypes of 35S:ΔC transgenic plants were similar to those observed in plants grown under stressful conditions (Stanton et al., 2000; Kim et al., 2007), suggesting that the NTM2 gene is related to plant stress responses.

Figure 1. NTM2 is a plasma membrane-bound NAC transcription factor. A, NTM2 constructs used. Numbers indicate amino acid (aa) residue positions. The TM was predicted using the ARAMEMNON membrane protein database (http://aramemnon.botanik.uni-koeln.de/). B, Transgenic plants overexpressing NTM2 gene constructs driven by the CaMV 35S promoter. Ler plants were used for transformation. Five-week-old plants grown in soil under long days were photographed (top panel). Transcript levels were determined by qRT-PCR using RNA samples extracted from 2-week-old whole plants grown on a MS-agar plate (bottom panel). Biological triplicates were averaged. Error bars represent se. The y axis is presented on a logarithmic scale for better comparison of fold changes. C, Subcellular localization of NTM2 and ΔC proteins. The GFP-NTM2 and GFP-ΔC fusion constructs were expressed transiently in Arabidopsis protoplasts and visualized by fluorescence microscopy (top panels) and differential interference contrast microscopy (bottom panels). Bars = 20 μm. D, Transcriptional activation activity assays in Arabidopsis protoplasts. The GAL4 transient expression assays were carried out using Arabidopsis protoplasts, as described previously (Miura et al., 2007). Vector control, Transformation with the effector vector without gene inserts; ARF5M and ARF1M, transformations with the effector vectors containing the ARF5M gene (activator control) and the ARF1M gene (repressor control), respectively (Tiwari et al., 2003). Five measurements were averaged. Error bars indicate se. Statistical significance was determined by Student’s t test (* P < 0.01, ** P < 0.005).
To obtain clues to the role played by NTM2, the effects of abiotic stresses and growth hormones on NTM2 gene expression were examined by quantitative real-time reverse transcription (qRT)-PCR. The NTM2 gene was slightly induced only by high salinity among the stress conditions examined when whole plants were used for total RNA extraction (Fig. 2A). Notably, expression studies using the shoot and root samples revealed that whereas the NTM2 gene was uninfluenced by high salinity in the shoot, it was induced approximately 8-fold in the roots (Fig. 2B). This result is also consistent with the strong induction of ANAC069 by NaCl in the roots (Jiang and Deyholos, 2006). The NTM2 gene was also induced by ABA (Fig. 2C), particularly in the root (Fig. 2D). These observations suggest that the NTM2 gene plays a role in salt stress responses, which is likely to be mediated by ABA.

We also examined the effects of high salinity on NTM2 gene expression using a promoter-GUS gene fusion, in which the GUS-coding sequence was fused to the 3′ end of the NTM2 gene promoter sequence covering an approximately 2-kb region upstream of the transcription start site. The promoter-GUS construct (pNTM2-GUS) was transformed into Ler plants. Whereas GUS activity was uninfluenced by high salinity in the leaves, it was elevated significantly in the roots (Fig. 2E), further supporting the predominant induction of the NTM2 gene in the roots under high salinity.

**ntm2-1 Seedling Growth Is Less Sensitive to High Salinity**

The phenotypes of 35S:ΔC transgenic plants and the effects of salt on NTM2 expression suggested that the NTM2 gene might be involved in the plant response to high salinity. To examine this hypothesis, we analyzed the phenotypes and salt responsiveness of a transposon insertional NTM2 knockout mutant (ntm2-1; CSHL-ET8732), which was obtained from the Arabidopsis Genetrap Database at Cold Spring Harbor Laboratory (Supplemental Fig. S2).

The ntm2-1 mutant did not exhibit any discernible phenotypes compared with control plants when grown under normal growth conditions (Fig. 1B). However, it responded differentially to high salinity. The ntm2-1 seedling growth was influenced to a lesser degree by salt (Fig. 3A). In particular, unlike control seedlings, leaf yellowing was not observed in the mutant seedlings under high salinity (Fig. 3B). Primary root growth of the ntm2-1 mutant was also less sensitive to high salinity (Fig. 3, C and D), supporting that the NTM2 gene is related to salt responses.

We also examined the effects of ABA on seedling growth. Notably, the effects of ABA on seedling growth were similar in control and ntm2-1 mutant plants (Fig. 3E). Furthermore, salt induction of the NTM2 gene still occurred in the roots of abi2-1, an ABA-insensitive mutant with disrupted ABA signaling (Zhu, 2002; Fig. 3F), as observed in control plants treated with salt (Fig. 2D). These observations indicate that the role of the NTM2 gene is related to salt responses.

**Figure 2.** The NTM2 gene is induced by high salt. Transcript levels were determined by qRT-PCR. Biological triplicates were averaged. Error bars indicate se. Statistical significance was determined by Student’s t test (* P < 0.01). Two-week-old plants grown on MS-agar plates were used for extraction of total RNA or subsequent treatments. A, Effects of abiotic stress conditions on NTM2 gene transcription. Plants were exposed to cold (4°C, 3 h), drought (DR; 3 h), or NaCl (150 mM, 6 h). Whole plants were used for extraction of total RNA. M3 and M6, Mock treatments for 3 and 6 h, respectively. B, Effects of high salinity on NTM2 gene transcription in the shoots (SH) and roots (RO). Plants were soaked for 6 h in MS liquid cultures supplemented with 150 mM NaCl, and the shoot and root samples were harvested separately for extraction of total RNA. C, Effects of growth hormones on NTM2 gene transcription. Plants were transferred to MS liquid cultures containing appropriate concentrations of growth hormones, such as ABA (50 μM, 6 h), IAA (20 μM, 6 h), GA (50 μM, 6 h), or PAC (50 μM, 6 h). Whole plants were used for extraction of total RNA. D, Effects of ABA on NTM2 gene transcription in the shoots and roots. Plants were treated with ABA as described in C, and the shoot and root samples were harvested separately for extraction of total RNA. E, Effects of high salinity on the promoter activities of the NTM2 gene. Two-week-old transgenic plants expressing the pNTM2-GUS fusion grown on MS-agar plates were incubated for 6 h in MS liquid cultures supplemented with 150 mM NaCl and subjected to GUS staining.
NTM2 gene in plant salt responses does not depend on ABA.

**Germination of ntm2-1 Seeds Is Resistant to High Salinity**

One of the plant developmental processes affected by salt is seed germination. Soil salinity represses seed germination either by imposing osmotic stress (Perruc et al., 2007) or by reducing GA biosynthesis (Achard et al., 2006; Kim et al., 2008). We found that the NTM2 gene is induced by high salinity. Therefore, it was hypothesized that the NTM2 gene might be related to seed germination under high salinity.

We first examined the expression pattern of the NTM2 gene in germinating seeds of transgenic plants expressing the pNTM2-GUS construct. Reporter gene expression was detected at a low level in germinating seeds under normal conditions (Fig. 4A). In contrast, it was highly induced in the emerging radicle under high salinity.

We next examined the germination phenotypes of the ntm2-1 mutant seeds under high salinity. Germination of control seeds was delayed in the presence of 100 to 150 mM NaCl (Fig. 4B), as reported previously (Achard et al., 2006; Kim et al., 2008). In contrast, that of the ntm2-1 mutant seeds exhibited reduced sensitivity to high salinity. When counted 4 d after cold imbibition, whereas germination of control seeds was reduced by approximately 70% in the presence of 150 mM NaCl, that of the ntm2-1 seeds was reduced by approximately 40% (Fig. 4C). Germination of the 35S:ΔC transgenic seeds was reduced by more than 90% under identical conditions. To confirm the role of the NTM2 gene in germination under high salinity, the ntm2-1 mutant was transformed with the NTM2 gene with its own promoter. The resultant pNTM2-NTM2/ntm2-1 plants were phenotypically similar to control plants (data not shown). Furthermore, its germination phenotype was also similar to that of control seeds under high salinity (Fig. 4D), indicating that the NTM2 gene plays a role in salt regulation of seed germination.

We also examined the effects of ABA and GA on the germination phenotypes of the ntm2-1 seeds. Germination of the ntm2-1 seeds was influenced by ABA in a pattern similar to that observed in control seeds (Fig. 4E). The germination phenotypes of control and ntm2-1 seeds were also similar in the presence of the GA biosynthetic inhibitor paclobutrazol (PAC; Sankhla et al., 2006; Fig. 4F). Together, these observations indicate that the NTM2-mediated salt signaling in seed germination is not related to ABA and GA, which is in agreement with the ABA independence of the NTM2 gene in seedling growth (Fig. 3, C and D).

**Auxin Modulates the Germination of ntm2-1 Seeds under High Salinity**

Arabidopsis mutants containing lower levels of active auxin exhibit reduced growth and, in many cases, also show enhanced resistance to environmental stresses (Wang et al., 2001; Park et al., 2007; Shibasaki et al., 2009). Auxin has also been implicated in seed germination under stressful conditions (Liu et al., 2007). We found that plant growth was severely retarded in the 35S:ΔC transgenic plants. In addition, seedling growth and seed germination of the ntm2-1...
mutant were resistant to high salinity. Therefore, we examined whether the NTM2 gene is related to auxin.

Under normal growth conditions, germination of control seeds was uninfluenced to a discernible level by 1 μM IAA (Fig. 5A). Interestingly, when counted 2 d after cold imbibition under high salinity, the repressive effects of high salinity on germination were exaggerated by IAA in control seeds (Fig. 5B). In contrast, germination of ntm2-1 seeds was influenced to a lesser degree by auxin. To further examine the role of IAA in salt regulation of seed germination, germination assays were carried out in the presence of 150 mM
NaCl and varying concentrations of IAA. The results showed that germination of the ntm2-1 seeds was less sensitive to auxin (Fig. 5C), which is consistent with the reduced sensitivity of ntm2-1 seed germination. These observations indicate that whereas auxin does not contribute to seed germination under normal growth conditions, it delays seed germination under high salinity.

The relationship between auxin and NTM2-mediated salt signaling during seed germination was also explored by employing the DR5-GUS reporter system (Ni et al., 2001). A relatively high level of GUS activity was detected in the emerging radicles of germinating seeds (Fig. 5D), as has been reported previously (Ni et al., 2001; Liu et al., 2007). When seeds of the DR5-GUS plants were allowed to germinate on half-strength Murashige and Skoog-agar plates (hereafter referred to as MS-agar plates) containing 100 mM NaCl, the GUS activity was elevated in the emerging radicles, as observed on those supplemented with 1 μM auxin. Notably, the GUS activity was further elevated in the emerging radicles when both NaCl and IAA were included in the assays, showing that elevation of the GUS activity in the emerging radicles goes well with delayed seed germination.

We next examined whether the suppressive effects of auxin on germination were also applicable to other salt stress responses, such as seedling growth. We found that growth retardation and leaf yellowing still occurred in the salt-treated control plants regardless of IAA (Fig. 5E). In addition, ntm2-1 seedling growth under high salinity was also uninfluenced by auxin, indicating that auxin is not related to the NTM2-mediated salt signaling during seedling growth.

**IAA30 Is Involved in the NTM2-Mediated Salt Signaling during Seed Germination**

Our data indicated that auxin is linked with the NTM2 gene in seed germination under high salinity. To further investigate the salt-auxin signaling connection, we examined the expression of genes involved in auxin signaling and metabolism, such as Transport Inhibitor Response1 (TIR1) and its potential homologs, Aux/IAAs, and GH3s, in the ntm2-1 mutant. Among the genes examined, the Aux/IAA30 gene (hereafter referred to as IAA30) was most prominently influenced by high salinity (Fig. 6A; Supplemental Fig. S3). It was induced by approximately 6-fold by 150 mM NaCl, as inferred from the digital northern data available in the Genevestigator database (https://www.genevestigator.com/gv/index.jsp). Notably, the salt induction of IAA30 largely disappeared in the ntm2-1 mutant (Fig. 6A). In addition, gene expression studies using the shoot and root samples revealed that whereas the IAA30 gene was not discernibly affected by high salinity in the shoots, it was induced approximately 6-fold in the roots (Supplemental Fig. S4), like the NTM2 gene. These observations indicate that salt induction of the IAA30 gene at least in part depends on NTM2.

In addition to the IAA30 gene, other Aux/IAA and GH3 genes, such as IAA11, IAA19, and GH3:4, were also induced by high salinity, but not in the ntm2-1 mutant (Fig. 6A; Supplemental Figs. S3 and S5), suggesting that the NTM2-mediated salt signaling is linked to multiple auxin signaling pathways.

The NTM2-dependent salt induction of IAA30 was further examined by histochemical staining using the pIAA30-GUS fusion construct, in which a GUS-coding sequence was transcriptionally fused to the IAA30 gene promoter sequence, consisting of approximately 1.9 kb upstream of the transcriptional start site. GUS staining assays of transgenic plants expressing the pIAA30-GUS fusion construct revealed that GUS activity was elevated after salt treatments in the roots (Supplemental Fig. S6). In contrast, no elevation of GUS activity was observed in the ntm2-1 mutant background.

Kinetic measurements of the IAA30 transcript revealed that its induction was initiated within 1 h after salt treatments, reaching the plateau at a time point of 3 h (Fig. 6B). In the ntm2-1 mutant, the inductive effects of salt on IAA30 expression were significantly reduced, as observed in histochemical staining assays (Supplemental Fig. S5), showing that the salt induction of the IAA30 gene depends on the NTM2 gene.

We next asked whether the NTM2 dependence of the IAA30 gene expression occurred in the germinating seeds. Cold-imibed seeds were allowed to germinate for 30 h under high salinity, and the IAA30 transcript levels were measured in the germinating seeds. The results showed that whereas the transcript level was elevated in the salt-treated control seeds, no induction of the IAA30 gene was observed in the germinating ntm2-1 mutant (Fig. 6C), supporting that the IAA30 gene is a component of NTM2-mediated salt signaling in seed germination. Meanwhile, we observed that the inductive effects of auxin on the IAA30 gene decreased by approximately 50% in the ntm2-1 mutant (Fig. 6D), suggesting that the NTM2 gene contributes to the auxin induction of IAA30 expression.

To further examine the relationship between auxin and NTM2-mediated salt signaling in seed germination, the IAA30 gene was overexpressed driven by the CaMV 35S promoter in the ntm2-1 mutant and the germination phenotypes of the resultant transgenic seeds (30OX/ntm2-1) were analyzed. Under high salinity, the germination percentage of the transgenic seeds was lower than that of the ntm2-1 seeds but was comparable to that of control seeds (Fig. 6E), demonstrating that the effects of auxin on seed germination under high salinity are mediated at least in part by the IAA30 gene.

**NTM2 Binds to the IAA30 Gene Promoter**

We found that the IAA30 gene is induced in the 35S:ΔC transgenic plants. In addition, salt induction of the IAA30 gene requires NTM2. Therefore, we asked whether NTM2 directly regulates the IAA30 gene.

Nucleotide sequence analysis revealed that the IAA30 gene promoter contained a conserved sequence region...
which is similar to the known NAC-binding sequence (Olsen et al., 2005a). We carried out electrophoretic mobility shift assays to examine whether NTM2 binds to the conserved sequence (IAA30-BS). The recombinant ΔC protein was prepared as a maltose-binding protein (MBP)-ΔC in an *Escherichia coli* expression system. The MBP-ΔC protein bound to the IAA30-BS sequence (Fig. 6F). In addition, its binding was reduced significantly in the presence of excess amounts of unlabelled competitor DNA. However, this interaction was unaffected by the addition of the mutated competitor DNA (mIAA30-BS), showing that NTM2 binds specifically to the IAA30 gene promoter.

To examine the transcriptional activation activity of NTM2 in planta, we carried out transient coexpression assays in Arabidopsis protoplasts using a series of GUS reporter constructs and the p35S:ΔC effector plasmid (Fig. 6G, top panel). Cotransformation with the pIAA30-BS reporter increased GUS activity approximately 3-fold (Fig. 6G, bottom panel). In contrast, cotransformation with the pmIAA30 reporter did not influence GUS activity, indicating that the NTM2 protein functions as a transcriptional activator of the IAA30 gene by binding directly to the gene promoter.

The NTM2 protein is associated with the plasma membranes. Our data indicate that it activates the IAA30 gene under high salinity, necessitating that it is released from the plasma membranes upon exposure to high salinity. To examine this, the GFP-NTM2 gene fusion was expressed transiently in Arabidopsis protoplasts and they were exposed to high salinity. We observed that whereas GFP signals were localized predominantly in the plasma membranes (Fig. 1C), a large portion of GFP signals were detected in the indicated time periods before harvesting plant materials.

The IAA30 gene is involved in the NTM2-mediated salt regulation of germination. In A to D, transcript levels were determined by qRT-PCR. Biological triplicates were averaged. Error bars indicate s.e. Statistical significance was determined by Student’s *t* test (*P* < 0.01). Two-week-old plants grown on MS-agar plates were used for treatments with NaCl and IAA. Whole plants were used for extraction of total RNA. M, Mock; S, NaCl. A, Effects of high salinity on IAA gene transcription. Plants were transferred to MS liquid cultures supplemented with 150 mM NaCl and soaked for 3 h before harvesting plant materials. B, Kinetic expression pattern of the IAA30 gene under high salinity. Plants were soaked in MS liquid cultures supplemented with 150 mM NaCl for the indicated time periods before harvesting plant materials. C, IAA30 gene transcription in germinating seeds under high salinity. Cold-imibed seeds were allowed to germinate for 30 h, and total RNA was extracted from the germinating seeds. D, Kinetic expression pattern of the IAA30 gene after auxin treatments. Plants were transferred to MS liquid cultures supplemented with 10 μM IAA and incubated for the indicated time periods before harvesting plant materials. E, Germination phenotypes of the *ntm2-1* mutant overexpressing the IAA30 gene. The *ntm2-1* mutant was transformed with the IAA30 gene driven by the CaMV 35S promoter (30OX/ntm2-1). Germination assays were carried out as described in Figure 4B. F, Electrophoretic mobility shift assays on ΔC binding to a conserved sequence in the IAA30 gene promoter. The recombinant ΔC protein was prepared as a MBP-ΔC fusion in *E. coli* cells. The minus (–) lane is a control without adding ΔC protein. Approximately 0.5 μg of the ΔC protein was used for each assay. The conserved nucleotides underlined were mutated in the mIAA30-BS sequence. Unlabeled DNA fragments were added as competitors. G, Transcriptional activation activity assays in Arabidopsis protoplasts. The IAA30-BS or mIAA30-BS sequence was fused upstream to a 35S minimal TATA promoter (Min35S)-GUS reporter construct (top panel). The reporter and the 35S:ΔC effector constructs were cotransformed into Arabidopsis protoplasts. Luciferase gene expression was used to normalize the GUS activity. Five measurements were averaged (bottom panel). Error bars indicate s.e. Statistical significance was determined by Student’s *t* test (*P* < 0.01). Nos, Terminator in the pCAMBIA 1305.1 vector.
Germination Is Delayed by Auxin under High Salinity

We found that germination was suppressed by auxin under high salinity. Therefore, it was suspected that the effects of salt on germination would be exaggerated in plants containing higher levels of active auxin.

We first examined the germination phenotype of transgenic plants overexpressing the YUCCA3 (YUC3) gene, which encodes an auxin biosynthetic enzyme (Zhao et al., 2001). As inferred from assays on the effects of auxin on germination under high salinity, germination of the 35S::YUC3 transgenic seeds was more severely suppressed by high salt than that of control seeds (Fig. 7A). In contrast, seed germination of the axr2-1 mutant, which has a gain-of-function mutation in the IAA7 gene (Nagpal et al., 2000), was uninfluenced to a discernible level by high salt. In addition, the IAA7 gene was uninfluenced by salt (data not shown), suggesting that the effect of auxin on seed germination under high salinity is not related to the IAA7 gene.

Altogether, our observations demonstrate that auxin signals are incorporated into the NTM2-mediated salt signal transduction pathway during seed germination (Fig. 7B). In this signaling cross talk, the IAA30 gene incorporates auxin and salt signals into the germination process.

DISCUSSION

NTM2-Mediated Salt Signaling in Seed Germination

Soil salinity influences a broad spectrum of physiological and developmental events in plants, from seed germination to flowering initiation (Achard et al., 2006; Jamil et al., 2006). High salinity imposes ionic and osmotic stresses on plants (Zhu, 2002; Munns and Tester, 2008). In earlier stages of plant responses, a rapid osmotic phase is caused by the osmotic pressure of high salinity in the soil. After salts accumulate above a threshold level within the plant, a slower ionic phase occurs because of the toxic effects of salt ions.

Molecular genetic and physiological studies have identified numerous genes and molecular mechanisms underlying resistance responses to salt stress. In particular, roles of ABA have been extensively studied in salt regulation of seed germination (Zhu, 2002). Furthermore, it has been shown that the ABA-mediated salt signaling during the germination process is also interconnected with other growth hormone signaling, such as GA and ethylene (Kucera et al., 2005; Holdsworth et al., 2008).

In this work, we found that a NAC transcription factor, NTM2, constitutes a salt signaling pathway functioning in seed germination. Although the NTM2 gene was induced by ABA to some degree, its role in seed germination and seedling growth under high salinity was independent of ABA. Instead, the NTM2-mediated salt signaling was linked to auxin signaling. The NTM2 gene was induced by high salinity primarily in the roots, a major plant organ that perceives soil salinity. Germination of the ntm2-1 mutant seeds was less sensitive to high salinity, and the salt resistance response disappeared in the ntm2-1 mutant complemented with a wild-type NTM2 gene, confirming the role of NTM2 in the salt regulation of seed germination.

Concerning the role of NTM2 in salt signaling, an additional factor that should be considered is its association with the plasma membranes. Whereas the full-size NTM2 protein having a putative TM motif in the C-terminal region was localized at the plasma membranes, the ΔC form lacking the TM motif was found mostly in the nucleus. Transgenic plants overexpressing the full-size NTM2 protein exhibited no discernible phenotypes, but those overexpressing the ΔC form showed distinct phenotypes, as observed with other membrane-bound NAC proteins (Kim et al., 2006, 2008; Seo et al., 2010). Therefore, it is possible that proteolytic release of the NTM2 protein from the plasma membranes would be an additional step regulated by high salinity in addition to the salt effects on NTM2 gene transcription.
The NTM2 protein is released from the plasma membranes under high salinity. It is known that membrane-bound transcription factors are released proteolytically from the membranes by either intramembrane proteases or ubiquitin/proteasome-dependent processing (Seo et al., 2008). All the NAC membrane-bound transcription factors characterized so far, such as NTM1, NTL6, and NTL8, are processed by calpain- or metalloprotease-like activities (Kim et al., 2006, 2008; Seo et al., 2010). It will be interesting to examine whether NTM2 processing is also mediated by specific proteases or by ubiquitin-mediated processing.

**Auxin and Seed Germination**

Our data demonstrate that auxin signals are interconnected with NTM2-mediated salt signaling during seed germination, in which the IAA30 gene plays a role (Fig. 7B). Whereas auxin does not influence the germination process under normal growth conditions, it acts as a negative regulator of seed germination under high salinity. Consistent with this notion, seed germination of the YUC3-overexpressing plants was more sensitive to high salinity. Histochemical assays using the DR5-GUS reporter system also showed that elevation of GUS activity in the radicles is correlated with delayed seed germination. We observed that GUS activity was elevated by high salinity as well as by auxin in the emerging radicle. It was further elevated when the seeds were allowed to germinate in the presence of both auxin and salt, the combination of which significantly delays seed germination.

However, the relationship between auxin and salt signaling during seed germination does not seem to be simple. The germination process is completed with the protrusion of the radicle through the seed coat (Finch-Savage and Leubner-Metzger, 2006), suggesting that growth potential of the emerging radicle is essential for the completion of the germination process. Therefore, a question to be answered is how the positive regulatory role of auxin on the emergence of the radicles is compromised under high salinity.

The Aux/IAA proteins are short-lived transcription factors that act as repressors of early auxin response genes by forming heterodimers with ARFs that act as positive regulators (Reed, 2001). Auxin enhances the interaction of the Aux/IAA proteins with TIR1, a component of the SCF E3 ligase complex, and directs degradation of the Aux/IAA proteins, resulting in activation of the ARF-regulated genes (Tan et al., 2007). Canonical Aux/IAA proteins are distinguished by having four conserved sequence motifs, designated domains I, II, III, and IV. It is notable that a few Aux/IAA members, including IAA30, IAA31, and IAA20, lack domain II (Dreher et al., 2006), which mediates the interaction with TIR1 (Woodward and Bartel, 2005). Accordingly, the noncanonical Aux/IAA proteins have a longer half-life (Remington et al., 2004). These noncanonical, long-lived Aux/IAA members have been suggested to play distinct roles in auxin response and signaling (Remington et al., 2004; Dreher et al., 2006).

The IAA30 gene is auxin inducible, unlike IAA20 and IAA31 (Remington et al., 2004; Dreher et al., 2006), and expressed mainly in the root apical meristem. The IAA30-overexpressing plants exhibit dwarfed growth with stunted primary root growth because of disturbed root apical meristem activity (Sato and Yamamoto, 2008). We found that it is also induced by high salt. However, the inductive effect of salt disappeared in the ntm2-1 mutant, indicating that salt induction of the IAA30 gene depends on NTM2. Notably, the inductive effect of auxin on IAA30 expression was reduced in the ntm2-1 mutant, further supporting the correlation between the IAA30-mediated auxin signal and the NTM2-mediated salt signal. Previous data and our own data suggest that high salinity, when applied together with auxin, elevates the level of active auxin and thus induces the IAA30 gene to a level that confers an inhibitory effect in the radicle. As a result, the growth potential of the radicle would be reduced, resulting in repression of the germination process.

It has been reported that endogenous auxin contents are elevated in the radicle after cold inhibition in several plant species (Bialek and Cohen, 1989; Ni et al., 2001; Liu et al., 2007), which is necessary for the growth potential of the radicle. However, further elevation of endogenous auxin contents by salt stress in the radicle would also activate negative regulators of auxin signaling, such as IAA30 (Remington et al., 2004). Therefore, it is envisioned that the IAA30-mediated cross talk between salt stress and auxin signals is an adaptation strategy that ensures the germination of seeds only under favorable growth conditions.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Arabidopsis (Arabidopsis thaliana) lines used were in the Ler background. The transposon insertion mutant ntm2-1 (CSHL-ET8732), obtained from the Arabidopsis Genetrap Database at Cold Spring Harbor Laboratory, was in the Ler ecotype. Plants were grown in a controlled culture room set at 22°C with a relative humidity of 60% under long days (16 h of light and 8 h of dark) with white light illumination (120 μmol photons m⁻² s⁻¹) provided by fluorescent FLR40D/A tubes (Osram). The absence of gene expression in the ntm2-1 mutant was verified by RT-PCR before use.

**Arabidopsis Transformation**

To produce transgenic plants overexpressing the NTM2, ΔTM, and ΔC genes, individual cDNAs were amplified by RT-PCR using SuperScript II reverse transcriptase (Invitrogen) and the Pfu Turbo DNA polymerase (Stratagene). The PCR products were subcloned into the pB2GW7 expression vector (Invitrogen; Karimi et al., 2002). Agrobacterium tumefaciens-mediated Arabidopsis transformation was performed according to a modified floral dip method (Clough and Bent, 1998). Homozygous transgenic lines were obtained by selection for three or more consecutive generations.

**Analysis of Transcript Levels**

Total RNAs were extracted from appropriate plant materials using the RNasy Plant Mini Kit (Qiagen) or from germinating seeds according to the

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Park et al.
procedure described previously (Suzuki et al., 2004). They were pretreated with an RNase-free DNase I to eliminate contaminating genomic DNA and cleaned up using the Qiagen Plant Total RNA Isolation Kit. Transcript levels were determined by qRT-PCR carried out in 96-well blocks using the Applied Biosystems 7500 Real-Time PCR System and the SYBR Green I Master Mix in a volume of 20 μL. The PCR primers were designed using the Primer Express software installed in the system and listed in Supplemental Table S1. The two-step thermal cycling profile used was 15 s at 94°C and 1 min at 68°C. Reactions were carried out in biological triplicates using plant samples harvested separately for each run. The comparative ΔΔCt method was used to evaluate relative quantities of each amplified product. The threshold cycle (Ct) was automatically determined for each reaction by the system set with default parameters. PCR specificity was determined by melt curve analysis of amplified products using the standard method installed in the system.

Histochemical Staining

Transgenic plants overexpressing the DRS-GUS reporter, in which GUS gene expression is driven by an artificial DRS auxin-responsive cis-element (Ni et al., 2001), were used. To examine the effects of IAA and high salt on GUS expression in germinating seeds, the transgenic seeds were germinated on MS-agar plates supplemented with 10 μM IAA or 150 mM NaCl or both. Transgenic plants overexpressing the pNTM2-GUS and pIAA30-GUS fusions, in which GUS gene expression is driven by the NTM2 and IAA30 gene promoters, respectively, were subject to salt treatments. Plants grown for 2 weeks on MS-agar plates were soaked for 6 h in MS liquid cultures supplemented with 150 mM NaCl.

For histochemical detection of GUS activities, plant materials were incubated in 90% acetone for 15 min on ice, washed twice with rinsing solution [50 mM sodium phosphate, pH 7.2, 0.5 mM K3Fe(CN)6, and 0.5 mM K4Fe(CN)6], and subsequently incubated at 37°C for 1 to 3 h in fresh rinsing solution containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid). The tissues were subsequently dipped in ethanol and visualized using a DIMIS-M digital camera (IMTECH).

Germination Assays

Routinely, 2-week-old, air-dried seeds were used for germination assays. Seeds were imbibed on MS-agar plates at 4°C for 3 d in complete darkness and allowed to germinate at 22°C under long days. Emergence of a visible radicle was used as a morphological marker for germination. To examine the effects of IAA, PAC, ABA, and NaCl, MS-agar plates supplemented with 0.1 to 10 mM IAA, 5 to 100 mM PAC, 0.5 to 2 mM ABA, or 50 to 150 mM NaCl were used for the germination assays. Approximately 50 to 60 seeds were routinely counted for each measurement, and three independent measurements were averaged.

Transient Expression Assays in Arabidopsis Protoplasts

For transcriptional activation activity assays, we employed a GAL4 transient expression system using Arabidopsis protoplasts (Miura et al., 2007). The N TM2 gene constructs were fused in-frame to the 3’ end of the GAL4 DNA-binding domain-coding sequence in the effector plasmid. The effector plasmid, the reporter plasmid containing the GUS reporter gene, and the plasmid containing the Renilla luciferase gene, which is used to normalize the measurements, were cotransformed into Arabidopsis protoplasts by a polyethylene glycol (PEG)-mediated transformation method (Yoo et al., 2007). GUS activities were assayed by the fluorometric method as described previously (Jefferson et al., 1987). Five measurements were averaged and statistically treated for individual N TM2 gene constructs.

To examine the transcriptional activation activity of the ΔC protein on the IAA30 gene, several reporter and effector plasmids were prepared. The pMin35S reporter plasmid contains a minimal CaMV 35S promoter (the –56 to –8 sequence region, including the TATA box) and the GUS reporter gene. In the pIAA30 reporter plasmid, a putative NAC-binding sequence (IAA30-BS) identified from the IAA30 gene promoter was fused to the 35S minimal promoter. The IAA30-BS sequence was mutated, resulting in mIAA30-BS, to verify specific binding of the ΔC protein. To construct the p35S-ΔC effector plasmid, the ΔC gene sequence was subcloned into a plant expression vector containing the CaMV 35S promoter. The reporter and effector plasmids were cotransformed into Arabidopsis protoplasts by a PEG-mediated transformation method.

Subcellular Localization of N TM2 Proteins

A GFP-coding sequence was fused in-frame to the 5’ end of the N TM2 gene sequences. The gene fusions were subcloned into the p2FGW7 expression vector (Invitrogen) and transformed into Arabidopsis protoplasts by a PEG-mediated method. The N TM2 proteins were visualized by differential interference contrast microscopy and fluorescence microscopy.

Electrophoretic Mobility Shift Assays

The ΔC gene sequence was subcloned into the pMAL-c2X Escherichia coli expression vector (NEB) containing a MBP-coding sequence. The MBP-ΔC fusion protein was purified according to the manufacturer’s instructions using the pMAL Protein Fusion and Purification System (no. E8000S). A 27-tp IA A30-BS DNA fragment was end labeled with [32P]dATP using T4 poly-nucleotide kinase. Labeled probes were incubated for 30 min at 25°C with 0.5 μg of the recombinant MBP-ΔC protein in binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol) supplemented with 100 ng of poly(dI-dC) in the presence or absence of competitor DNAs. The reaction mixtures were electrophoresed on 6% native polyacrylamide gels. The gels were dried on Whatman 3MM paper and exposed to x-ray films.

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers IAA30 (A0Jdg62100) and N TM2 (A1hgt1356).

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of N TM1 and N TM2 proteins.

Supplemental Figure S2. Mapping of the transposon insertion site and absence of N TM2 gene expression in the ntm2-1 mutant.

Supplemental Figure S3. Expression patterns of TIR-related and GH3 genes under high salinity.

Supplemental Figure S4. Effects of high salinity on IAA30 gene expression in the shoots (SH) and roots (RO).

Supplemental Figure S5. Kinetic expression patterns of IAA11, IAA19, and GH3.4 genes under high salinity.

Supplemental Figure S6. Effects of high salinity on the promoter activities of the IAA30 gene.

Supplemental Figure S7. High salinity-induced nuclear localization of N TM2.

Supplemental Table S1. List of PCR primers used in this work.

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LITERATURE CITED


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