

# Integration of Auxin and Salt Signals by the NAC Transcription Factor NTM2 during Seed Germination in *Arabidopsis*<sup>1[W]</sup>

Jungmin Park<sup>2</sup>, Youn-Sung Kim<sup>2</sup>, Sang-Gyu Kim, Jae-Hoon Jung, Je-Chang Woo, and Chung-Mo Park\*

Department of Chemistry (J.P., Y.-S.K., J.-H.J., C.-M.P.) and Plant Genomics and Breeding Institute (C.-M.P.), Seoul National University, Seoul, Korea 151-742; Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, D-07745 Jena, Germany (S.-G.K.); and Department of Biology, Mokpo National University, Jeonnam, Korea 534-729 (J.-C.W.)

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*.

Seed germination is a plant-specific developmental process during which an embryonic plant grows to form a seedling. It is critical for the establishment of plant growth in soil. As sessile organisms, most plant species cannot leave the place where their seeds germinate throughout their life span. Therefore, seeds possess an array of strategies by which they constantly monitor intrinsic and environmental conditions to determine when to germinate (Finkelstein et al., 2008).

The most critical external factors that influence germination include ambient temperature, water availability, oxygen, and soil salinity. Light intensity and quality also play a role in seed germination (Finch-Savage and

Leubner-Metzger, 2006). These environmental signals are integrated into internal developmental programs, such as growth hormone signaling, to regulate the timing of germination (Finkelstein et al., 2008). Therefore, identification of signaling molecules and elucidation of underlying molecular mechanisms linking external cues with internal growth hormonal signals are crucial for understanding the germination process.

The roles of many signaling molecules, particularly abscisic acid (ABA) and GA, have been studied for decades using mutants with defective germination phenotypes, mostly in *Arabidopsis* (*Arabidopsis thaliana*). Whereas ABA is required for seed dormancy and thus acts as a negative regulator of germination, GA promotes seed germination. Consequently, whereas mutants having defects in ABA biosynthesis exhibit a reduction in seed dormancy, those with mutations in ABA catabolic pathways show enhanced seed dormancy (Okamoto et al., 2006). Soil salinity influences seed germination in an ABA-dependent manner at least partly by repressing GA biosynthesis (Achard et al., 2006; Kim et al., 2008). Consistent with the promotive role of GA in germination, mutants lacking GA biosynthesis or defective in GA signaling do not germinate even when external conditions are favorable (Peng and Harberd, 1997; Kim et al., 2008).

Auxin plays diverse roles in virtually all aspects of plant growth and developmental processes (Weijers

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<sup>2</sup> These authors contributed equally to the article.

\* Corresponding author; e-mail cmpark@snu.ac.kr.

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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 also support its role in seed germination. 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 endogenous contents of free indole-3-acetic acid (IAA) are elevated, those of conjugated forms are reduced 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 hyposensitive 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.

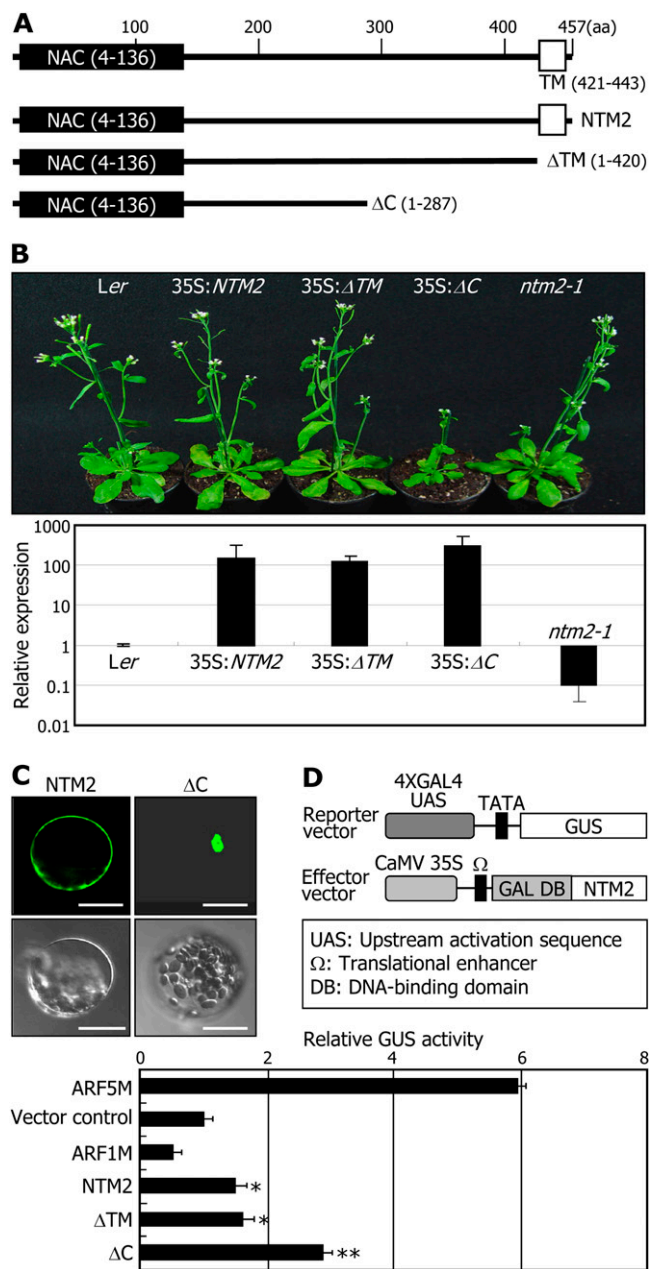
## RESULTS

### 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  $\Delta$ TM construct included residues 1 to 420 and lacked the TM motif. The  $\Delta$ C 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  $\Delta$ TM form (35S: $\Delta$ TM) were phenotypically indistinguishable from control plants (Fig. 1B). In contrast, those overexpressing the  $\Delta$ C 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  $\Delta$ C form



**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  $\Delta$ C proteins. The GFP-NTM2 and GFP- $\Delta$ C fusion constructs were expressed transiently in Arabidopsis protoplasts and visualized by fluorescence microscopy (top panels) and differential interference

contrast microscopy (bottom panels). Bars = 20  $\mu$ m. 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 (\*  $P < 0.01$ , \*\*  $P < 0.005$ ).

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  $\Delta$ C 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  $\Delta$ TM proteins possess discernible levels of transcriptional activation activities, showing that the NTM2 protein is a transcriptional activator (Fig. 1D, bottom panel). The  $\Delta$ C protein exhibited the highest activity, which was much higher than that of the  $\Delta$ TM protein, suggesting that membrane release itself is not adequate for the transcriptional activation activity. It is also envisioned that the C-terminal sequence of  $\Delta$ TM, which is missing in  $\Delta$ C, 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: $\Delta$ C 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.

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.

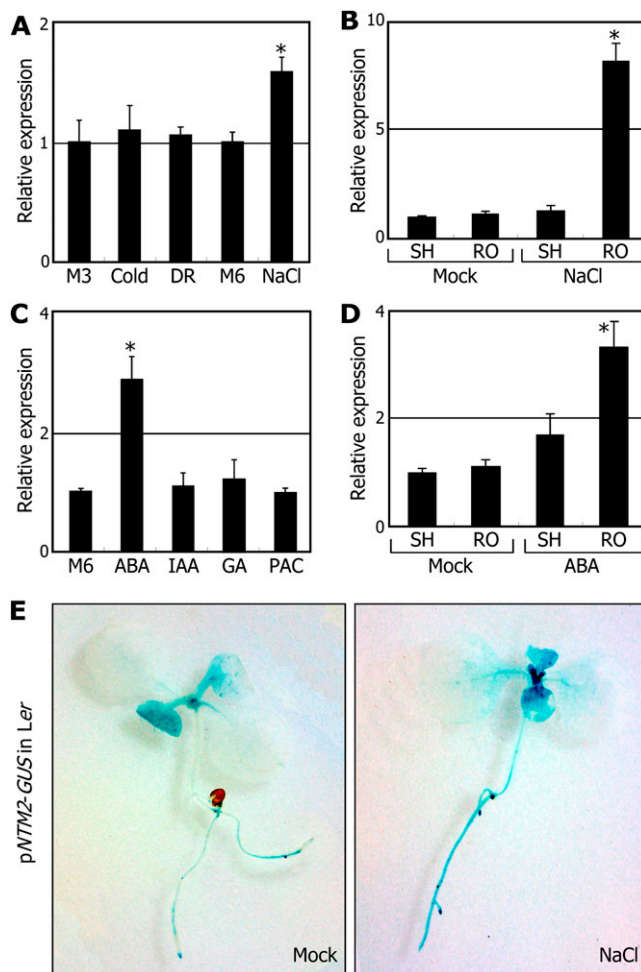
We also examined the effects of high salinity on *NTM2* gene expression using a promoter-*GUS* gene fusion, in which the *GUS*-coding sequence was fused to the 3' end of the *NTM2* gene promoter sequence covering an approximately 2-kb region upstream of the transcription start site. The promoter-*GUS* 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

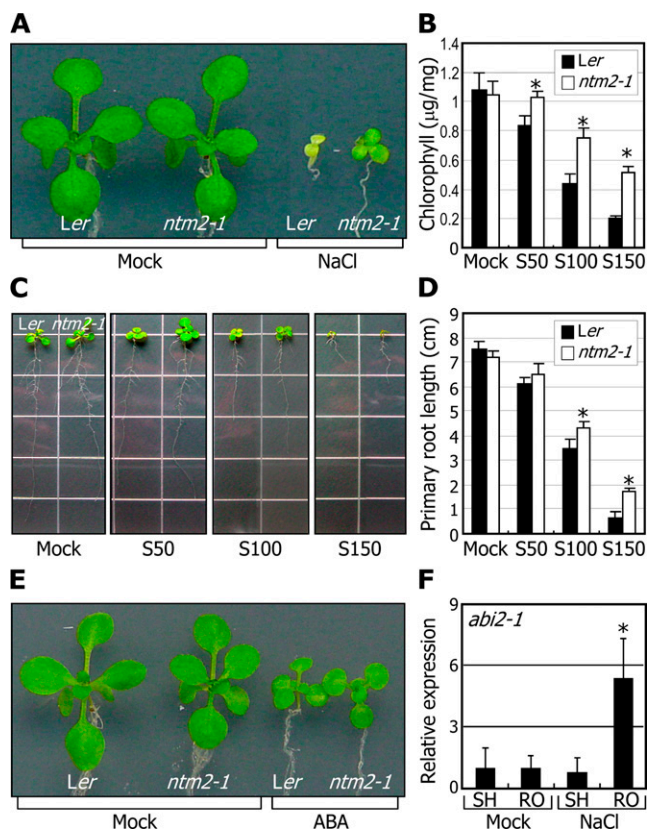
The phenotypes of 35S: $\Delta C$  transgenic plants and the effects of salt on *NTM2* expression suggested that the *NTM2* gene might be involved in the plant response to high salinity. To examine this hypothesis, we analyzed the phenotypes and salt responsiveness of a transposon insertional *NTM2* 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



**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  $\mu$ M, 6 h), IAA (20  $\mu$ M, 6 h), GA (50  $\mu$ M, 6 h), or PAC (50  $\mu$ M, 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.



**Figure 3.** The *ntm2-1* mutant exhibits reduced sensitivity to high salinity. A, Effects of high salinity on *ntm2-1* seedling growth. Three-day-old seedlings grown on MS-agar plates were transferred to fresh MS-agar plates supplemented with 150 mM NaCl and further grown for 2 weeks. B, Measurements of chlorophyll contents. Aerial parts of 2-week-old seedlings grown on MS-agar plates containing various concentrations of NaCl were used for extraction of chlorophylls. Ten measurements were averaged. Statistical significance was determined by Student's *t* test (\*  $P < 0.01$ ). Error bars indicated SE. S50, S100, and S150, NaCl at 50 mM, 100 mM, and 150 mM, respectively. C and D, Effects of high salinity on primary root growth. Three-day-old seedlings grown on MS-agar plates were transferred to fresh MS-agar plates supplemented with various concentrations of NaCl and further grown for 10 d (C). Primary root lengths of 20 seedlings were measured and averaged (D). Error bars represent SE (*t* test; \*  $P < 0.01$ ). E, Effects of ABA on *ntm2-1* seedling growth. Plants were treated as described in A, but 0.6  $\mu$ M ABA was used instead of NaCl. F, Effects of high salinity on *NTM2* gene transcription in the *abi2-1* mutant. Two-week-old plants grown on MS-agar plates were transferred to MS liquid cultures supplemented with 150 mM NaCl and soaked for 6 h. The shoot (SH) and root (RO) samples were harvested separately for extraction of total RNA. Levels of the *NTM2* transcripts were determined by qRT-PCR. Biological triplicates were averaged. Error bars indicate SE (*t* test; \*  $P < 0.01$ ).

*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.

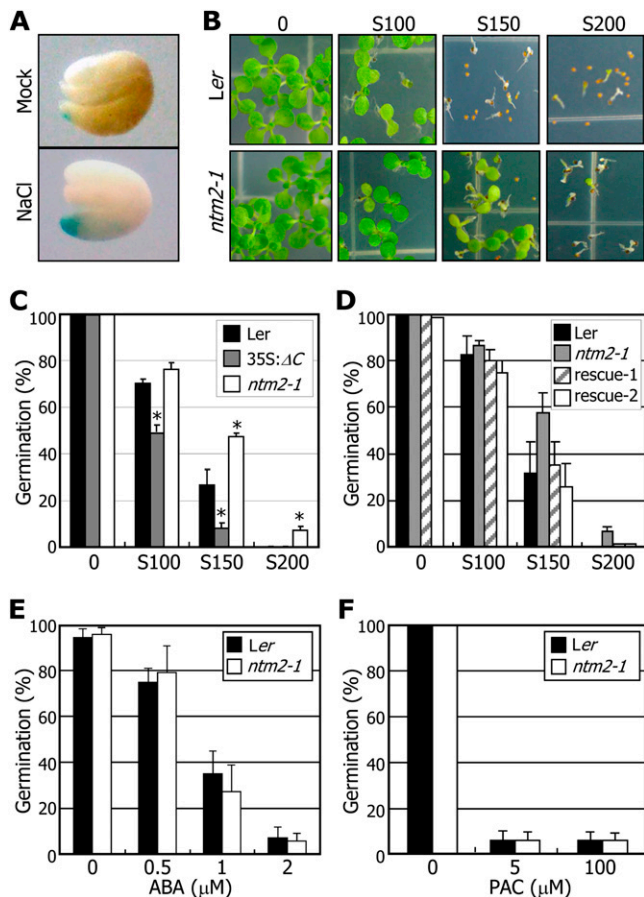
We first examined the expression pattern of the *NTM2* gene in germinating seeds of transgenic plants expressing the p*NTM2*-*GUS* construct. Reporter gene expression was detected at a low level in germinating seeds under normal conditions (Fig. 4A). In contrast, it was highly induced in the emerging 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: $\Delta$ C 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 p*NTM2*-*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: $\Delta$ C transgenic plants. In addition, seedling growth and seed germination of the *ntm2-1*

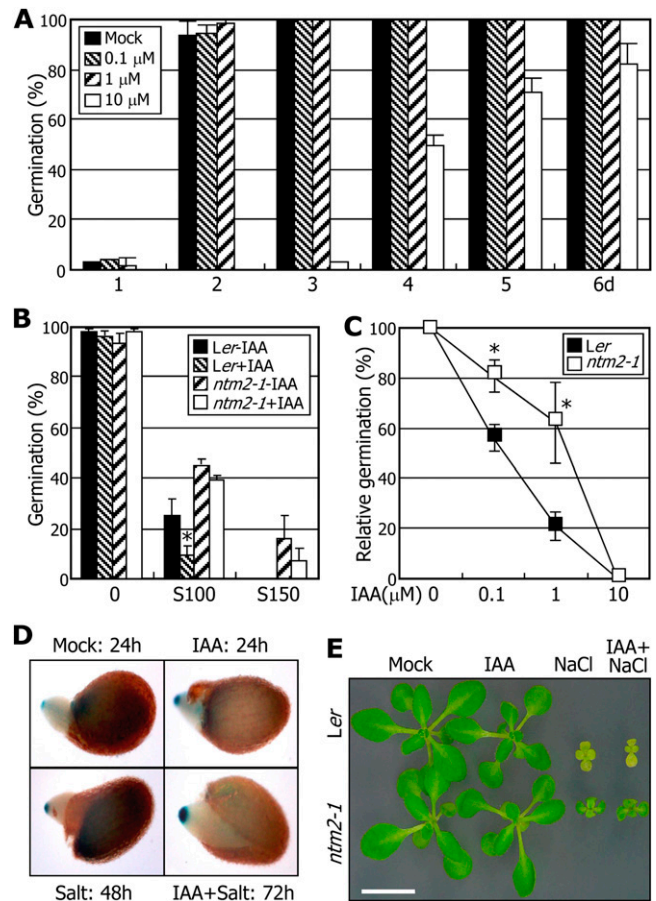


**Figure 4.** Germination of the *ntm2-1* seeds is less sensitive to high salinity. **A**, *NTM2* expression in germinating seeds. Seeds of transgenic plants expressing the p*NTM2*-*GUS* reporter were cold imbibed and allowed to germinate for 2 d on MS-agar plates containing 150 mM NaCl before *GUS* staining. **B** and **C**, Effects of high salinity on the germination of *ntm2-1* seeds. Cold-imbibed seeds were germinated on MS-agar plates containing varying concentrations of NaCl and photographed 7 d after cold imbibition (**B**). Germination percentages were calculated 4 d after cold imbibition (**C**). Radicle emergence was used as a morphological marker for germination. Three measurements, each consisting of approximately 50 to 60 seeds, were averaged for each plant group. Error bars indicate  $SE$  (\*  $P < 0.01$ ). S100, S150, and S200, NaCl at 100 mM, 150 mM, and 200 mM, respectively. **D**, Complementation of the *ntm2-1* mutant. The *ntm2-1* mutant was transformed with the *NTM2* gene with its own promoter, resulting in p*NTM2*:*NTM2*/*ntm2-1* (rescue). Two independent lines were subject to germination assays as described in **B**. **E**, Effects of ABA on the germination of *ntm2-1* seeds. Germination assays were carried out as described in **B**, but ABA was used instead of NaCl. **F**, Effects of PAC on the germination of *ntm2-1* seeds. Germination assays were carried out as described in **B**, but PAC was used instead of NaCl.

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  $\mu$ M IAA (Fig. 5A). Interestingly, when counted 2 d after cold imbibition under high salinity, the repressive effects of high salinity on germination were exagger-

ated 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



**Figure 5.** Auxin influences the germination of the *ntm2-1* seeds under high salinity. **A**, Effects of auxin on the germination of *Ler* seeds. Seeds were cold imbibed and germinated on MS-agar plates supplemented with varying concentrations of IAA. Germination assays were carried out as described in Figure 4B. **B** and **C**, Effects of auxin on the germination of *ntm2-1* seeds under high salinity. Seeds were cold imbibed and germinated on MS-agar plates supplemented with 1 mM IAA and varying concentrations of NaCl (**B**) or on MS-agar plates supplemented with 150 mM NaCl and varying concentrations of IAA (**C**). Germination assays were carried out as described in Figure 4B, except that germination percentages were calculated 2 d after cold imbibition. Error bars indicate  $SE$ . Statistical significance was determined by Student's *t* test (\*  $P < 0.01$ ). S100 and S150, NaCl at 100 mM and 150 mM, respectively. In **C**, germination percentages were calculated relative to those on NaCl at 150 mM without auxin. **D**, Effects of auxin and salt on *DR5*-*GUS* expression. Cold-imbibed seeds of *DR5*-*GUS* plants were allowed to germinate for the indicated time periods on MS-agar plates supplemented with 10  $\mu$ M IAA or 150 mM NaCl or both. Germinating seeds at similar germination stages were subjected to *GUS* staining. **E**, Effects of auxin on *ntm2-1* seedling growth under high salinity. Seedlings were grown for 2 weeks on MS-agar plates supplemented with 10  $\mu$ M IAA or 100 mM NaCl or both. Bar = 10 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  $\mu$ M 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.

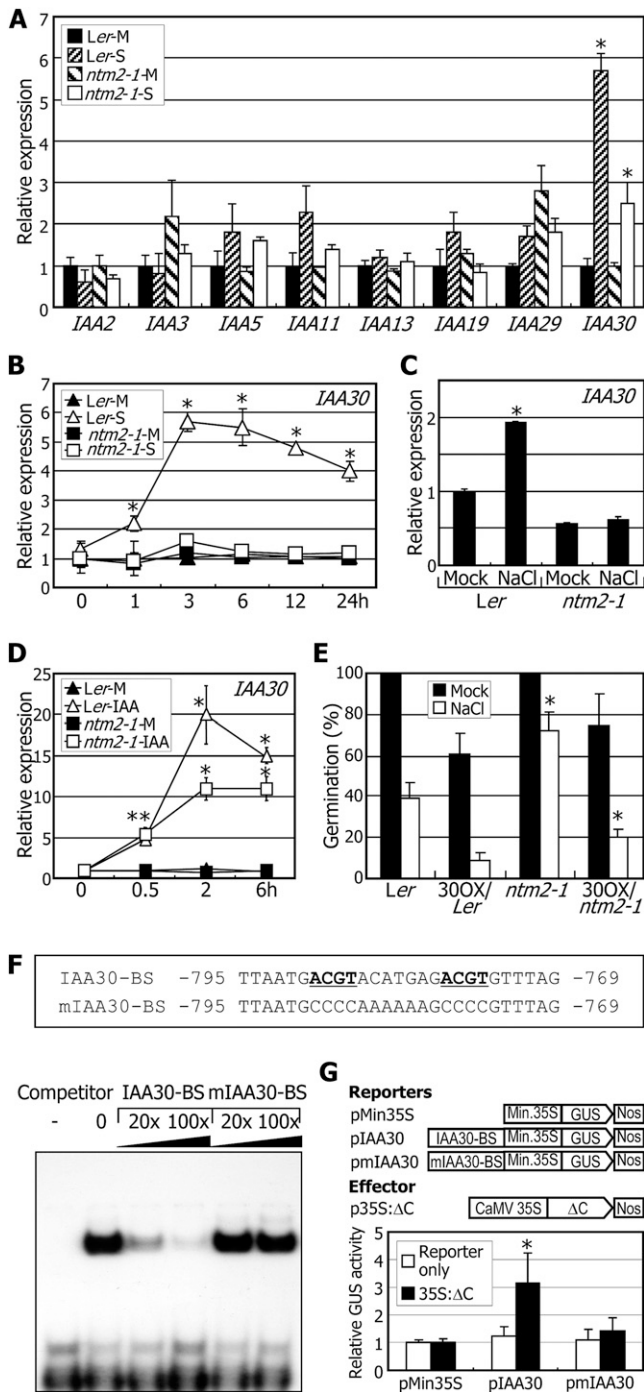
We next asked whether the NTM2 dependence of the *IAA30* gene expression occurred in the germinating seeds. Cold-imbibed seeds were allowed to germinate for 30 h under high salinity, and the *IAA30* transcript levels were measured in the germinating seeds. The results showed that whereas the transcript 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: $\Delta$ C 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



**Figure 6.** 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 *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 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

(Fig. 6F), 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, this binding was reduced significantly in the presence of excess amounts of unlabeled 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

the indicated time periods before harvesting plant materials. C, *IAA30* gene transcription in germinating seeds under high salinity. Cold-imbibed 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 *se*. Statistical significance was determined by Student's *t* test (\* *P* < 0.01). Nos, Terminator in the pCambia 1305.1 vector.



nucleus after salt treatments (Supplemental Fig. S7), indicating that NTM2 processing is induced by high salinity.

### 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

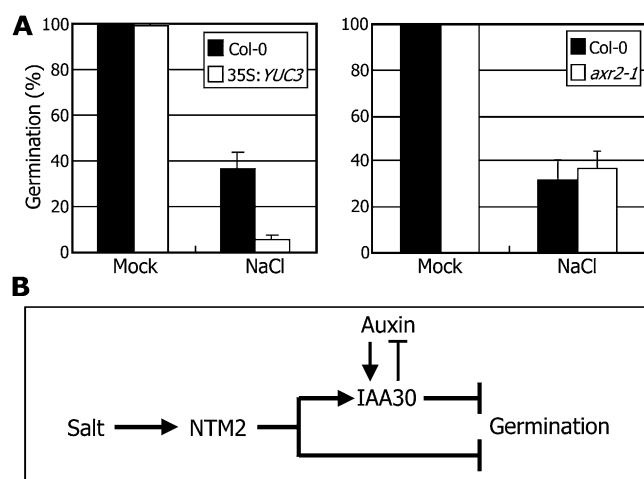
### NTM2-Mediated Salt Signaling in Seed Germination

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  $\Delta C$  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  $\Delta C$  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.



**Figure 7.** Auxin interacts with NTM2-mediated salt stress signals during seed germination. A, Germination phenotypes of auxin-related mutants under high salinity. Seeds of *axr2-1* mutants and transgenic plants overexpressing the *YUC3* gene (At1G04610) driven by the CaMV 35S promoter were cold imbibed and germinated on MS-agar plates supplemented with 150 mM NaCl. Germination assays were carried out as described in Figure 4B. Error bars indicate SE. Statistical significance was determined by Student's *t* test (\*  $P < 0.01$ ). Col-0, Ecotype Columbia. B, Schematic working model of NTM2 during seed germination under high salinity. A part of the NTM2-mediated salt signals is mediated by *IAA30*, which functions as a negative regulator of auxin signaling.

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 non-canonical, 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 imbibition 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  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) 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*,  $\Delta\text{TM}$ , and  $\Delta\text{C}$  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 under the control of the CaMV 35S promoter using the Gateway vector system (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 RNeasy Plant Mini Kit (Qiagen) or from germinating seeds according to the

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  $\mu$ L. 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  $\Delta\Delta C_T$  method was used to evaluate relative quantities of each amplified product. The threshold cycle ( $C_T$ ) 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 DR5-GUS reporter, in which *GUS* gene expression is driven by an artificial DR5 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  $\mu$ M 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  $K_3Fe(CN)_6$ , and 0.5 mM  $K_4Fe(CN)_6$ ], and subsequently incubated at 37°C for 1 to 3 h in fresh rinsing solution containing 2 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (Duchefa). They were subsequently dipped in ethanol and visualized using a DIMIS-M digital camera (JMTECH).

## 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  $\mu$ M IAA, 5 to 100  $\mu$ M PAC, 0.5 to 2  $\mu$ M 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 *NTM2* 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 *NTM2* gene constructs.

To examine the transcriptional activation activity of the  $\Delta C$  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  $\Delta C$  protein. To construct the p35S: $\Delta C$  effector plasmid, the  $\Delta C$  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 NTM2 Proteins

A GFP-coding sequence was fused in-frame to the 5' end of the *NTM2* gene sequences. The gene fusions were subcloned into the p2FGW7 expression vector (Invitrogen) and transformed into Arabidopsis protoplasts by a PEG-mediated method. The *NTM2* proteins were visualized by differential interference contrast microscopy and fluorescence microscopy.

## Electrophoretic Mobility Shift Assays

The  $\Delta C$  gene sequence was subcloned into the pMAL-c2X *Escherichia coli* expression vector (NEB) containing a MBP-coding sequence. The MBP- $\Delta C$  fusion protein was purified according to the manufacturer's instructions using the pMAL Protein Fusion and Purification System (no. E8000S). A 27-bp IAA30-BS DNA fragment was end labeled with [ $^{32}$ P] $\gamma$ -ATP using T4 polynucleotide kinase. Labeled probes were incubated for 30 min at 25°C with 0.5  $\mu$ g of the recombinant MBP- $\Delta C$  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 data libraries under accession numbers IAA30 (At3g62100) and *NTM2* (At4g01550).

## Supplemental Data

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

**Supplemental Figure S1.** Amino acid sequence alignment of *NTM1* and *NTM2* proteins.

**Supplemental Figure S2.** Mapping of the transposon insertion site and absence of *NTM2* 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 *NTM2*.

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

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