

Exploring membrane-associated NAC transcription factors in *Arabidopsis*: implications for membrane biology in genome regulation

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ABSTRACT

Controlled proteolytic cleavage of membrane-associated transcription factors (MTFs) is an intriguing activation strategy that ensures rapid transcriptional responses to incoming stimuli. Several MTFs are known to regulate diverse cellular functions in prokaryotes, yeast, and animals. In *Arabidopsis*, a few NAC MTFs mediate either cytokinin signaling during cell division or endoplasmic reticulum (ER) stress responses. Through genome-wide analysis, it was found that at least 13 members of the NAC family in *Arabidopsis* contain strong α -helical transmembrane motifs (TMs) in their C-terminal regions and are predicted to be membrane-associated. Interestingly, most of the putative NAC MTF genes are up-regulated by stress conditions, suggesting that they may be involved in stress responses. Notably, transgenic studies revealed that membrane release is essential for the function of NAC MTFs. Transgenic plants overexpressing partial-size NAC constructs devoid of the TMs, but not those overexpressing full-size constructs, showed distinct phenotypic changes, including dwarfed growth and delayed flowering. The rice genome also contains more than six NAC MTFs. Furthermore, the presence of numerous MTFs is predicted in the whole transcription factors in plants. We thus propose that proteolytic activation of MTFs is a genome-wide mechanism regulating plant genomes.

INTRODUCTION

Transcriptional responses are regulated at multiple steps, including expression of genes encoding transcription factors

and translocation of transcription factors from the cytoplasm to the nucleus. In many cases, transcription factors are stored in their dormant forms in the cytoplasm, and upon stimulation, they are activated and localized into the nucleus. Regulated activation of preexisting dormant transcription factors certainly provides an efficient way of gene regulation and is considered to be an adaptive strategy that undertakes prompt responses to environmental changes (1–4).

There are several mechanisms for activating dormant transcription factors. In one example, they are activated by post-translational modifications, such as protein phosphorylation. This has been proven in the case of the STAT transcription factors that are activated through JAK-mediated phosphorylation (2,3). In another example, such as the NF- κ B/Rel transcription factors that are anchored to I κ B α , the dormant transcription factors are activated after the degradation of the cytoplasmic anchors (5). The most striking example is the activation of membrane-associated transcription factors (MTFs) that are expressed as dormant precursors and integrated into the intracellular membranes. They are activated by controlled proteolytic cleavage through either one of two distinct, but biochemically related pathways. In regulated ubiquitin (Ub)/proteasome-dependent processing (RUP), they are ubiquitinated and partially degraded by the 26S proteasome in a tightly controlled manner, resulting in the release of transcriptionally active forms (6). In regulated intramembrane proteolysis (RIP), active forms are released by specific membrane-associated proteases (2,4).

It is remarkable that Ub-mediated protein degradation is directly linked to the activation of dormant proteins as well as to the inactivation of unnecessary proteins. One example of RUP is the activation of the SPT23/MGA2 transcription factor in yeast. SPT23/MGA2 is necessary for *OLE1* expression in the *OLE* pathway that regulates membrane fluidity (6). It is expressed as a dormant ER/nuclear membrane-associated precursor (p120), from which a transcriptionally active form (p90) is released by RUP. The RUP-mediated p120 processing is abolished by unsaturated fatty acids. However,

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors

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RUP-mediated MTF activation has not yet been reported in higher eukaryotes.

Several MTFs have been shown to be activated by RIP in prokaryotes and animals, among which the SRE-binding protein (SREBP) transcription factor has been most extensively studied (1). The RIP activity has also been implicated in the activation of an *Arabidopsis* NAC MTF, NTM1 (www.plantcell.org/cgi/doi/10.1105/tpc.106.043018) (7). After release from the ER/nuclear membranes by calpain or its functional homolog, the activated NTM1 form enters the nucleus and activates a subset of CDK inhibitor genes, e.g. KRPs, thereby resulting in reduced cell division (8). One additional NAC MTF has been recently characterized in *Arabidopsis*. A transcription factor AtbZIP60 has been predicted to be membrane-associated and regulate ER stress responses (9). Although the nature of the activation process is unknown, it is apparent that membrane release is essential for the AtbZIP60 function. To our knowledge, NTM1 is the only plant MTF whose activation mechanism and physiological role have been studied in detail (7).

NTM1 belongs to the NAC transcription factor family that are unique to plants (10,11). The NAC proteins contain a highly conserved NAC DNA-binding domain (DBD) that consists of approximately 160 residues in their N-terminal regions. The transcriptional activities reside in their C-terminal regions, although the C-terminal sequences are quite diverse. The NAC family is one of the largest transcription factor families in plant genomes. There are approximately a hundred of NAC transcription factors in each of the *Arabidopsis* and rice genomes (12,13). Several NAC members have been functionally studied in floral development (14), apical meristem formation (15), growth hormone signaling (16,17), ER stress responses (9) and cell-cycle control (7). However, most NAC transcription factors have not yet been functionally characterized, and the protein structures of the NACs have not been thoroughly examined.

In this study, we analyzed the protein structures of the *Arabidopsis* and rice NACs through the ARAMEMNON membrane protein database (18) and found that more than 13 NAC members in *Arabidopsis* and 6 NAC members in rice possess strong α -helical TMs like the NTM1 structure. The putative membrane-associated NACs were designated *NTLs* for *NTM1*-like in this report. Molecular and transgenic studies revealed that membrane release is a prerequisite for the *NTL* function and that the *NTLs* play regulatory roles in diverse plant growth and developmental processes, such as stress signaling and flowering initiation. Furthermore, a significant number of plant transcription factors were predicted to be anchored to the intracellular membranes, thus indicating that proteolytic activation of MTFs is a regulatory scheme that occurs widely in plant genomes.

MATERIALS AND METHODS

Bioinformatics softwares

The amino acid sequences of the *NTLs* were compared using BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>), and their phylogenetic relationships were inferred using PHYLIP (<http://evolution.genetics.washington.edu/phylip.html>). Protein domain predictions were performed using the softwares available

in the ExPASy database (<http://us.expasy.org/tools/>). TMs were predicted from the NAC proteins and other transcription factors using the ARAMEMNON membrane protein database (<http://aramemnon.botanik.uni-koeln.de/>) (18). The *NTL* gene structures were obtained from the TAIR and MIPS databases (<http://www.arabidopsis.org/>, <http://mips.gsf.de/>).

Plant materials, growth conditions and *Arabidopsis* transformation

The *Arabidopsis thaliana* lines used were in the Columbia ecotype (Col-0). Plants were grown in a controlled culture room at 23°C and a relative humidity of 60% under long day (LD) conditions (16 h light and 8 h dark) with white light illumination (110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). *Agrobacterium*-mediated transformation of *Arabidopsis* plants was carried out by a modified floral dip method (19).

Transcript level analysis

Semiquantitative RT-PCR was employed for analysis of transcript levels. Total RNA samples were extracted from plant materials using the RNeasy Plant Total RNA Isolation Kit (Qiagen). Prior to use, the total RNA samples were extensively pretreated with RNase-free DNase I to eliminate any contaminating genomic DNA. The first-strand cDNA was synthesized from 1–2 μg of total RNA in a 20 μl reaction volume using the Superscript II reverse transcriptase (Invitrogen), and 2 μl of the reaction mixture was subsequently used for RT-PCR runs in a 50 μl reaction volume. The RT-PCR runs consisted of 15–35 cycles, depending on the linear range of PCR amplification for each gene. Each PCR cycle included incubations at 94°C for 1 min, at 55°C for 30 s, and at 72°C for 3 min. One additional cycle at 72°C for 7 min was run after the last cycle to allow trimming of incomplete polymerizations. Whenever possible, positive and negative control genes were included in the reactions to assure the feasibility of the assay conditions.

RT-PCR-based Southern blot hybridizations were carried out using gene-specific probes that had been labeled with digoxigenin-UTP by the DIG RNA Labeling Kit according to the procedure provided by the manufacturer (Roche Applied Science).

Abiotic stress treatments

Wild-type *Arabidopsis* plants that were grown for 2 weeks on Murashige and Skoog (MS)-agar plates were used for all abiotic stress treatments. For cold and heat treatments, plants were incubated at either 4°C for 24 h or at 37°C for 1 h before harvesting plant materials, respectively. For drought stress treatments, plants were dehydrated on dry 3 MM filters for the indicated time periods. To examine the effects of NaCl on the *NTL* expression, plants were transferred to a hydroponic growth medium containing 100 or 200 mM NaCl and gently shaken for 5 h before harvesting plant materials.

For methylmethanesulfonate (MMS) treatments, plants were transferred to a hydroponic growth medium containing 100 p.p.m. MMS and incubated for 1–19 h. For hydrogen peroxide treatments, plants were transferred to a hydroponic growth medium containing 50 mM H_2O_2 and subjected to vacuum for 5 min followed by sudden release. They were

subsequently incubated under normal growth conditions for the indicated time periods.

Flowering time measurements

The *NTL8* transgenic plants were grown under LD conditions for flowering time measurements. The number of rosette leaves at floral initiation and the days to the first floral bud formation were used to measure flowering times.

Tissue-specific expression of *NTL6*

A GUS (β -glucuronidase)-coding sequence was transcriptionally fused to the *NTL6* promoter (*pNTL6*, base positions +1 to -1980). The *pNTL6-GUS* construct was transformed into *Arabidopsis* plants, and homozygotic plants were subjected to GUS staining. The stained plants were examined by bright-field microscopy.

Analysis of MTF processing

Six copies of the *myc*-coding sequences were in-frame fused to the 5' ends of the full-size *Arabidopsis* and rice *NTL* genes. The *myc-NTL* gene fusion constructs were directly infiltrated into *Nicotiana benthamiana* leaves as described previously (20), and the leaves were incubated at room temperature for 24 h. The plant leaves were then ground in liquid nitrogen, and total proteins were extracted in 1 \times SDS-PAGE buffer. The protein samples were analyzed on 12% SDS-PAGE gels and blotted onto Hybond-P⁺ membranes (Amersham-Pharmacia Biotech). The blots were hybridized with a polyclonal anti-*myc* antibody (Santa Cruz Biotechnology).

Transcriptional activity assays and cell fractionation analysis

The transcriptional activity assays were carried out as described previously (7) using the pGBKT7 vector and the yeast strain AH109 (Clontech, Palo Alto, CA). The cell fractionation assays were carried out as described previously using transgenic *Arabidopsis* plants overexpressing a *myc-NTL8* gene fusion under the control of the CaMV 35S promoter.

RESULTS

Arabidopsis genome contains more than thirteen NAC MTFs

RIP-mediated MTF activation has been characterized in several prokaryotic and animal transcription factors (1). RUP mediates the activation of the SPT23/MGA2 transcription factor in yeast (6). In plants, NTM1 is the only MTF that has been characterized at the molecular level (7). NTM1 belongs to the plant-specific NAC transcription factor family. However, it is unique among the characterized NAC proteins in that it contains a strong TM and is associated with the intracellular membranes. Meanwhile, a variety of genome-wide gene expression analysis have shown that a number of NACs are influenced by various biotic and abiotic stresses (21–23), suggesting that they may be involved in plant stress responses and signaling. Considering the physiological nature of the MTF activation to be an adaptive strategy for quick responses to adverse growth conditions, we anticipated that

other NAC members might also be regulated by the membranes like NTM1.

To examine this hypothesis, the protein structures of all the *Arabidopsis* NAC proteins were analyzed using the ARAMEMNON membrane protein database (18). Interestingly, a significant number of NAC proteins had a structural organization similar to that of NTM1: a NAC domain was present in their N-terminal regions and a strong α -helical TM was found in their C-terminal regions. For further analysis, we selected 13 members whose TMs had the hydrophobicity values of >0.85 (Figure 1A). The NTM1-like (*NTL*) genes are distributed throughout the five chromosomes with the exception of *NTL12* and *NTL13* that are located close together on chromosome 4. Furthermore, the *NTL* gene structures are quite diverse and have varying numbers of exons, ranging from three to seven (Figure 1B).

Although the sizes of the *NTL* proteins are quite variable, consisting of 335–652 residues, they all have a common structural organization in which the NAC domains are located in their N-terminal regions and the TMs are located in their far C-terminal regions (Figure 1C). However, the sequences that lie between the NAC domains and the TMs differ in amino acid sequences and apparently represent the size variations among the *NTL* proteins. Membrane-free NACs that have been functionally characterized so far consist of ~320–330 residues (24–26). A transcriptionally active form of NTM1 also contains 322 residues (7). These indicate that the C-terminal sequences removed by the cleavage events vary among the *NTLs*.

NTLs are classified into four phylogenetic subgroups

To determine the phylogenetic relationships among the thirteen *NTLs*, their amino acid sequences were analyzed using PHYLIP. Since the sequence similarities are restricted to the NAC domains in most *NTLs*, the phylogeny would mainly reflect the sequence similarities within their NAC domains. The *NTLs* were evidently classified into four phylogenetic subgroups (Figure 2A). However, the phylogenetic relationships were not strictly related to the protein sizes, gene structures or chromosomal locations of the *NTLs* (Figure 2B). Furthermore, the *NTL* pairs that showed close phylogenetic relationships did not always exhibit similar expression profiles (see below). Collectively, these observations suggest that each *NTL* may have a distinct role, although some degree of functional redundancy among them cannot be excluded.

The whole NAC proteins in *Arabidopsis* and rice have been classified into two major groups, I and II, that include 14 and 4 subgroups, respectively (10). Interestingly, the majority of the *NTLs* are clustered within a few subgroups of group I, such as NAC2, TIP, and OsNAC8 (Supplementary Figure S1). The *NTLs* may be related with the functional distinction of these NAC subgroups.

Each *NTL* gene exhibits a distinct expression pattern

To obtain insights into the physiological roles of the *NTLs*, their expression patterns were examined in terms of tissue specificity and growth stage-dependence. The transcript levels of the *NTLs* were so low that they could not be discernibly detected by northern blot hybridization. We therefore employed semiquantitative RT-PCR to compare the *NTL*

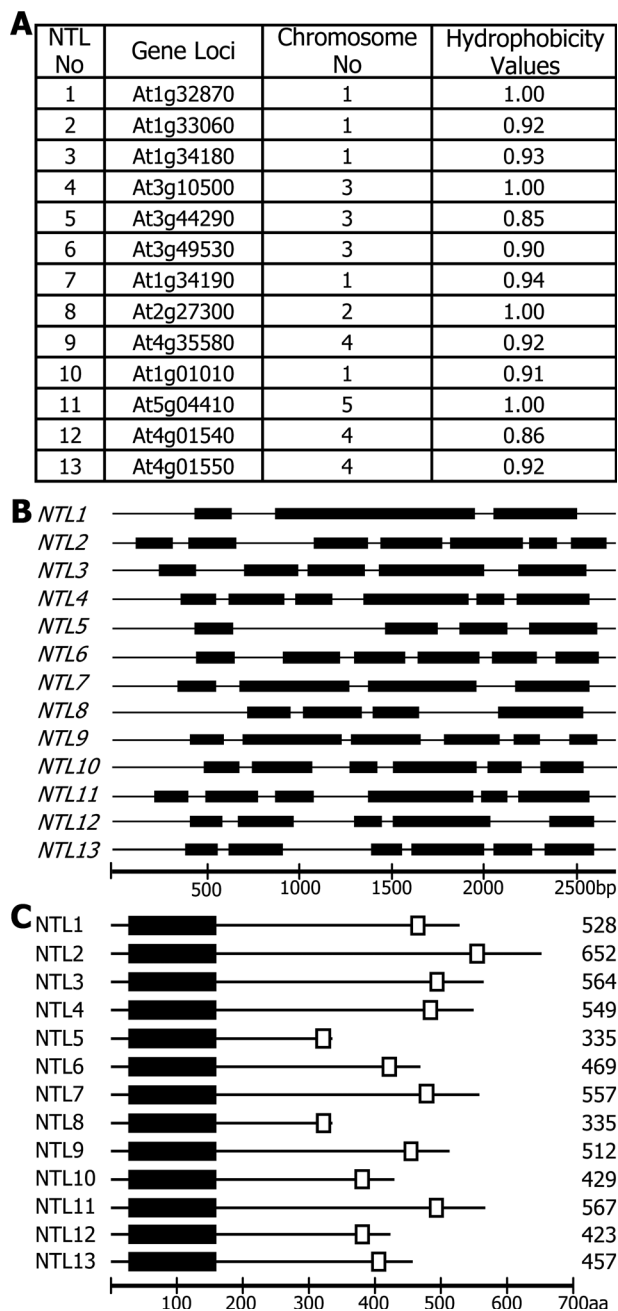


Figure 1. *NTL* genes and protein structures of *NTLs*. (A) *Arabidopsis NTL* genes. The presence of the TMs in the *Arabidopsis* NAC transcription factors was examined in the membrane protein database ARAMEMNON. At least thirteen NAC members were predicted to be MTFs. *NTL12* and *NTL13* are equivalent to NTM1 and NTM2, respectively (7). (B) *NTL* gene structures. They consist of 3–7 exons. (C) Protein structures of *NTLs*. The highly conserved NAC domains are present in their N-terminal regions (black boxes). The α -helical TMs (open boxes) are located in their far C-terminal regions.

transcript levels. While most *NTLs* were expressed to relatively high levels in leaves, stems and in roots, the expression levels were low in flower and shoot apex tissues (Figure 3A). Together with their induction by stress conditions (see below), this observation suggests that *NTLs* may affect growth and developmental processes of vegetative organs in response to external signals.

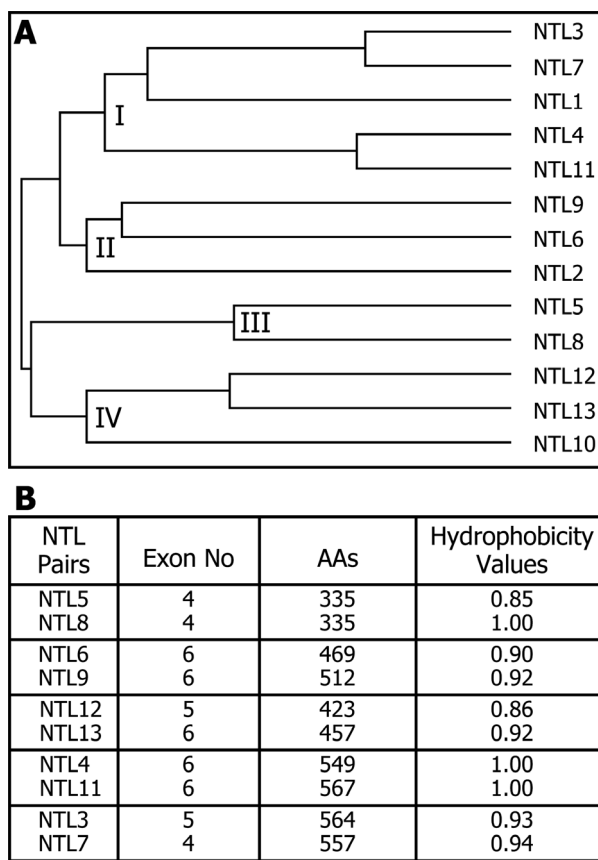


Figure 2. Phylogenetic analysis of *NTLs*. (A) Phylogenetic relationships. The phylogenetic relationships were inferred using the PHYLIP program. The *NTL* proteins are apparently classified into 4 phylogenetic subgroups (I–IV). (B) *NTL* pairs with close relationships.

NTL4, *NTL7* and *NTL11* were expressed to relatively high levels, and the expression levels were further elevated throughout the life span (Figure 3B). In contrast, the expression levels of *NTL2* and *NTL9* were low, and they were constantly expressed from germination to flowering. *NTL3*, *NTL5*, *NTL6* and *NTL10* were expressed to low levels in young seedlings, but the levels rapidly increased as plants grew. The diverse tissue-specific and growth stage-dependent expression profiles may represent distinct roles of individual *NTLs*.

NTL genes are affected by abiotic stresses

Numerous transcriptome studies have shown that many NAC genes are regulated by diverse biotic and abiotic stresses (21–23), suggesting that they may have a role in stress responses and signalings. Furthermore, the expression of many *NTLs* are altered in stressed plants (21,27,28). We observed that most *NTLs* are highly expressed in vegetative organs that are more susceptible to abiotic stress conditions (Figure 3A).

To further examine the effects of abiotic stresses on the *NTL* expression, wild-type plants were subjected to various stress conditions, including drought, cold, heat and high-salinity, and the *NTL* expression was analyzed by semiquantitative RT–PCR runs. Interestingly, *NTLs* responded differentially to different abiotic stresses (Figure 3C and D).

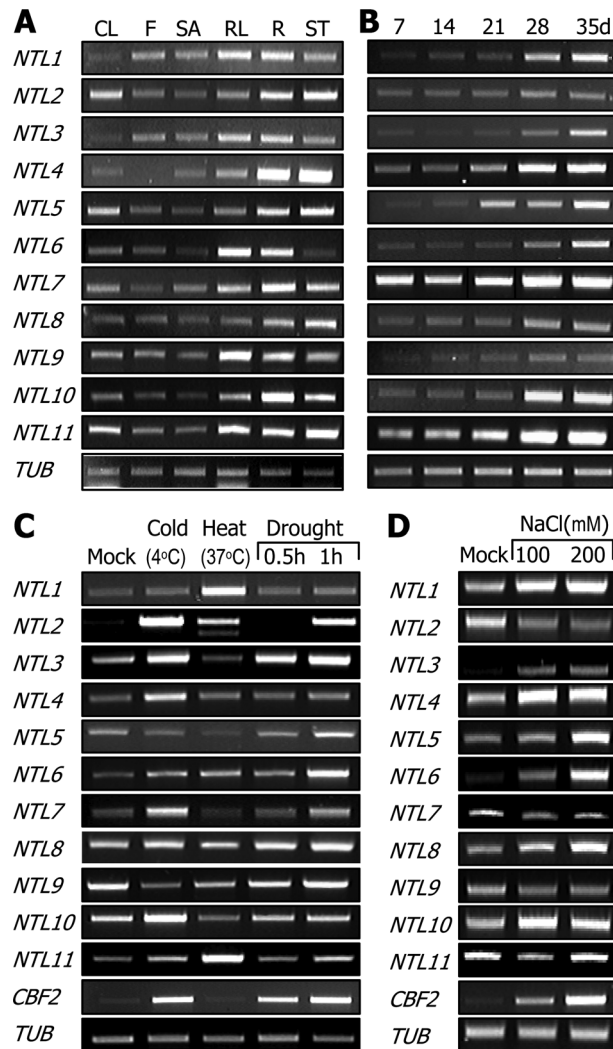


Figure 3. Expression patterns of *NTLs* and their responses to abiotic stresses. (A) Tissue-specific expression patterns. Total RNA samples were separately prepared from cauline leaves (CL), flowers (F), shoot apices (SA), rosette leaves (RL), roots (R) and stems (ST). A tubulin gene (*TUB*) was included as a control for constitutive expression. (B) Growth stage-dependent expression patterns. Plants were harvested at the indicated time intervals throughout the life span for total RNA extraction. (C) Effects of abiotic stresses on *NTL* expression. Plants grown for 2 weeks on MS-agar plates were incubated at 4°C (cold) for 12 h or 37°C (heat) for 1 h before harvesting plant materials. To examine the effects of drought stress, plants were dehydrated on dry 3 MM paper for 30 or 60 min. *CBF2* was included as a positive control. (D) NaCl effects on *NTL* expression.

NTL1 and *NTL11* were induced primarily by heat (37°C), and *NTL4* and *NTL7* were induced specifically by cold (4°C). Meanwhile, *NTL3* and *NTL6* were dramatically up-regulated by NaCl in a similar kinetics to that of *CBF2* (Figure 3D). Notably, some *NTLs*, such as *NTL2* and *NTL3*, were broadly influenced by cold, drought, and NaCl. The various expression profiles may be associated with the distinct roles of individual *NTLs* in diverse abiotic stress responses.

Notably, most of the *NTLs* affected by various stress conditions (21–23) were also influenced in similar patterns in our assay conditions (Supplementary Figure S2), supporting the feasibility of our assay system and regulatory roles for *NTLs* in stress signaling in plants.

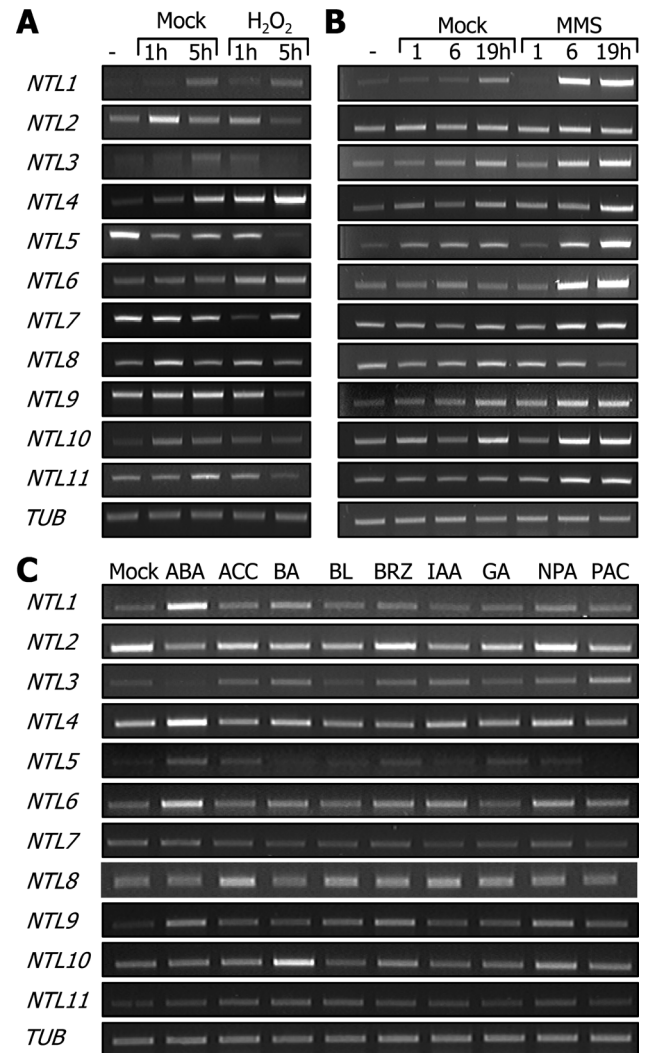


Figure 4. Regulation of *NTL* expressions by H_2O_2 , MMS and growth hormones. (A) Effects of H_2O_2 . Plants were subjected to a vacuum/release cycle in hydroponic MS media supplemented with 50 mM H_2O_2 and subsequently grown under normal growth conditions for the indicated time periods. (B) Effects of MMS. Plants were incubated in hydroponic MS media containing 100 p.p.m. MMS for the indicated time periods. (C) Growth hormone effects. Plants that were grown for 2 weeks on MS-agar plates were incubated in hydroponic MS media supplemented with appropriate growth regulators. They were used at the final concentrations of 100 μ M for ABA and indole-3-acetic acid (IAA), 50 μ M for 1-aminocyclopropane-1-carboxylic acid (ACC), BA and paclobutrazol (PAC), 20 μ M for gibberellic acid (GA) and 1-N-naphthylphthalamic acid (NPA), and 1 μ M for brassinolide (BL) and brassinazole (BRZ).

NTL genes are activated by MMS

Responses of the *NTL* genes to hydrogen peroxide (H_2O_2) were not prominent except in the case of *NTL4* that was slightly but reproducibly induced by this treatment. *NTL2*, *NTL9* and *NTL11* were only marginally repressed by the treatment (Figure 4A). It appears that *NTLs* are not directly involved in responses to reactive oxygen species (ROS).

Plant responses to genotoxic agents, including MMS and cyclophosphamide (CP) that alkylate nucleophilic bioorganic molecules, are typically illustrated by cell cycle arrest, induction of DNA repair systems, and activation of stress

signaling mediators (27,28). To examine the effects of MMS on the *NTL* expression, wild-type plants were treated with 100 p.p.m. MMS. Many *NTLs*, including *NTL1*, *NTLs* 3–6, *NTL9* and *NTL11*, were significantly induced by MMS (Figure 4B). This result further supports the notion that *NTLs* regulate stress responses or signaling.

Consistent with the *NTL* induction by abiotic stress treatments, *NTL1*, *NTL4*, *NTL6* and *NTL9* were significantly induced by abscisic acid (ABA) (Figure 4C), a primary signaling molecule that mediates abiotic stress responses (29–32). Notably, *NTL10* was induced by N-benzyladenine (BA), suggesting a role in cytokinin signaling. The *NTL10* function is likely to be different from those of other *NTLs*.

Membrane release is essential for the *NTL6* function

Our results strongly support the idea that the *NTLs* are intimately related to abiotic stress responses. To further examine this, we decided to produce a series of transgenic plants that overexpress *NTLs* and examine any phenotypic alterations. Based on the effects of abiotic stress conditions on the *NTL* expression patterns, *NTL6* and *NTL8* were chosen for transgenic studies. While *NTL6* was prominently affected by diverse abiotic stresses and MMS, *NTL8* was not discernibly affected by heat, cold or drought (Figures 3 and 4). It was induced only by high-salinity. In addition, *NTL6* and *NTL8* belong to different phylogenetic groups (Figure 2A). It was thus anticipated that *NTL6* and *NTL8* might play distinct roles.

To investigate whether membrane release is essential for the *NTL6* function, two partial-size constructs were also included in the transgenic assays. One construct (*6ΔC*) was devoid of the TM and adjacent sequence region and thus assumed to be structurally equivalent to other nuclear NACs. The other partial-size construct (*6ΔTM*) lacked only the TM (Figure 5A). Transgenic plants overexpressing either a full-size *NTL6* (*35S::NTL6*) or the *6ΔTM* construct (*35S::6ΔTM*) did not show any detectable phenotypic changes (Figure 5B). Surprisingly, the transgenic plants overexpressing *6ΔC* (*35S::6ΔC*) exhibited severe phenotypic alterations, such as dwarfed growth and curled leaves with slightly serrated margins. Most of the transgenic lines obtained from each transformation consistently displayed the phenotypes shown in Figure 5B and C, supporting that the phenotypes of the transgenic plants are caused by the overexpression of the *NTL6* constructs. These observations indicate that membrane release is essential for the *NTL6* function, similar to the cases of NTM1 and AtbZIP60 (7,9). Notably, the *35S::6ΔC* transgenic plants resembled wild-type plants grown under abiotic stress conditions in that they exhibited reduced growth and morphological alterations (29,32). Furthermore, the phenotype of the *35S::6ΔC* transgenic plants is also consistent with the responses of *NTL6* to abiotic stresses (Figures 3 and 4). Meanwhile, a T-DNA insertional knockout mutant displayed a phenotype essentially identical to that of wild-type plants (data not shown). This may be either due to functional redundancy among the *NTLs* or related to the low transcription of *NTL6* in normal growth conditions (see Discussion). Overexpression of the transgenes in the transgenic plants was confirmed by RT-PCR runs (Figure 5B).

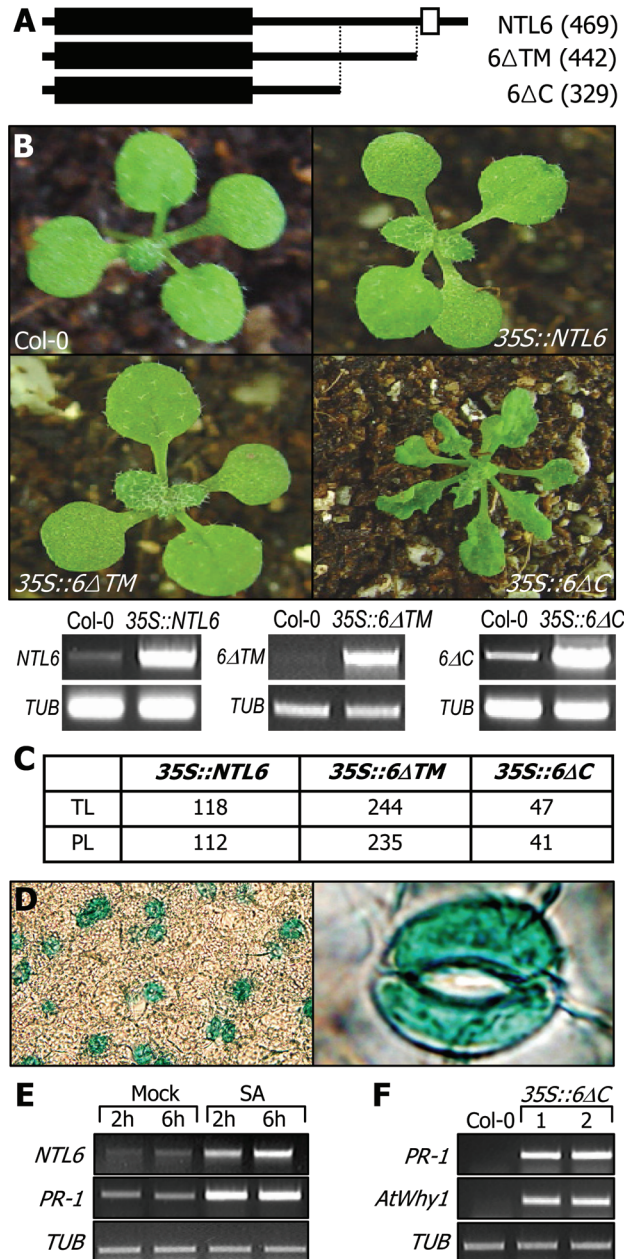


Figure 5. *NTL6* transgenic plants. (A) *NTL6* constructs used for *Arabidopsis* transformation. The numbers in parentheses are those of amino acid residues. (B) *NTL6* transgenic plants. Ten-day-old plants are displayed. Overexpression of the transgenes was confirmed by RT-PCRs. (C) Statistics of transgenic plants. TL indicates the number of total transgenic lines obtained, and PL indicates the number of transgenic plants showing the phenotype shown in (B). (D) Localized expression of *NTL6* in guard cells. (E) *NTL6* induction by SA. Ten-day-old wild-type plants were treated with SA at a final concentration of 0.1 mM for 2 h or 6 h. (F) Defense gene induction in the *35S::6ΔC* transgenic plants. Two representative transgenic plants (1 and 2) were analyzed.

To get more insights into the role of *NTL6* at the cellular level, a GUS-coding sequence was transcriptionally fused to the promoter (*pNTL6*) of *NTL6*, and the *pNTL6-GUS* construct was transformed into *Arabidopsis* plants. Notably, the GUS activity was predominantly detected in the guard cells of the transgenic plants (Figure 5D) in addition to the vascular tissues (data not shown). Consistent with the

high-level expression of *NTL6* in root tissue (Figure 3A), the GUS activity was also detected to a significant level in the vascular cylinders of primary and lateral roots (data not shown). *NTL6* may have a role in defending against dehydration occurring under abiotic stress conditions, such as drought, cold and high-salinity (32).

Some stress symptoms are commonly observed in plants grown under both biotic and abiotic stress conditions. These include growth retardation, morphological alterations and reduced metabolic activities (33). Furthermore, some *NTLs*, including *NTL6*, are also influenced by some bacterial infections (21). We therefore hypothesized that *NTL6* might also be related to biotic stress responses. We first examined whether the *NTL6* expression was influenced by SA, a critical signaling molecule that mediates biotic stress responses in plants (34). Interestingly, *NTL6* was significantly induced by SA, like *pathogenesis-related-1* (*PR-1*) (Figure 5E). Furthermore, the transcript levels of *PR-1* and *AtWhy1*, which are activated by SA-mediated signals and play important roles in pathogen resistance (35), were greatly elevated in the *35S::6ΔC* transgenic plants (Figure 5F). These observations indicate that *NTL6* is linked to the SA-dependent signaling as well as to the ABA-mediated abiotic stress responses.

NTL8 regulates flowering time

NTL8 was chosen for transgenic studies based on the following characteristics. It is distinct among the *NTL* genes in that it does not detectably respond to most of the stress treatments examined except for the slight induction by NaCl (Figure 3D). It may not be directly related to stress responses, although it is still possible that *NTL8* may affect certain aspects of plant growth and development under specific stress conditions. In addition, *NTL8* is the smallest of the thirteen *NTLs* (Figure 1C). It was thus impractical to discriminate between the ΔC and ΔTM constructs (Figure 6A). Therefore, we included only the ΔC construct in addition to a full-size construct in the transformation experiments.

We obtained two distinct homozygotic lines of the *35S::8ΔC* transgenic plants with different phenotypes (Figure 6B). One line (*35S::8ΔC-1*) was late flowering with apparently normal leaf morphology (Figure 6C). In contrast, the other line (*35S::8ΔC-2*) exhibited severely dwarfed growth, similar to the *35S::6ΔC* transgenic plants (Figure 5B), and delayed flowering. Although the molecular mechanism underlying the differential ΔC transgene expressions in these two lines is unclear, the transgene expression was significantly higher in *35S::8ΔC-2* (Figure 6D). These results suggest that *NTL8* does not simply regulate flowering time but modulates it, probably in response to stress signals. The *NTL8*-mediated flowering time control may be related to the altered flowering phenotype frequently observed in stressed plants (31,32). Consistent with the delayed flowering phenotype, flowering time genes, including *FLOWERING LOCUS T* (*FT*), *FRUITFUL* (*FUL*) and *CAULIFLOWER* (*CAL*), were down-regulated in the *35S::8ΔC-2* transgenic plants (Figure 6E) as well as in the *35S::8ΔC-1* transgenic plants (data not shown).

Transgenic studies also suggest that the biochemical mechanism underlying the *NTL8* function may be different from that of the *NTL6* function. Transgenic plants

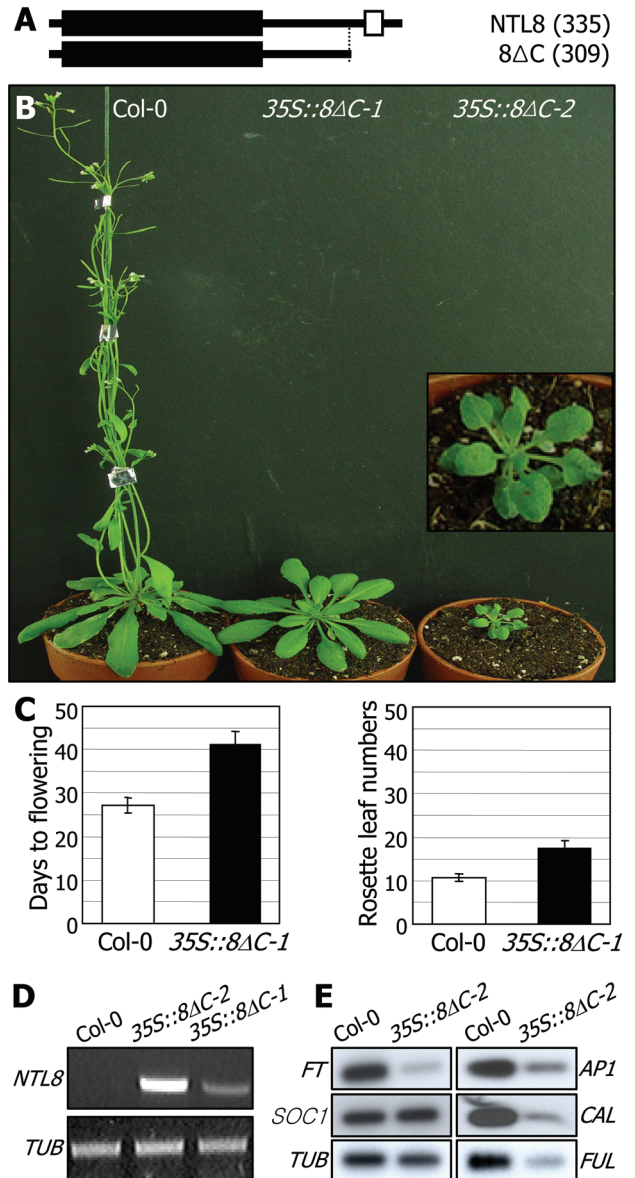


Figure 6. *NTL8* transgenic plants. (A) *NTL8* constructs used for *Arabidopsis* transformation. (B) *NTL8* transgenic plants. Two types of the *35S::8ΔC* transgenic plants were isolated: one type has a normal morphology but is late flowering (*35S::8ΔC-1*), and the other type shows reduced growth as well as severe late flowering (*35S::8ΔC-2*). Inlet shows an enlarged view of the *35S::8ΔC-2* transgenic plant. (C) Flowering time measurements of the *35S::8ΔC-1* transgenic plants. Thirty plants were measured and averaged for each plant group. Bars denote standard error of the mean. Statistical significance was determined using a student *t*-test ($P < 0.01$). (D) ΔC transgene expression. The severity of phenotypic alterations in the *35S::8ΔC* transgenic plants is proportional to the level of the ΔC transgene expression. (E) Expression of flowering time genes. The transcript levels were analyzed by RT-PCR-based Southern blot hybridization.

overexpressing the full-size *NTL8* (*35S::NTL8*) showed similar phenotypic changes to those of the *35S::8ΔC* transgenic plants (Figure 6B). This is in contrast to that exhibited by the *35S::NTL6* transgenic plants (Figure 5). Growth was retarded, and flowering was significantly delayed in the *35S::NTL8* transgenic plants like the *35S::8ΔC* transgenic plants (data not shown). This is probably related to the distinct nature of the *NTL8* processing. While the *NTL6*

processing may be an inducible event, the *NTL8* processing may be constitutive but blocked by certain endogenous or external signals. Consistent with this view, whereas the full-size *NTL6* was predominantly detected in total cellular extracts, both the full-size and processed *NTL8* forms were detected (Figure 7B).

Processed *NTL* forms are transcriptional activators

Our observations suggest that *NTLs* are released from the membranes and localized into the nucleus, where they may function as transcriptional activator like other known NAC transcription factors.

To examine this hypothesis, the *NTL8* gene constructs were in-frame fused to the GAL4 DBD in the yeast expression vector pGBKT7. The GAL4-*NTL8* fusions were expressed in a yeast strain containing a reporter gene (*LacZ*), and α -galactosidase activity was measured. The yeast cells expressing a full-size *NTL8* showed *LacZ* activation (data not shown). Notably, the 8 Δ C and even the C-terminal region of 8 Δ C also activated the *LacZ* reporter gene to a level even higher than that by the full-size *NTL8* (Figure 7A). However, the NAC domain alone did not activate it, clearly demonstrating that *NTL8*, and possibly other *NTLs* too, is a transcriptional activator.

NTM1 is membrane-associated, and western blot analysis has shown that total cellular extracts contain two NTM1-specific polypeptides: the full-size, membrane-associated form and the processed, nuclear form (7).

To investigate whether the *NTL* proteins are processed in a similar manner, six copies of the *myc*-coding sequences were fused in-frame to the 5' ends of some of the *Arabidopsis* and rice *NTL* genes. The *myc-NTL* gene fusions were transiently expressed in *N.benthamiana* leaves after direct infiltration. Western blot analysis using a polyclonal anti-*myc* antibody revealed that total cellular extracts prepared from the leaves infiltrated with the *myc-NTL8* and *myc-osNTL3* constructs contained two specific bands in each case (Figure 7B). Size estimations predicted that the upper bands were the full-size forms, and the lower bands were the processed forms. In contrast, only a single band, which is apparently the full-size *NTL*, was detected in each of the cellular extracts prepared from the *myc-NTL6* and *myc-NTL10* leaves. These results suggest that the *NTL* processing might be either repressible or inducible. The processing of *NTL8* and *osNTL3* may occur constitutively under normal growth conditions, but it would be blocked by certain incoming stimuli. In contrast, the processing of *NTL6* and *NTL10* would be an inducible event. This interpretation is also consistent with the phenotypes of the *NTL6* and *NTL8* transgenic plants (Figures 5 and 6). While transgenic plants with the full-size *NTL6* construct were identical to wild-type plants (Figure 5B), those overexpressing the full-size *NTL8* construct exhibited a similar phenotype to that of the 35S::8 Δ C transgenic plants (Figure 6B).

Membrane release of *NTL6* is induced by ABA

We further examined the membrane-association of *NTLs* through cell fractionation assays using transgenic *Arabidopsis* plants overexpressing a *myc-NTL8* fusion. Total cellular extract contained two major *NTL8*-specific bands

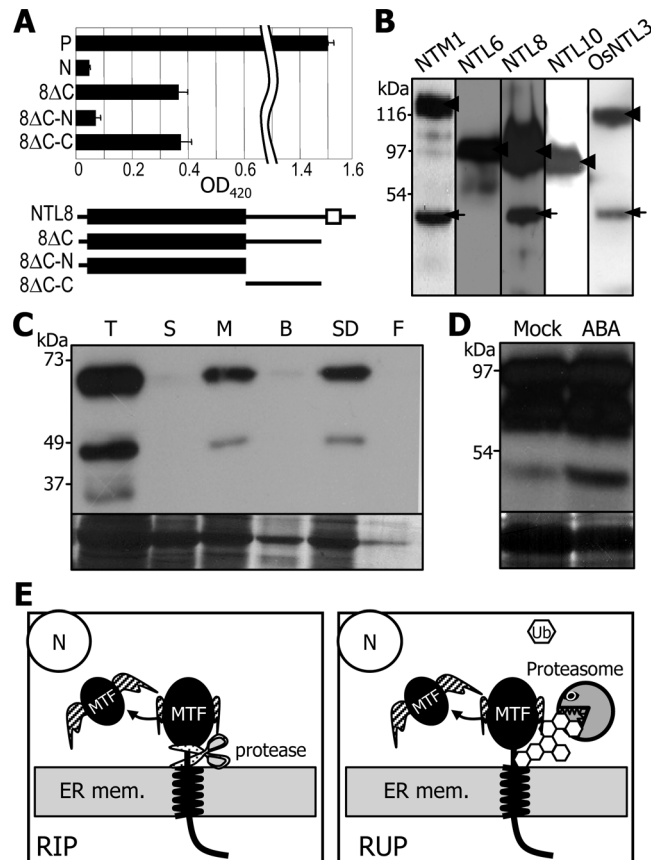


Figure 7. Processing of *Arabidopsis* and rice *NTLs*. (A) Analysis of transcriptional activities. P, positive control (full-size GAL4); N, negative control (DNA-binding domain); 8 Δ C-N and 8 Δ C-C, N-terminal and C-terminal regions of 8 Δ C, respectively. Three independent measurements were averaged ($P < 0.01$). (B) *NTL* processing. The *myc-NTL* gene fusion constructs were infiltrated into *N.benthamiana* leaves. The full-size (arrow heads) and the processed (arrows) *NTLs* were immunologically detected using an anti-*myc* antibody. (C) Membrane-association of *NTL8*. Aliquots of each fraction, which have been processed as described previously (7), were subject to western blot analysis (upper panel) using an anti-*myc* antibody. The Coomassie-stained membrane is displayed at the bottom. T, total extract; S, soluble fraction; M, membrane fraction; B, buffer-extracted fraction; SD, SDS-extracted fraction; F, final membrane fraction. (D) ABA effects on *NTL6* processing. Plants were treated with 100 μ M ABA for 6 h. A part of Coomassie-stained gel is shown at the bottom. (E) Two putative mechanisms for releasing MTFs. Controlled cleavage of MTFs either by intramembrane proteases (RIP) or ubiquitin/proteasome-dependent processing (RUP) is schematically shown. This diagram is modified from Hoppe *et al.* (2).

(Figure 7C). The upper band was predicted to be the full-size *NTL8* protein and exclusively detected in the membrane fractions. The lower band was close to that of 8 Δ C and other nuclear NACs, suggesting that it was a processed form. Unexpectedly, the processed form was not detected in the soluble fractions. This might be due to an unstable nature of the processed form, as has been observed with the soluble forms of other MTFs(2,7). Similar results were obtained with *NTL6* (data not shown). These observations demonstrate that *NTLs* are associated with the membranes.

The next question was what signals trigger the membrane release of *NTLs*. To answer the question, transgenic plants overexpressing a *myc-NTL6* fusion were treated with various stresses and growth hormones, and total cellular extracts were analyzed by western blot analysis. Whereas most of

the treatments did not exhibit any discernible effects on the *NTL6* processing, ABA significantly induced it (Figure 7D). It was unexpected that SA did not have any effects, since *PR* genes were up-regulated in the *35S::6ΔC* transgenic plants (Figure 5). *NTL6* may have a role in mediating ABA signals in biotic stress responses.

Some rice NACs are also membrane-associated

The rice genome contains 74 NAC transcription factors, which comprise one of the largest transcription factor families in this plant species (12). To examine whether the membrane-association of the NACs in rice, the protein structures of the rice NACs were analyzed in a similar way as with the *Arabidopsis* NACs using the ARAMEMNON database. At least six NAC members contained strong TMs, with hydrophobicity values of higher than 0.85, in positions similar to those of the *Arabidopsis* *NTLs* (Supplementary Figure S3). It is evident that membrane regulation of the NAC MTFs is conserved at least in two plant species, *Arabidopsis* and rice.

In this work, we analyzed the protein structures of the NAC transcription factors and studied the physiological roles of selected *Arabidopsis* *NTLs*. A similar regulatory mechanism is also envisioned for other MTFs in plants. Our data also suggest that membrane regulation of transcription factors is not an exceptional mechanism but a genome-wide strategy for gene regulation functioning widely in plant genomes. The MTFs are activated either by RIP or RUP (Figure 7E). It is also possible that both activities may be required for the regulation of a certain MTF. For example, a MTF may be released by intramembrane proteases, but the stability of the released form may further be regulated by the 26S proteasome, like the NTM1 processing (7).

DISCUSSION

MTF activation is an efficient way of achieving rapid transcriptional responses

Plants are constantly exposed to a variety of biotic and abiotic stresses in nature. Therefore, they have developed versatile systems for accurate stress signal perception and transduction. Taking this into account, the MTF activation strategy that ensures more rapid transcriptional responses to environmental fluctuations would be an efficient way to maximize plant survival under adverse growth conditions (1–3).

Here, we demonstrated through protein structural analysis and transgenic studies that a subset of plant-specific NAC transcription factors is membrane-associated. The *NTL* genes are possibly related to plant stress responses. They are influenced by diverse stress conditions, and transgenic plants overproducing constitutively active *NTL* forms exhibit typical phenotypic alterations that are frequently observed in plants grown under stress conditions.

Several NAC transcription factors have been functionally studied in diverse plant growth and developmental processes, including floral development (14), apical meristem formation (15) and growth hormone signaling (7,16,17). There are up to 13 NAC MTFs, including NTM1 and NTM2 (18), in *Arabidopsis*. The *NTLs* certainly belong to a distinct set of

the NAC transcription factors whose functions are modulated by their release from the intracellular membranes. It is notable that *NTLs* are influenced by various stresses. The transgenic plants overexpressing constitutively active *NTL6* or *NTL8* forms (6ΔC or 8ΔC, respectively) exhibit reduced growth and altered leaf morphologies, indicative of hypersensitive responses to environmental stresses (29,32), suggesting that the *NTLs* may mediate diverse aspects of stress responses or signaling. It is envisioned that membrane regulation of the *NTL* activation is a unique biochemical scheme that plants use to efficiently defend against environmental stresses.

Arabidopsis genome contains numerous MTFs

The *Arabidopsis* and rice genomes contain 1533 and 1336 transcription factor genes, respectively, covering 5–6% of each genome (12,13). The *Arabidopsis* genome contains 109 members of the NAC transcription factors, and the rice genome contains 74 NAC members. It is therefore estimated that ~10% of the NAC members are membrane-associated in each genome.

Surprisingly, ARAMEMNON-assisted protein structure analysis of the whole transcription factors in the *Arabidopsis* genome predicted that a considerable number of transcription factors from most of the major transcription factor families possess strong TMs in their C-terminal regions (Supplementary Figure S4) (18). For example, >4 members are predicted to be membrane-associated in each of the bHLH, C2H2, MADS and SBP families, and six members of the bZIP family contain the TMs. These observations are also consistent with the computational prediction study on transcription factors in eukaryotes (36). More than 76 transcription factors have been estimated to be membrane-associated. The wide occurrence of the TMs in most transcription factor families in *Arabidopsis* further supports that the MTF processing is a genome-wide mechanism. Furthermore, numerous proteins, which contain known DBDs but have not been classified as transcription factors because of the presence of the TMs, would also function as transcription factors. More extensive analysis of plant genomes may lead to the identification of a greater number of transcription factor genes than has originally been estimated.

MTF may be involved in various growth and developmental processes

The MTFs are released from the membranes by either one of the two activation mechanisms: RIP and RUP. In RIP, a set of intramembrane proteases, including S1P and S2P, work sequentially to release the MTFs in a stepwise manner (1,37,38) (Figure 7C). A few S1P and S2P homologues are also present in the *Arabidopsis* genome. Furthermore, NTM1 is cleaved by calpain, a membrane-associated cysteine protease, or its functional homologue (7). Notably, an *Arabidopsis* mutant in which a calpain protease gene had been inactivated showed a strikingly similar phenotype to that of the NTM1 mutant (7,39). The previous and our observations suggest that the membrane-bound calpain protease or its functional homologues may also have a role in the activation of the *NTLs* and other MTFs. Considering the large number of the MTFs in plant genomes, the battery of membrane-associated

proteases that regulate the MTF activation would be much larger.

The SPT23/MGA2 transcription factor in yeast is the only example that has been unequivocally shown to be activated by RUP (6). Although the RUP pathway has not yet been reported in higher eukaryotes, the 26S proteasome activity is likely to be involved in the regulation of the MTF function. It has recently been demonstrated that NTM1 is released from the membranes by proteolytic cleavage but the protein stability of the active NTM1 form is further modulated by the 26S proteasome. It is also envisioned that the RUP pathway may activate at least some of the *NTLs* and other MTFs in plants. Recent studies have shown that Ub-mediated proteolytic cleavage exerts a role in various growth hormone signaling pathways (40–42). There are more than a thousand of E3 ligases that determine substrate specificities in the *Arabidopsis* genome (43). It is possible that individual *NTLs* or MTFs may be processed or the protein stability may be regulated by distinct E3 ligases in different growth hormone signaling pathways, particularly those mediated by SA and ABA.

NTM1 exerts a role in cell-cycle control (7). We showed that *NTL6* regulates stress responses in plants. *NTL8* regulates flowering time, apparently by repressing a floral integrator (*FT*) (44,45). Furthermore, the *NTL8* function might also be linked to stress signaling pathways. The membrane-associated *Arabidopsis* transcription factor, AtbZIP60, regulates ER stress responses in a way unique to plants (9). These observations suggest that MTFs, including *NTLs*, may play various roles in a wide-variety of plant growth and developmental processes in plants.

Knockout mutants that had T-DNA insertions within the open reading frames of *NTL6* and *NTL8* were similar to wild-type plants. This would be caused by functional redundancy among the *NTLs* or between the *NTL*-mediated pathways and other stress signaling pathways. We observed that most *NTLs* are induced by stress conditions. Alternatively, this may be related to the expression patterns of the *NTLs*. They are expressed to very low levels in normal growth conditions but induced under stress conditions. Further works are required to discriminate these two possibilities. For example, multiple mutants would help answer the question. In addition, it will also be interesting to examine whether the knockout mutants exhibits reduced resistance to biotic and abiotic stresses compared to wild-type plants.

Taken together, our data indicate that NAC MTF activities are apparently regulated at the post-translational level as well as at the transcriptional level. We are under way to investigate how stress-related growth hormones, such as ABA and SA, and stress conditions affect the *NTL* processing and how they are released from the membranes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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