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

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Received September 1, 2006; Revised and Accepted November 8, 2006

**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-κB/Rel transcription factors that are anchored to IkBα, 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,
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. KRP3, 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 μmol photons m⁻²s⁻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 H₂O₂ 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 1x 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 pGBK7 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 3SS 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 ARAMEMON 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
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.

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).
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.

**NTL genes are activated by MMS**

Responses of the NTL genes to hydrogen peroxide (H₂O₂) 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 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 AthZIP60 (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.](image-url)

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
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::8ΔC 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 8AC and 8ΔTM constructs (Figure 6A). Therefore, we included only the 8AC construct in addition to a full-size construct in the transformation experiments.

We obtained two distinct homozygotic lines of the 35S::8AC transgenic plants with different phenotypes (Figure 6B). One line (35S::8AC-1) was late flowering with apparently normal leaf morphology (Figure 6C). In contrast, the other line (35S::8AC-2) exhibited severely dwarfed growth, similar to the 35S::6AC transgenic plants (Figure 5B), and delayed flowering. Although the molecular mechanism underlying the differential AC transgene expressions in these two lines is unclear, the transgene expression was significantly higher in 35S::8AC-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::8AC-2 transgenic plants (Figure 6E) as well as in the 35S::8AC-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 overexpressing the full-size NTL8 (35S::NTL8) showed similar phenotypic changes to those of the 35S::8AC 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::8AC 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 pGBK7T7. 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 8AC and even the C-terminal region of 8AC 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::8AC 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 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 8AC 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

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**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); 8AC-N and 8AC-C, N-terminal and C-terminal regions of 8AC, 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 (arrowheads) and the processed (arrows) NTLs were immunologically detected using an anti-myc antibody. (C) Membrane-association of NTL8, Alisquots 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 μM 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).
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::NAC 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 factor genes 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 (6AC or 8AC, 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, AthZIP60, 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.

ACKNOWLEDGEMENTS

The authors thank Mrs Younghhee Choi for proofreading the manuscript. This work was supported by the Brain Korea 21, Biogreen 21 (20050301034456), National Research Laboratory (2005-01039) Programs, and by grants from the Plant Signaling Network Research Center and from KOSEF (R08-2004-000-10066-0 to Y.-S.K.) and KRF (2005-070-C00129). Funding to pay the Open Access publication charges for this article was provided by Korea Science & Engineering Foundation (KOSEF) and Korea Research Foundation (KRF).

Conflict of interest statement. None declared.

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