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Probing protein structural requirements for activation of membrane-bound NAC transcription factors in *Arabidopsis* and rice

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ABSTRACT

Many transcription factors are stored in a dormant state through association with the cellular membranes in plants. Upon stimulation by internal and environmental signals, they are activated through proteolytic cleavage events either by membrane-associated proteases or by ubiquitination-dependent proteasome activities. Controlled proteolytic activation of the dormant, membrane-bound transcription factors (MTFs) is an intriguing way of ensuring rapid transcriptional responses to abrupt environmental changes. However, the underlying activation mechanisms and protein structural requirements are largely unknown in most cases. Here, we analyzed the primary and secondary structures of the NAC MTFs, particularly of the amino acid sequences surrounding the putative cleavage sites. Interestingly, the putative biologically active forms have strong hydrophilic motifs at their C-termini. Accordingly, transgenic *Arabidopsis* plants overexpressing the truncated forms having the C-terminal hydrophilic motifs exhibited distinct phenotypes. The finding was also applicable to rice NAC MTFs. Among the full-size OsNTL2 protein and a series of truncated OsNTL2 forms lacking the C-terminal transmembrane motif, only the OsNTL2 form (Os2ΔC4) having a strong hydrophilic peak at the C-terminus exhibited a high transcriptional activation activity when assayed in yeast cells. Our findings will provide insights into how plant MTFs are activated to release the biologically active forms.

1. Introduction

Recent studies have shown that numerous eukaryotic transcription factors are integrated into the intracellular membranes [1–3]. Upon stimulation by developmental and external signals, the dormant, membrane-bound transcription factors (MTFs) are proteolytically activated, and the transcriptionally active MTF forms are translocated into the nucleus, where they regulate expression of target genes [4–6]. The MTFs are activated through distinct proteolytic cleavage events mediated either by specific proteases integrated into the membranes or by ubiquitination-dependent proteasome activities. Accumulating evidence indicates that controlled proteolytic activation of the membrane-anchored transcription factors serves as a adaptive strategy that ensures prompt transcriptional responses to abrupt environmental changes [3,4].

Several MTFs have recently been identified and functionally characterized in *Arabidopsis* and rice, including a few members of the bZIP (basic leucine zipper) and NAC (NAM/ATAF1/2/CUC2) transcription factors [2,3,7,8]. AtbZIP60 is the first plant MTF that has been functionally studied [9]. It has been shown that endoplasmic reticulum (ER) stress triggers the activation of the ER membrane-associated AtbZIP60 protein [10].

A recent study has shown that at least 13 NAC members, collectively designated NTM1-likes (NTLs), are membrane-associated via the C-terminal, α-helical transmembrane motif [11,12]. Among them, the best-studied one is the NAC with Transmembrane Motif 1 (NTM1) protein. The activated NTM1 form regulates cell division by inducing a subset of cyclin-dependent kinase inhibitor genes [11]. NTL8 processing is induced by high salt and regulates flowering transition and seed germination under high salinity [13,14]. Notably, transgenic approaches using truncated NTM1 and NTL8 forms lacking the C-terminal transmembrane motifs have shown that membrane release is a prerequisite for the NAC MTF (NTL) activities.

Extensive analysis of the *Arabidopsis* genome has estimated that approximately 10% of the transcription factors are associated with

Abbreviations: bZIP, basic leucine zipper; ER, endoplasmic reticulum; MTF, membrane-bound transcription factor; NAC, NAM/ATAF1/2/CUC2; NTM1, NAC with transmembrane motif 1.

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the cellular membranes [12,15], supporting the notion that controlled activation of MTFs is an important component of gene regulatory network. The proteases responsible for the proteolytic activation of a few bZIP MTFs have been determined, and a functional correlation between proteolytic activation and roles in plant stress responses have been demonstrated [7,8]. However, the biochemical mechanisms and protein structural requirements underlying the proteolytic cleavage events are unknown in most cases, particularly in the case of the NAC MTF activation.

In this work, we employed a series of biochemical approaches in conjunction with phenotypic analysis of transgenic plants overexpressing full-size and truncated NAC MTF forms to obtain insight into how plant NAC MTFs are activated. We found that a prominent hydrophilic peak exists near the putative cleavage sites. In addition, transgenic *Arabidopsis* plants overexpressing truncated NAC MTF forms having the hydrophilic peak at the C-termini exhibited distinct phenotypes. In contrast, expression of other truncated forms or full-size ones did not cause any discernible phenotypic changes, indicating that the C-terminal hydrophilic peak is important for the NAC MTF activities.

2. Materials and methods

2.1. Plant materials and growth conditions

All *Arabidopsis thaliana* lines used were in the Columbia background (Col-0). Plants were grown in a controlled culture room at 22 °C with a relative humidity of 55% under long days (16-h light and 8-h dark) with white light illumination (120 μmol/m^{2}s) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea).

2.2. Construction of NTL expression constructs

To generate transgenic *Arabidopsis* plants overexpressing the *NTL* genes, the PCR products were subcloned into the pB2GW7 vector under the control of the CaMV 35S promoter using the GATAWAY™ cloning system (Invitrogen, Paisley, UK). The PCR primers used were: NTL1ΔC-F; 5′-**AAAAAGCAGGCT**ATGGACTTGTCGGTTGAG, NTL1ΔC-R; 5′-**AGAAAGCTGGGT**TCAACTAGACTCGTCTTTTGA, NTL2ΔC-F; 5′-**AAAAAGCAGGCT**ATGAACCAAATAAAAAACAAAACTTTA, NTL2ΔC-R; 5′-**AGAAAGCTGGGT**TCAAGGAGCAGTGCCTTG, NTL4ΔC-F; 5′-**AAAAAGCAGGCT**ATGGGTCGTGGCTCAGTA, and NTL4ΔC-R; 5′-**AGAAAGCTGGGT**TCATTGTTTTGAACAAGATGC. They contained vector recognition sequences (bold) at the 5′ ends for efficient subcloning. All the expression constructs were confirmed by restriction enzyme mapping and direct DNA sequencing.

2.3. Arabidopsis transformation

The *Arabidopsis NTL* gene sequences were subcloned into the pB2GW7 vector using the GATAWAY™ cloning system. In the constructs, the *NTL* gene expression is driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter. Transgenic *Arabidopsis* plants were produced by a modified floral dip method [16]. Homozygous transgenic lines were isolated through two additional generations after the primary selection and confirmed by analysis of segregation ratios.

2.4. Bioinformatics softwares

The amino acid sequences of the NTL proteins were obtained from the *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/). They were compared using the BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequence of the rice OsNTL2 was obtained from the Rice Genome Annotation Project database, Michigan State University (http://rice.plantbiology.msu.edu/). Hydrophobicity plots were obtained using the ARAMEMNON membrane protein database (http://aramemnon.botanik.uni-koeln.de/) [15].

2.5. Transcriptional activation activity assays in yeast cells

The transcriptional activation activity assays were carried out as previously described [11] using the pGBKT7 vector and the yeast strain AH109 (Clontech, Palo Alto, CA). For more accurate comparison of the transcription activation activities, β-galactosidase activities were also measured. Five measurements were averaged and statistically treated using a Student's *t*-test.

2.6. NTL4 processing

Six copies of the MYC-coding sequence were fused in-frame to the 5′ end of a full-size *NTL4* or a truncated *4ΔC2* gene, and the fusion constructs were transformed into *Arabidopsis*. The transgenic plants grown for 3 weeks on MS-agar plates were harvested. Whole plants were used for extraction of total proteins. Plant materials were ground in liquid nitrogen, and total protein extracts were suspended in SDS-PAGE sample loading buffer, analyzed on 10% SDS-PAGE gels, and blotted onto Hybond-P$^+$ membranes (Amersham-Pharmacia, Amersham, UK). The blots were hybridized with an anti-MYC antibody (Santa Cruz Biotech, Santa Cruz, CA).

2.7. Subcellular localization of the NTL4 proteins

The green fluorescence protein (GFP)-coding sequence was fused in-frame to the 5′ end of the *NTL4* and *NTL4ΔC2* gene sequences, and the gene fusion constructs were transiently expressed in *Arabidopsis* protoplasts as previously described [17]. After incubation for 16 h at room temperature in the dark, the protoplasts were observed using the Multi-photon Confocal Laser Scanning microscope (LSM510 NLO, Carl Zeiss, Germany).

3. Results

3.1. Protein structural analysis of the NAC MTF (NTL) proteins

We have recently examined the physiological roles of individual *Arabidopsis NTL* genes by constructing transgenic plants overproducing truncated NTL forms devoid of the transmembrane motifs [12]. In the transgenic approaches, we used *NTL* gene sequences encoding truncated NTL proteins consisting of approximately 330 residues, which are similar in size to the known nuclear NAC transcription factors. Interestingly, while transgenic plants overexpressing truncated gene sequences of NTM1, NTM2, NTL6, NTL8, and NTL9 exhibited distinct phenotypic changes, those overproducing similarly truncated forms of other NTLs, such as NTL1, NTL2, and NTL4, did not show any new phenotypes [12], showing that cuts at arbitrary sites in the removal of the transmembrane motifs do not always suffice for the NTL activation and thus proteolytic cleavage should occur at the proper sites in individual NTLs.

To obtain insights into the biochemical mechanisms underlying the NTL activation, we analyzed the protein structures of NTM1, NTM2, NTL6, NTL8, and NTL9, since overexpression of their truncated forms causes a broad range of distinct phenotypic changes, such as dwarfed growth with serrated leaf margin, delayed flowering and seed germination under high salinity, and distorted leaf morphology [12]. There were no discernible sequence similarities between the C-terminal regions of the truncated forms (Fig. 1A). In addition, comparison of their full amino acid sequences showed that sequence similarities were confined to the NAC domains (data not shown), suggesting that the NTL processing does not depend on the primary sequences.