Plant Signaling & Behavior 5:5, 481-483; May 2010; © 2010 Landes Bioscience

A membrane-bound NAC transcription factor as an integrator of biotic and abiotic stress signals

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Key words: arabidopsis, membrane-bound transcription factor (MTF), ABA, salt stress, seed germination

Abbreviations: ABA, abscisic acid; ABI, ABA insensitive; ABRE, ABA-responsive element; DRE, dehydration-responsive element; MTF, membrane-bound transcription factor; NAC, NAM/ATAF1/2/CUC2; NTL, NTM1-like; PR, pathogenesis-related

Transcription factors are central components of gene regulatory networks that mediate virtually all aspects of growth and developmental processes in biological systems. The activity of transcription factors is regulated at multiple steps, such as gene transcription, posttranscriptional RNA processing, posttranslational modification, protein-protein interactions and controlled protein turnover. Controlled activation of dormant, membrane-bound transcription factor (MTF) is an intriguing regulatory mechanism that ensures quick transcriptional responses to environmental fluctuations in plants, in which various stress hormones serve as signaling mediators. NTL6 is proteolytically activated upon exposure to cold and induces expression of the *Pathogenesis-Related* (*PR*) genes. The membrane-mediated cold signaling in inducing pathogen resistance is considered to be an adaptive strategy that protects plants against infection by hydrophilic pathogens frequently occurring during cold season. We found that NTL6 also mediates abscisic acid (ABA) regulation of abiotic stress responses in Arabidopsis. NTL6 is proteolytically activated by ABA. Transgenic plants overexpressing a nuclear NTL6 form (35S:*6ΔC*) exhibited a hypersensitive response to ABA and high salinity in seed germination. Taken together, these observations indicate that NTL6 plays an integrative role in plant responses to both biotic and abiotic stress conditions.

ABA regulates diverse aspects of plant growth and developmental processes as well as stress response, such as seed germination, post-germinative growth, lateral root development and regulation of stomatal aperture.[1] It induces adaptive responses under high salinity, drought and cold. Genes and molecular mechanisms governing ABA signaling have been extensively studied by molecular genetic and biochemical approaches.[1,2]

Genes regulated by abiotic stress conditions encode a variety of signal transduction components, including transcription factors, kinases and phosphatases. They also encode cell protection proteins, such as osmolyte biosynthetic enzymes, chaperones and late embryogenesis abundant (LEA) proteins.[2] While many stress-inducible genes are regulated by ABA, other genes are unaffected in ABA biosynthetic or signaling mutants, indicating that both ABA-dependent and -independent signaling schemes are involved in plant stress responses.[2,3]

It has been demonstrated that signaling pathways governing plant responses to high salinity, water deficit, and low temperatures share common targets and unified regulatory mechanisms.[1-3] It is therefore widely perceived that there are active signaling crosstalks among the stress signaling pathways. Expression of the *Dehydration-Responsive Element Binding protein 1* (*DREB1*)/*C-repeat Binding Factor* (*CBF*) genes is regulated by cold-specific stimuli. However, the *DREB2* genes are induced by salt and drought but not by cold. In addition, transcription of the *DREB1D/CBF4* gene is induced by osmotic stress. Because the DREB/CBF transcription factors binds to the dehydration-responsive element/C-repeat (DRE/CRT) sequence, it seems that most of the DREB/CBF-mediated signals are converged at the gene promoters containing the DRE/CRT sequence.[2]

The cis-acting elements existing in gene promoters are one of the major sites for signal convergence. The ABA-responsive element (ABRE) is a typical cis-acting element that mediates ABA-dependent signaling.[2,3] On the other hand, the DRE/CRT element is a cis-acting element that mediates ABA-independent signaling.[2,3] Interestingly, the promoter of the *Responsive to Dessication 29A* (*RD29A*) gene contains four DRE-like sequences and one ABRE element. Although more than 2 copies of the ABRE element are required for proper ABA-mediated signal transduction, the DRE-ABRE interactions seem to be feasible to mediate webs of ABA signalings. As inferred by the presence of a series of distinct cis-acting elements in the gene promoter, the *RD29A* gene is induced by ABA, dehydration and cold. The induction patterns in response to dehydration and cold stresses are still maintained in the ABA biosynthetic and signaling mutants, indicating that the *RD29A* gene integrates both the ABA-dependent and ABA-independent signalings via the interaction of the cis-acting elements.[2]

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Submitted: 12/24/09; Accepted: 12/24/09
Previously published online: www.landesbioscience.com/journals/psb/article/11083

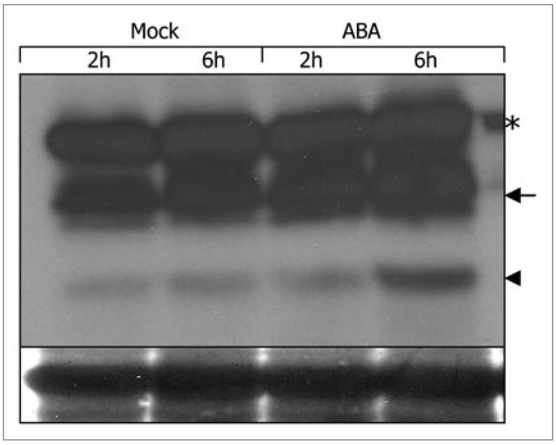

Figure 1. Effects of ABA on NTL6 processing. Six copies of the MYC-coding sequence were fused in-frame to the 5' end of a full-size *NTL6* gene, and the *MYC-NTL6* gene fusion was overexpressed under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter in Arabidopsis. The resultant 35S:*NTL6* transgenic plants have been described previously.[18] Two-week-old 35S:*NTL6* transgenic seedlings grown on MS-agar plates were transferred to MS liquid cultures supplemented with 20 μM ABA and gently shaken for the indicated time periods before harvesting plant materials. Harvested plant materials were ground in liquid nitrogen, and total cellular extracts were suspended in SDS-PAGE sample loading buffer. The protein samples were analyzed on 10% SDS-PAGE gels and blotted onto Hybond-P$^+$ membranes (Amersham-Pharmacia). The NTL6 proteins were detected using an anti-MYC antibody (Santa Cruz Biotech). The full-size (arrow), posttranslationally modified (asterisk), and processed (arrowhead) NTL6 forms are indicated. A part of the Coomassie blue-stained gel is displayed at the bottom as loading control. h, hours.

Several potential ABA receptors have been reported in recent years. According to the proposed model, ABA perception occurs in diverse cellular locations, including the cytosol, the nucleus and the plasma membranes. Accordingly, a range of proteins, such as the chloroplast protein Mg chelatase H subunit and the START domain-containing proteins PYR/PYL/RCARs, contribute to ABA sensing.[4,5] The PYR1-mediated ABA perception is a well-defined example. The ABA-bound PYR1 protein disrupts the interaction between the SnRK kinase and the PP2C phosphatase by directly docking the active site of PP2C. Inhibition of the PP2C-mediated dephosphorylation of SnRK by PYR1 activates downstream events of the ABA signal transduction cascades.[5]

It has been suggested that extracellular perception of ABA is a critical event that initiate ABA signaling. Therefore, the plasma membrane-localized ABA receptors, such as the G-protein-coupled receptors (GPCRs), may play a critical role in ABA perception and downstream ABA signaling.[6] The role of *GPA1*, which is the sole Gα subunit gene in Arabidopsis, has been demonstrated in the membrane-mediated ABA signaling.[7] In addition, the GPCR-type G proteins (GTGs) are also involved in ABA perception.[6] The GTG1 and GTG2 proteins possess ABA-binding activity, and the *gtg1gtg2* double mutant exhibits a hyposensitive response to ABA.[6]

The plasma membrane is the primary site for perception of external signaling molecules, such as growth hormones and ligands. Consequently, membrane-bound proteins are intimately related with signal perception and transduction from the plasma membranes to the nucleus. One interesting example is the modulation of transcription factor activities by chemical and physical properties of the membranes. A group of transcription factors are membrane-associated in plants.[8] The MTFs are stored as dormant forms in association with the membranes. Upon stimulation by intrinsic and extrinsic signals, they are proteolytically activated via regulated intramembrane proteolysis (RIP) and/or regulated ubiquitin/proteasome-dependent processing (RUP).[9] The processed forms of the MTFs are translocated to the nucleus, where they regulate expression of target genes.[9,10]

Several NAC (NAM/ATAF1/2/CUC2) and basic leucine zipper (bZIP) transcription factors in Arabidopsis have been shown to be associated with the plasma membranes or with the endoplasmic reticulum (ER) membranes.[11-15] These MTFs are type II membrane proteins with their N-termini localized in the cytoplasmic side.[8] They are activated by membrane-associated proteases, such as the Site-1-Protease (S1P), via the RIP mechanism.[8] It is notable that the MTFs are involved in diverse biotic and abiotic stress responses. A genome-wide screening has revealed that at least 13 members of the NAC transcription factors are membrane-tethered and has been collectively termed as NTLs (NTM1-Like) in Arabidopsis.[11] The roles of the NTLs have been demonstrated in plant responses to high salinity, osmotic stress and cold. NTL8 mediates salt-regulation of flowering initiation and germination.[13] NTL9 regulates leaf senescence in response to osmotic stress.[16] Several bZIP MTFs, including bZIP60 and bZIP28, have been shown to play a role in ER stress responses.[15,17] It is now widely accepted that sequestration of transcription factors from the nucleus provide a way of quick responses to environmental fluctuation.[8,9]

Among the NTLs characterized so far, NTL6 is of particular interest. Processing of the plasma membrane-anchored NTL6 is triggered within 30 minutes after exposure to cold.[18] The activated, nuclear NTL6 form regulates a subset of the *PR* genes, such as *PR1*, *PR2* and *PR5*, by directly binding to the gene promoters, indicating that NTL6 mediates cold-induced pathogenesis.[18]

We recently found that ABA also promoted NTL6 processing as well as *NTL6* transcription (**Fig. 1**).[11] Other stress conditions and growth hormones, other than ABA, did not affect the NTL6 processing. It is notable that while NTL6 processing was rapidly triggered by cold, it was initiated 6 h after ABA treatments, suggesting that ABA-mediated NTL6 processing would be a secondary event occurring in cold-treated plants. The previous and our own data support that controlled activation of NTL6 is a molecular event that incorporate both biotic and abiotic stress signals.[18]

Seed germination is very sensitive to high salinity, and ABA plays a crucial role in this developmental process. To examine whether the ABA effects on NTL6 processing are related with seed germination under high salinity, we examined the effects of high salt on the germination of the seeds of the 35S:*6ΔC* transgenic plants and of the *NTL6* RNAi (6RNAi) plants.[18] Seeds were imbibed for 3 days at 4°C before germination assays. Radicle emergence was used as a visible marker for seed germination. While the germination of the 35S:*6ΔC* and 6RNAi seeds

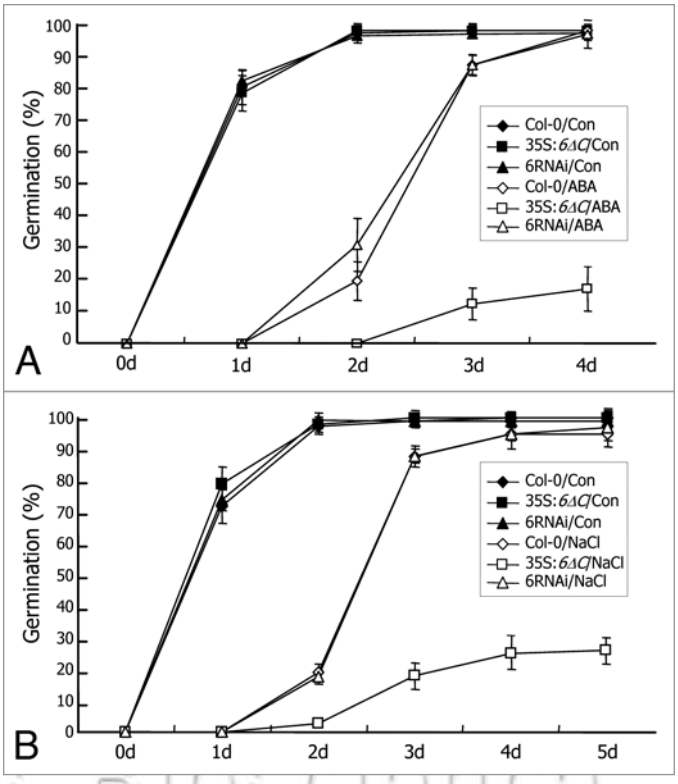

Figure 2. Effects of ABA and NaCl on seed germination. Transgenic plants overexpressing a transcriptionally active NTL6 form (35S:*6ΔC*) and RNAi plants with reduced *NTL6* gene expression (6RNAi) have been described previously.[18] Seeds were germinated on MS-agar plates supplemented either with 1 μM ABA or with 150 mM NaCl. Radicle emergence was used as a morphological marker for germination. Approximately 50 seeds were counted and averaged. Bars indicate standard error of the mean. d, days after cold-imbibition. Con, control.

were indistinguishable from that of the wild-type (WT) seeds under normal growth conditions, that of the 35S:*6ΔC* seeds was significantly delayed in the presence of 150 mM NaCl or 1 μM ABA (**Fig. 2**), showing that NTL6 is involved in ABA-mediated salt stress signaling that regulates seed germination.

Our observations entail that plant MTFs, such as NTL6, function as signaling integrators that mediate hormonal signaling crosstalks. ABA perception at the plasma membranes would be closely related with NTL processing. Considering the kinetics of NTL6 processing under cold and in the presence of ABA, it is likely that the effects of ABA on NTL6 processing are not direct. Instead, other signaling components and signal perception schemes may mediate the ABA effects.

Taken together, it is evident that NTL6 plays a role in ABA-regulated seed germination under high salinity in addition to its role in cold-induced pathogenesis. It will be interesting to examine how the molecular and cellular events occurring during ABA perception is linked with NTL6 processing and whether the ABA effects are directly linked with cold-induced pathogenesis. Assays of NTL6 processing in various ABA-related mutants and their responses to pathogen infection will provide clues as to the underlying molecular mechanisms governing ABA regulation of NTL6 processing.

Acknowledgements

This work was supported by the Brain Korea 21, Biogreen 21 (20080401034001), and National Research Laboratory Programs and by grants from the Plant Signaling Network Research Center, the Korea Science and Engineering Foundation (2007-03415), and from the Agricultural R&D Promotion Center (309017-5), Korea Ministry for Food, Agriculture, Forestry and Fisheries.

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