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The *Arabidopsis* NAC transcription factor NTL4 participates in a positive feedback loop that induces programmed cell death under heat stress conditions

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ABSTRACT

Programmed cell death (PCD) is an integral component of plant development and adaptation under adverse environmental conditions. Reactive oxygen species (ROS) are one of the most important players that trigger PCD in plants, and ROS-generating machinery is activated in plant cells undergoing PCD. The membrane-bound NAC transcription factor NTL4 has recently been proven to facilitate ROS production in response to drought stress in *Arabidopsis*. In this work, we show that NTL4 participates in a positive feedback loop that bursts ROS accumulation to modulate PCD under heat stress conditions. Heat stress induces *NTL4* gene transcription and NTL4 protein processing. The level of hydrogen peroxide (H_2O_2) was elevated in 35S:*4ΔC* transgenic plants that overexpress a transcriptionally active nuclear NTL4 form but significantly reduced in *NTL4*-deficient *ntl4* mutants under heat stress conditions. In addition, heat stress-induced cell death was accelerated in the 35S:*4ΔC* transgenic plants but decreased in the *ntl4* mutants. Notably, H_2O_2 triggers *NTL4* gene transcription and NTL4 protein processing under heat stress conditions. On the basis of these findings, we conclude that NTL4 modulates PCD through a ROS-mediated positive feedback control under heat stress conditions, possibly providing an adaptation strategy by which plants ensure their survival under extreme heat stress conditions.

1. Introduction

Programmed cell death (PCD) is a tightly regulated adaptation process in living organisms [1,2]. In plants, regulated destruction of cellular components is induced by a variety of endogenous and environmental signals, leading to removal of damaged cells and tissues for efficient recycling of nutrients and resources and proper plant development via the PCD pathway [1,3]. PCD is also intimately related with many physiological responses to developmental and environmental stimuli in plants, such as hypersensitive response (HR), tracheary element development, leaf senescence, and abiotic stress-induced cell death [1,3].

One of the major players triggering PCD in plants is reactive oxygen species (ROS) [4]. ROS trigger PCD in response to various biotic and abiotic stresses [5]. Under biotic stress conditions, HR is induced in the infected plant area by accumulating a large amount of ROS to block uncontrolled spread of microbial pathogens [6,7]. It is also well known that abiotic stress-induced ROS also cause PCD. When plants are exposed to drought stress, ROS burst occurs through the activation of ROS-generating enzymes, resulting in the induction of PCD [8]. Heat stress also causes PCD. Under heat stress conditions, ROS triggers apoptotic-like PCD in *Arabidopsis* cell suspensions in a light-dependent manner [9]. The abiotic stress-induced PCD is regarded as one of the adaptive strategy for proper recycling of nutrients in dead plant organs [8].

Abbreviations: ABA, abscisic acid; ABRE-like, ABA-responsive element; Atrboh, *ARABIDOPSIS THALIANA RESPIRATORY BURST OXIDASE HOMOLOG*; AS-1, activation sequence-1; CaMV, Cauliflower Mosaic Virus; DAB, 3,3′-diaminobenzidine; eIF4A, EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1; GFP, green fluorescent protein; GUS, β-glucuronidase; H_2O_2, hydrogen peroxide; HSE, heat shock element; MTF, membrane-bound transcription factor; NAC, NAM, ATAF1/2, and CUC2; PCD, programmed cell death; ROS, reactive oxygen species; RT-qPCR, quantitative real-time RT-PCR; SA, salicylic acid.

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Proteolytic activation of membrane-bound transcription factors (MTFs) is perceived as a versatile regulatory mechanism that ensures prompt transcriptional responses to abrupt environmental changes in plants [10]. The most extensively studied MTFs are the plant-specific NAC (NAM, ATAF1/2, and CUC2) TF members, which are associated with intracellular membranes, such as nuclear membranes, plasma membranes, and endoplasmic reticulum [11–13]. Recent studies have shown that the NAC MTFs mediate plant responses to various biotic and abiotic stresses, such as salt, cold, and pathogen attack [11,13,14].

Recently, it has been reported that one of the NAC MTFs, NTL4 (At3g10500), plays an important role in the drought-induced leaf senescence by promoting ROS production [8]. Drought stress signals up-regulate *NTL4* gene transcription and membrane release of the plasma membrane-bound NTL4 TF in an abscisic acid (ABA)-dependent manner. The nuclear NTL4 form directly binds to the promoters of *Atrboh* (*ARABIDOPSIS THALIANA RESPIRATORY BURST OXIDASE HOMOLOG*) genes, which encode NADPH oxidases that are involved in ROS production [8]. Accordingly, transgenic plants overexpressing a nuclear NTL4 form (35S:*4ΔC*) produce more ROS, but ROS content is reduced in *NTL4*-deficient *ntl4* mutants under drought stress conditions. Whereas the *ntl4* mutants are resistant to drought stress, the 35S:*4ΔC* transgenic plants exhibit drought-sensitive phenotype, supporting the role of NTL4 in the ROS regulation of drought stress response.

In this work, we found that NTL4 is closely associated with heat stress response by triggering hydrogen peroxide (H_2O_2) accumulation under heat stress conditions. Heat stress induces *NTL4* gene transcription and NTL4 protein processing, leading to H_2O_2 accumulation. Notably, H_2O_2, which accumulates in response to the NTL4-mediated heat stress signal, also promotes the proteolytic release of the NTL4 protein, constituting a positive feedback loop. Consequently, H_2O_2 accumulates to a high level in the 35S:*4ΔC* transgenic plants that exhibit elevated PCD response, but the level of H_2O_2 is reduced in the *ntl4* mutants that exhibit enhanced thermotolerance. These findings indicate that NTL4 plays an important role in heat stress response by mediating ROS-induced PCD under heat stress conditions.

2. Materials and methods

2.1. Plant materials and growth conditions

All of the *Arabidopsis thaliana* lines used were in Columbia (Col-0) background. Plants were grown in a controlled culture room at 23 °C with relative humidity of 60% under long day conditions (16-h light and 8-h dark) with white light illumination (120 μmol photons/s/m^2) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). The 35S:*4ΔC* transgenic plants have been described previously [8]. The *Arabidopsis* ABA-deficient *aba3-1* mutant and T-DNA insertional *NTL4*-deficient mutants *ntl4-1* (SALK-009578C) and *ntl4-2* (SALK-007900) were isolated from a mutant pool deposited in the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University). The SA-deficient *sid2* mutant and the *NahG* transgenic plant have been described previously [15].

2.2. Analysis of transcript levels

Relative mRNA levels were determined by quantitative real-time RT-PCR (RT-qPCR). Total RNA preparation, reverse transcription, and quantitative polymerase chain reaction were performed based on the rules that have been proposed to guarantee reproducible and accurate measurements of mRNA levels [16].

RT-qPCR reactions were performed in 96-well blocks using the Applied Biosystems 7500 Real-Time PCR System using the SYBR Green I master mix in reaction volume of 20 μl. The PCR primers were designed using the Primer Express software installed into the system and listed in Supplementary Table S1. The *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*eIF4A*) gene (At3g13920) was included in the reactions as internal control for normalizing the variations in the amounts of cDNA used [17]. RT-qPCR reactions were performed in biological triplicates using total RNA samples extracted from three independent replicate samples of plants grown under identical conditions. The comparative $\Delta\Delta C_T$ method was employed to evaluate relative quantities of each amplified product in the samples. The threshold cycle (C_T) was automatically determined for individual reactions by the system set with default parameters. The specificity of the RT-qPCR reactions was determined by melt curve analysis of the amplified products using the standard method installed in the system.

2.3. Histochemical assays

Histochemical assays were performed as described previously [8]. After β-glucuronidase (GUS) staining, plant materials were mounted on slide glasses and photographed using a DIMIS-M digital camera (JMTECH, Seoul, Korea).

2.4. Treatments with heat, ROS, and growth hormones

Seven-day-old plants grown on 1/2× Murashige and Skoog-agar plates (hereafter referred to as MS-agar plates) were used for treatments with abiotic stresses, chemicals, and growth hormones. For heat treatments, plants were transferred to a heat chamber set at 45 °C and incubated for various time durations. For hormone and ROS treatments, plants were transferred to MS-liquid medium containing 50 mM H_2O_2, 20 μM ABA, or 100 μM SA. Whole plants were used for extraction of total RNA.

2.5. Determination of H_2O_2 levels

Endogenous contents of H_2O_2 were measured as described previously [15]. Seven-day-old plants grown on MS-agar plates were used for the measurements using the Amplex Red hydrogen peroxide assay kit according to the procedure provided by the manufacturer (Molecular Probes, Eugene, OR). Fluorescence measurements were performed using the Cary Eclipse fluorescence spectrophotometer (Varian Associates, Palo Alto, CA).

DAB (3,3′-diaminobenzidine) staining was employed for the detection of H_2O_2 in plant tissues, as described previously [8]. Seven-day-old plants grown on MS-agar plates were treated by heat (45 °C) for 90 min and then subjected to DAB staining for 24 h with or without 10 mM ascorbic acid.

2.6. Detection of cell death

Trypan blue staining for the visualization of dead cells and electrolyte leakage assays were performed as described previously [8].

2.7. NTL4 processing

A MYC-coding sequence was fused in-frame to the 5′ end of the full-size *NTL4* gene (Supplementary Fig. S1A). The 35S:*MYC-NTL4* transgenic plants and immunological detection of NTL4 proteins have been described previously [8].

2.8. Subcellular localization of NTL4 proteins

A green fluorescent protein (GFP)-coding sequence (Supplementary Fig. S1B) was fused in-frame to the 5′ end of *NTL4* gene sequences, and the GFP–NTL4 fusion construct was transfected into