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Roles of ethylene receptor NTHK1 domains in plant growth, stress response and protein phosphorylation

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Abstract **Ethylene receptors sense ethylene and regulate downstream signaling events. Tobacco ethylene receptor NTHK1, possessing Ser/Thr kinase activity, has been found to function in plant growth and salt-stress responses. NTHK1 contains transmembrane domains, a GAF domain, a kinase domain and a receiver domain. We examined roles of these domains in regulation of plant leaf growth, salt-stress responses and salt-responsive gene expressions using an overexpression approach. We found that the transgenic *Arabidopsis* plants harboring the transmembrane domain plus kinase domain exhibited large rosettes, had reduction in ethylene sensitivity, and showed enhanced salt sensitivity. The transgenic plants harboring the transmembrane domain plus GAF domain also showed larger rosettes. Truncations of NTHK1 affected salt-induced gene expressions. Transmembrane domain plus kinase domain promoted *RD21A* and *VSP2* expression but decreased salt-induction of *AtNAC2*. The kinase domain itself promoted *AtERF4* gene expression. The GAF domain itself enhanced *Cor6.6* induction. Moreover, the NTHK1 functional kinase domain phosphorylated the HIS and ATP subdomains, and five putative phosphorylation sites were identified in these two subdomains. In addition, the salt-responsive element of the *NTHK1* gene was in the transmembrane-coding region but not in the promoter region. These results indicate that NTHK1 domains or combination of them have specific functions in plant leaf growth, salt-stress response, gene expression and protein phosphorylation.**

Keywords: Ethylene receptor; Domain; Salt-stress response; Protein phosphorylation

1. Introduction

Ethylene plays important roles in many aspects of plant growth and development, e.g., seed germination, asymmetric growth, root formation, flowering, senescence and ripening. It is also believed to function in biotic and abiotic stress responses [1,2]. Ethylene causes triple response in etiolated plant seedlings. Based on the triple response, a number of *Arabidopsis* mutants have been screened. Analyses of these ethylene response mutants have revealed components of the ethylene-signaling pathway, including ethylene receptors, a Raf-like protein kinase CTR1, a membrane protein EIN2 and a transcription factor EIN3 [3–6]. The CTR1 kinase activity has been confirmed and this protein may interact with ethylene receptors [7–10]. The membrane-associated EIN2 is related to Nramp-like metal-ion-transporters and plays a central role in the pathway [11]. EIN3 is a nuclear-localized transcription factor, whose stability is regulated by the proteasome-mediated pathway [12–15]. Since all these components are identified based on the analysis of the triple response, other components relating to other subsets of ethylene responses may be found [16]. Additional components may still be incorporated into this pathway [17].

In *Arabidopsis*, five ethylene receptors namely ETR1, ETR2, EIN4, ERS1 and ERS2 have been identified and all show similarity to the bacterial two-component histidine kinases [18–21]. Based on the structural features, the five receptors can be further classified into two subfamilies. Subfamily I contains ETR1 and ERS1. Subfamily II contains ETR2, EIN4 and ERS2. The subfamily I members have conserved histidine kinase domains whereas the subfamily II members have more diverged kinase domains when compared with the bacterial histidine kinases. All of the five ethylene receptors can bind ethylene [22–24] and all have kinase activity. Whereas the ETR1 has histidine kinase activity [25], the other four receptors contain Ser/Thr kinase activity, and ERS1 has both histidine kinase and Ser/Thr kinase activity under different assay conditions [26]. The roles of the ETR1 kinase domain and its kinase activity have been examined and may be subtle for ethylene signaling [27–30].

Homologous ethylene receptor genes have been isolated from many other plants, e.g., tomato [31], tobacco [32,33], muskmelon [34], rice [35] and wheat [36]. Each plant appears to contain several members of the ethylene receptor family. Although it is assumed that the functions of these receptors and their signaling components are similar to those of *Arabidopsis*, specificity may still be present among different plant species [37,38]. In tobacco, four ethylene receptors have been identified, i.e., NtETR1, NtERS1, NTHK1 and NTHK2 [32,33,39–41]. The first two receptors represents the subfamily I members whereas the latter two represents the subfamily II members showing around 50% identity to the EIN4 and ETR2 from *Arabidopsis*. Both the *NTHK1* and *NTHK2* genes are responsive to various stresses, with the *NTHK1* specifically induced by salt stress [32,33,41–43]. Both the NTHK1 and NTHK2 have Ser/Thr kinase activity, and NTHK2 may also have histidine kinase activity under different ion conditions

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[41,44]. Transgenic plants overexpressing NTHK1 exhibits large rosettes, reduced sensitivity to ethylene but enhanced sensitivity to salt stress at seedling stage [43,45]. NTHK1 also promotes expressions of salt-responsive marker genes [45]. Microarray analysis has identified novel receptor-like kinase and transcription factor genes that are salt-responsive and are regulated by NTHK1 and ethylene signaling [46,47].

NTHK1 contains several domains, including transmembrane domains, GAF domain, kinase domain and receiver domain. The functions of these domains in plant growth and stress response are not known. In the present study, we made several constructs harboring various truncated versions of the NTHK1 and examined the effects of the truncated NTHK1 on plant growth, salt-stress response and gene expressions using a heterologous *Arabidopsis* transgenic approach. Because *Arabidopsis* ethylene receptor gain-of-function mutant gene etr1-1 equivalently functions in a tobacco background [48], it is reasonable to assume that a tobacco ethylene receptor gene would similarly work in the *Arabidopsis* background. The mutant etr1-1 gene also confers dominant ethylene insensitivity in transgenic tomato [49], and mutant tomato ethylene receptors in transgenic *Arabidopsis* results in reduced ethylene sensitivity [50]. We find that the kinase domain of NTHK1 played a major role in promoting plant growth and generating stress responses. A specific mechanism for *NTHK1* mRNA accumulation, and the putative phosphorylated domains and phosphorylation sites in NTHK1 were also disclosed. Our results facilitate the understanding of the ethylene receptor functions in plants.

2. Materials and methods

2.1. Plant growth and stress treatments

Seeds of *Arabidopsis* (ecotype Columbia) and its ethylene-insensitive mutant *etr1-1* were treated with 70% ethanol for 5 min and then sterilized with 15% bleach (Kao incorporation, Tokyo, Japan). After washing for 5 times with sterile water, the seeds were plated on solidified MS medium [51]. The seeds were stratified at 4 °C for 2 d and then germinated at 23 °C under continuous illumination condition. For pot growth, seeds were sown in pots (8 × 10 cm) containing vermiculite soaked with 1/4 MS solution, and then germinated in growth chambers at 23 °C under continuous illumination.

Five-day-old seedlings from wild-type *Arabidopsis* (Col) and the *NTHK1* transgenic lines were transferred onto MS medium containing 0, 50, 100 and 200 mM of NaCl. Each plate was divided into several equal regions to grow the Col and the transgenic seedlings. After around seven days, the phenotypic change in these seedlings was observed. To examine gene expression, 12-day-old seedlings of *Arabidopsis* Columbia, various *NTHK1* transgenic lines were carefully pulled out from the plates and immersed in solution containing 100 mM NaCl for various times. The *NTHK1* transgenic seedlings of 12-day-old were also immersed in 50 μM of cycloheximide (CHX) for various times. The materials were collected for RNA extraction. ACC dose response was examined following previous description and the seedling length including hypocotyls and roots was measured for 25 to 30 seedlings [45].

2.2. Constructs and plant transformation

To express different truncated versions of NTHK1 in transgenic *Arabidopsis*, DNA fragments encoding the full-length NTHK1 (BK1, amino acids 1–762), NTHK1 without the receiver domain (ΔRD, amino acids 1–636), NTHK1 without ATP-binding motif and receiver domain (ΔATP, amino acids 1–445), the transmembrane domains plus the GAF domain (ΔHIS, amino acids 1–345), and NTHK1 without transmembrane domains (ΔTM, amino acids 145–762) were amplified from the original NTHK1 plasmid. For BK1, the sense primer NKF1 is 5′-TCCGGATCCATGTTAAGGACATTAGCATTAG-3′ and the antisense primer NKR1 is 5′-GCAGCTAGCACATCATCACGTGATTATGCTTG-3′. The primers for ΔRD are NKF1 and NKR2: 5′-AACGCTAGCCCCCTGGAGGAGTGTGG-3′. The primers for ΔATP are NKF1 and NKR3: 5′-GCAGCTAGCATGTAGCTGAA AATGCCTCAT-3′. The primers for ΔHIS are NKF1 and NKR4: 5′-GCAGCTAGCCAGAGCTCGATTTTGCTCCTC-3′. The primers for ΔTM are NKF2: 5′-AGGGGATCCATGCTGAAAAAGAAA ACTTGG-3′ and NKR1. All the DNA fragments were digested with *Bam*HI and *Kpn*I, and inserted into the *Bam*HI and *Kpn*I site of the pBIN438 vector. These versions were controlled by the 35S promoter. The constructs were transformed into *Arabidopsis* using infiltration method.

2.3. RNA isolation and Northern blot analysis

Total RNA isolation was performed following the description by Zhang et al. [52]. Total RNA (20–30 μg) was fractionated on a 1.0% agarose gel containing formaldehyde, blotted onto nylon membranes and hybridized as described previously [52]. Probes were labeled with α ^{32}P-dCTP by the random-priming method. All the Northern analyses were repeated for three times with independent RNA samples, and the results were consistent. Results from one set of the experiments were presented.

The gene-specific DNA fragments were amplified by PCR, confirmed by sequencing and used for probe-labeling. The primers used were as follows: for *AtERF4*, 5′-CTATCCGAGAATGGCCAAG-3′ and 5′-AACAACATGGGGTGAAACC-3′; for *Cor6.6*, 5′-ACATCA AAAACGATTTTACAAG-3′ and 5′-GAACTTAAACTAGATTTT GTTG-3′; Primers for other genes are as follows: for *VSP2* (At5g2 4770), 5′-CGGGAACGTAGCCGAACTCTTAGA-3′ and 5′-GAC-ATAGATATGAAGTGTATACAAGGG-3′; For *AtNAC2*, 5′- CTT AAAGCAACTACACAAGTC-3′ and 5′-ATCACAAAACACAGC-GAATG-3′; for *RD21A* (At4g11320), 5′-CTAAGTCCGTTGACTG-GAGAAACGA-3′ and 5′-GTTCTTGAGAGGGTATGAAGCTC GC-3′ and for *CHLBP* 5′-GATCCCGAGACCTTCGCTAGGAA-3′ and 5′-CGAAGTTGGTAGCGAAGGCCCATG-3′.

2.4. Scanning microscopy

For scanning electron microscopy, the procedures followed the description by Cao [45]. Briefly, samples were first fixed in 2.5% glutaraldehyde and then washed with phosphate buffer. The materials were further fixed in 1% Osmic acid (OsO4) and washed with phosphate buffer. After treatment with a series of ethanol, the samples were then treated with isopentyl acetate. The materials were then dried using a critical point dryer (CPD030), gold coated using a sputter coater (SCD005) and examined under a scanning electron microscope (Hitachi S-570).

2.5. Measurement of relative electrolyte leakage

Four-week-old pot-grown plants were subjected to salt treatment with increasing concentrations of NaCl (100, 200, 300 mM). The pots were placed in a tray containing the NaCl solution and the treatment with each concentration lasted 4 d. After 12-d treatment, the leaves were collected and used to evaluate the electrolyte leakage by determining their relative conductivity in solution. The conductivity was determined using a conductivity detector DDS-11A (Kangyi, Shanghai).

2.6. Expression of the truncated NTHK1 proteins and the phosphorylation assay

To express different truncated version of NTHK1 as fusions to glutathione *S*-transferase (GST) in yeast, DNA fragments encoding the GAF domain plus the putative kinase domain (GKD, amino acids 145–636), the kinase domain without the ATP-binding motif [GKD(ΔATP), amino acids 145–445], the kinase domain without the GAF domain [GKD(ΔGAF), amino acids 342–636], the receiver domain RD (amino acids 624–762), the GAF domain (amino acids 145–345), the HIS domain (amino acids 342–445) and the ATP-binding domain (ATP, amino acids 446–636) were amplified from the original NTHK1 plasmid. For GKD, GKD(ΔATP) and RD, the sense primer and the antisense primer were as described previously (Xie et al. [44]). The primers for GKD(ΔGAF) are 5′-TCCGGATCCCATATGAGAGAAACATTGGAG-3′ and 5′-AACGCTAGCCCCCTGGAGGAGTGTGG-3′. The primers for GAF are 5′-AGGGGATCCATGCTGAAAAAGAAAACTTGG-3′ and 5′-GCAGCTAGCCAGAGCTCGATTTTGCTCCTC-3′. The primers for HIS are 5′-TCCGGATCCCATATGAGAGAAAC-