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A low temperature-inducible protein AtSRC2 enhances the ROS-producing activity of NADPH oxidase AtRbohF

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ABSTRACT

Reactive oxygen species (ROS) produced by NADPH oxidases play critical roles in plant environmental responses. *Arabidopsis thaliana* NADPH oxidase AtRbohF-mediated ROS-production is involved in abiotic stress responses. Because overproduction of ROS is highly toxic to cells, the activity of AtRbohF needs to be tightly regulated in response to diverse stimuli. The ROS-producing activity of AtRbohF is activated by Ca^{2+} and protein phosphorylation, but other regulatory factors for AtRbohF are mostly unknown. In this study, we screened for proteins that interact with the N-terminal cytosolic region of AtRbohF by a yeast two-hybrid screen, and isolated AtSRC2, an *A. thaliana* homolog of SRC2 (soybean gene regulated by cold-2). A co-immunoprecipitation assay revealed that AtSRC2 interacts with the N-terminal region of AtRbohF in plant cells. Intracellular localization of GFP-tagged AtSRC2 was partially overlapped with that of GFP-tagged AtRbohF at the cell periphery. Co-expression of AtSRC2 enhanced the Ca^{2+}-dependent ROS-producing activity of AtRbohF in HEK293T cells, but did not affect its phosphorylation-dependent activation. Low-temperature treatment induced expression of the *AtSRC2* gene in Arabidopsis roots in proportion to levels of ROS production that was partially dependent on AtRbohF. Our findings suggest that AtSRC2 is a novel activator of Ca^{2+}-dependent AtRbohF-mediated ROS production and may play a role in cold responses.

1. Introduction

Increasing evidence indicates that reactive oxygen species (ROS) produced by NADPH oxidases (NOXs) function as signaling molecules in most eukaryotes. In plants, NOX-mediated ROS production has been shown to be involved in the regulation of developmental programs, pathogen defense responses, abiotic stress signaling and other processes [1,2]. The plant NOXs, also known as respiratory burst oxidase homologues (Rbohs), have six transmembrane (TM) helices as well as C-terminal intracellular FAD/NADPH-binding domains, and particularly an extended N-terminal region that contains two Ca^{2+}-binding EF-hand motifs [3,4]. The *Arabidopsis thaliana* genome contains ten *Rboh* genes (*AtRbohA–J*). *AtRbohF* has been shown to be involved in environmental stress responses [5–8].

Heterologous expression in human embryonic kidney 293 T (HEK293T) cells is a powerful tool to characterize plant Rbohs [9–12]. Ionomycin, a Ca^{2+} ionophore, induces Ca^{2+} influx into the cells and in turn induces AtRbohF-mediated rapid and transient ROS production in HEK293T cells [11]. This activation results from the binding of Ca^{2+} to the EF-hand motif(s) of AtRbohF. Concomitantly, calyculin A (CA), a Ser/Thr protein phosphatase inhibitor, induces phosphorylation of AtRbohD and activation of its ROS-producing activity [9]. The ROS-producing activity of AtRbohF is also activated by CA, suggesting its phosphorylation-dependent activation [11].

Because overproduction of ROS is highly toxic to cells, the activity of AtRbohF needs to be tightly regulated in response to diverse stimuli. Whereas the ROS-producing activities of many animal and fungal NOX proteins are regulated by several regulatory proteins, most of these regulators have not been found in plants and only a few proteins that interact with Rbohs have been identified [13]. Recently a calcineurin B-like protein (CBL)-interacting protein kinase 26 (CIPK26) has been shown to bind directly to AtRbohF and negatively modulate its activity [14]. However, the regulatory mechanism for controlling the ROS-producing activity of Rbohs including AtRbohF still remains largely unknown.

ROS have been implicated to be involved in the expression of numerous genes, including cold stress responsive transcription factor genes such as *ZAT12* and *DREB2A* [15,16]. ZAT12 (At5g59820), a zinc finger

Abbreviations: ABA, abscisic acid; AtRboh, *Arabidopsis thaliana* respiratory burst oxidase homologue; co-IP, co-immunoprecipitation; NOX, NADPH oxidase; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SRC2, soybean gene regulated by cold-2; TM, transmembrane

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protein, contributes to cold acclimation [17]. DREB2A (At5g05410) is a transcription factor that specifically interacts with a *cis*-acting dehydration-responsive element (DRE) that is involved in the cold- and dehydration-responsive gene expression. DREB2A participates in cold acclimation [18]. The MAP kinase cascade functions in ROS signaling and cold responses. Activity of mitogen-activated protein kinase 6 can be activated not only in response to low temperature treatment [19] but also by ROS [20].

In this study, we isolated AtSRC2, an *A. thaliana* homolog of soybean gene regulated by cold-2 (*SRC2*) as a novel protein interacting with AtRbohF. The expression of soybean *SRC2* is induced by low temperature treatment [21]. However, the functions of SRC2 and its homologs under cold stress had remained unknown. Here, we show that AtSRC2 binds to AtRbohF in plant cells and enhances its ROS-producing activity in HEK293T cells. Possible physiological implications of this novel mechanism for regulating Rboh-mediated ROS production are discussed.

2. Materials and methods

2.1. Plasmid construction

The coding DNA sequences (CDSs) of *AtRbohF* (At1g64060), *AtRbohF*^{N} (amino acid residues 1–387) and *AtSRC2* (At1g09070) were PCR-amplified from a cDNA library of *A. thaliana*. For the yeast two-hybrid screen, the CDSs of *AtRbohF*^{N} and *AtSRC2* were cloned into pGBKT7 and pGADT7 (Clonetech), respectively. For the co-immunoprecipitation (co-IP) analysis, the CDSs of either *AtRbohF*^{N} or *AtSRC2* were PCR-amplified with primer containing the 3×*FLAG* or 3×*Myc* sequences, respectively. Then the PCR fragments of 3×*FLAG*-tagged *AtRbohF*^{N} and 3×*Myc*-tagged *AtSRC2* were independently cloned into the multiple cloning site 1 of pRI201-AN (TaKaRa). For expression of GFP fusion proteins, the PCR-amplified *CaMV35S* promoter fragments, the *NOS* terminator fragments and the CDS of *AcGFP* (TaKaRa) were cloned into the pBluescript II SK (+) vector. Then the CDSs of *AtSRC2* and *AtRbohF* were PCR-amplified independently and cloned in frame with the N-terminal *AcGFP* tag. To measure ROS, 3×*FLAG* tagged *AtRbohF*, 3×*Myc* tagged *AtSRC2* or 3×*Myc* tagged *GFP* was first PCR-amplified. Subsequently, the PCR fragments of 3×*FLAG-AtRbohF*, 3×*Myc-AtSRC2* or 3×*Myc-GFP* were cloned into the *Bam*HI site of pEF1-MCS using an In-Fusion Cloning Kit (Clontech), resulting in *pEF1-3×FLAG-AtRbohF*, *pEF1-3×Myc-AtSRC2* and *pEF1-3×Myc-GFP*, respectively.

2.2. Yeast two-hybrid assay

A yeast two-hybrid assay was performed as described in [14]. For cDNA library construction, total RNA was isolated from 7-day-old seedlings of *A. thaliana* (Columbia) using the Matchmaker Library Construction & Screening Kits (Clontech) according to the manufacturer's protocol. To detect protein–protein interactions, a dilution series of the transformed cells were spotted on SD medium lacking tryptophan, leucine, and histidine. We independently replicated this experiment more than twice with similar results.

2.3. Co-immunoprecipitation analysis

Co-IP analysis was performed as described in [14]. Briefly, equal volumes of two Agrobacterium strains (FLAG-AtRbohF^{N} and Myc-AtSRC2) were mixed and co-infiltrated into the leaves of *Nicotiana benthamiana* plants. Total proteins were extracted from leaves two days after infiltration in the extraction buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM $CaCl_2$, 1 × Proteinase inhibitor cocktail (Roche) and 1 mM EDTA]. The protein extracts were incubated in anti-FLAG M2 affinity gel (Sigma-Aldrich) at 4 °C for 2 h. Immunoprecipitated proteins were analyzed by Western blotting using anti-c-Myc monoclonal antibodies (Wako) and anti-FLAG M2 monoclonal antibodies (Sigma-Aldrich).

2.4. Subcellular localization of AtSRC2 and AtRbohF

Microprojectiles were prepared with 7 μg of GFP-fusion constructs DNA per shot and bombarded into onion epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad) with 1100 psi rupture disks. To induce plasmolysis, cells were incubated in 0.5 M mannitol for 10–30 min. The expression and localization of the GFP fusion proteins in the cell were detected with a LSM5 EXCITER or LSM780 (Carl Zeiss). We independently replicated this experiment more than five times with similar results.

2.5. Measurement of ROS production in HEK293T cells

The ROS producing activity was assayed as described in [9]. Briefly, HEK293T cells were transiently transfected with GeneJuice transfection regent (Novagen) according to the manufacturer's instructions. ROS production was detected by a luminol-amplified chemiluminescence technique. Chemiluminescence was measured every 30 s using a microplate luminometer Centro LB960 (Berthold Technologies). ROS production was expressed in relative luminescence units (RLU). The maximum value of the luminescence unit (activity) was set at 1.0. Data are presented as the average of three samples in a representative experiment. We independently replicated this experiment more than five times with similar results.

2.6. Detection of ROS in plants and cold treatment

Ten-day-old Arabidopsis (Col-0) seedlings were vacuum-infiltrated with freshly prepared NBT solution [0.1 mg ml^{-1} 4-nitro blue tetrazolium chloride (NBT; Roche) in 0.1 M sodium phosphate buffer, pH 7.4], stained at room temperature in the dark for 2 h and then washed with 0.1 M sodium phosphate buffer (pH 7.4). For cold treatment of the plants, approximately two-week-old seedlings were treated at 4 °C for 5 h. The *atrbohF* mutant used in this study was *atrbohF*-F3 [5].

2.7. Preparation of total RNA and real-time PCR analysis

Total RNA was isolated from roots of 10-day-old seedlings. Real-time RT-PCR was performed using THUNDERBIRD qPCR mix (TOYOBO) and Applied Biosystems 7500 Real-Time PCR system. The primers used were as follows: *AtRbohF* (forward, 5′-AGCAGAACGAGCATCACCTT-3′; reverse, 5′-GGATTCGATCTCGGATTTCA-3′) and *TUB2* (forward, 5′-ATTCCCCCGTCTTCACTTCT-3′; reverse, 5′-GCACATTCAGCATCTGCTCGT-3′). Data were normalized by the level of *TUB2* mRNA expression in each sample.

2.8. Northern blotting

Total RNAs (10 μg) were electrophoresed, transferred to a nylon membrane and hybridized with a digoxigenin-labeled RNA probe specific for the *AtSRC2* transcript. The probe was generated with DIG RNA Labeling kit (SP6/T7) (Roche). Alkaline phosphatase-conjugated antibodies anti-DIG (Roche) and CDP-Star (Roche) were used for immunological detection of DIG.

3. Results and discussion

3.1. Isolation of AtSRC2 as an AtRbohF-interacting protein

To isolate proteins that interact with AtRbohF, we performed a yeast two-hybrid screen with an Arabidopsis cDNA library. The full-length AtRbohF was not suitable for this screen because it has six TM helices (Fig. 1A). Since the cytosolic N-terminal region of Rboh functions as a regulatory domain [9–12,22–24], we used the N-terminal region of AtRbohF (AtRbohF^{N}; amino acid residues 1–387) as the bait. Screening of 4.56×10^{6} yeast transformants resulted in the isolation of a fragment