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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

containing the C2 domain of AtSRC2 (amino acid residues 23–176) (Fig. 1A). The C2 domain has been reported to act as a module for protein–protein interaction or Ca^{2+}-dependent phospholipid-binding [25,26]. Cells co-expressing both the AtSRC2 fragment and AtRbohF^N were able to grow in the absence of histidine (Fig. 1B). These results indicate that AtSRC2 interacted with the N-terminal region of AtRbohF in yeast cells. The C2 domain of animal phospholipase C-delta 1 (PLC-δ1) binds its own EF-hand motif as an intramolecular interaction [27]. AtRbohF has two EF-hands in the N-terminal region (Fig. 1A), suggesting that the C2 domain of AtSRC2 may interact with the EF-hand motifs of AtRbohF.

3.2. Interaction of AtSRC2 with AtRbohF in plant cells

We performed co-IP experiments to examine the interaction between AtRbohF and AtSRC2 in plant cells. The N-terminal FLAG-tagged AtRbohF^N (FLAG-AtRbohF^N) and the N-terminal Myc-tagged full length AtSRC2 (Myc-AtSRC2) were transiently co-expressed in *N. benthamiana* leaves by agroinfiltration. The cell extracts from leaves were subsequently subjected to immunoprecipitation using anti-FLAG antibody, and the immunocomplexes were analyzed by Western blotting with anti-Myc antibody. As shown in Fig. 2, Myc-AtSRC2 was co-immunoprecipitated with FLAG-AtRbohF^N, suggesting that AtSRC2 interacts with AtRbohF in plant cells.

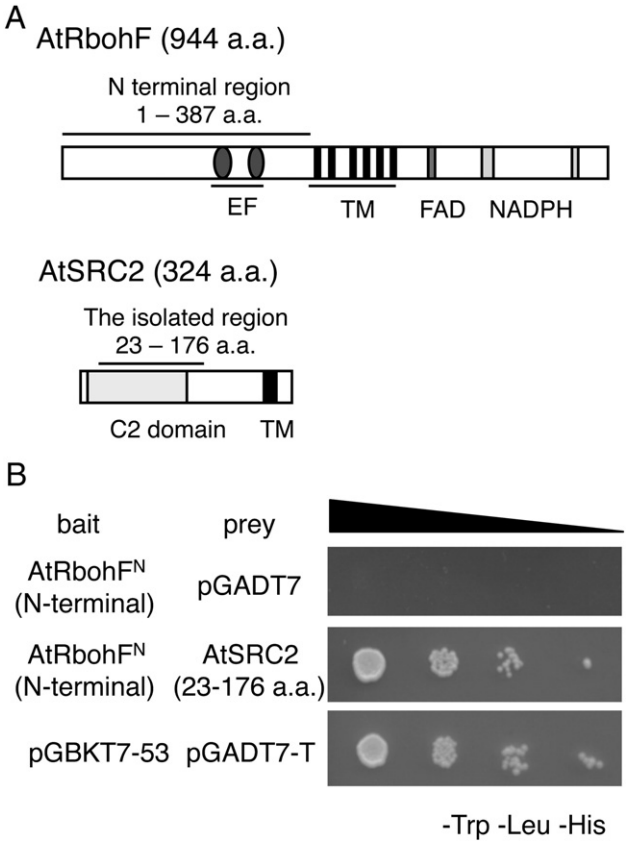

Fig. 1. Interaction of AtSRC2 with the N-terminal region of AtRbohF in a yeast two-hybrid assay. (A) Schematic representation of the domain structures of AtRbohF and AtSRC2. AtRbohF is a TM protein with six TM helices. The N-terminal region of AtRbohF contains two EF-hand motifs (EF), whereas the C-terminal region has FAD- and NADPH-binding domains. The region used for the yeast two-hybrid assay included amino acid residues 1–387 of AtRbohF. AtSRC2 has a C2 domain and a TM helix. The region isolated by the yeast two-hybrid screen includes amino acid residues 23–176 of AtSRC2. (B) Interaction of the N-terminal region of AtRbohF with the AtSRC2 fragment in a yeast two-hybrid assay. Protein interaction was determined by a growth assay on medium lacking tryptophan (Trp), leucine (Leu) and histidine (His). Dilutions (from 10^0 to 10^3) of transformed cells were spotted onto the plates. The interaction between pGBKT7-53 (p53) and pGADT7-T (SV40 large T-antigen) was used as a positive control. The pGADT7 empty vector was used as a negative control.

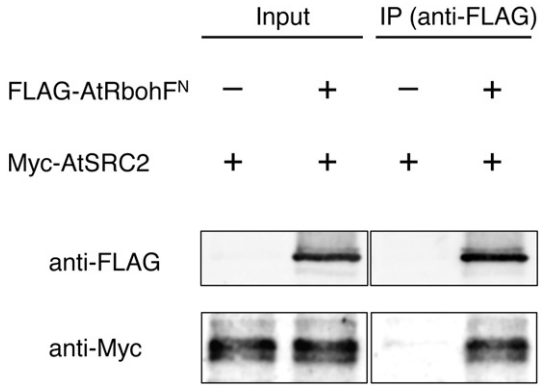

Fig. 2. Interaction of AtSRC2 with the N-terminal region of AtRbohF in plant cells. Co-IP assay in *N. benthamiana* leaf extracts. Myc-AtSRC2 (38 kDa) was transiently expressed with or without FLAG-AtRbohF^N (47 kDa) in *N. benthamiana* leaves by agroinfiltration. Protein extracts were immunoprecipitated with anti-FLAG antibodies and subjected to Western blotting using anti-FLAG or anti-Myc antibodies.

This observation suggests that AtSRC2 could be localized near AtRbohF in plant cells. To test this idea, we transiently expressed the N-terminal GFP-tagged AtSRC2 (GFP-AtSRC2) or AtRbohF (GFP-AtRbohF) in onion epidermal cells by biolistic bombardment and observed their intracellular localization of the proteins by confocal microscopy. Free GFP used as a control was distributed throughout the cytosol including the cytoplasmic strands and the nuclei (Fig. 3 upper). AtRbohF has been shown biochemically to be an intrinsic plasma membrane protein [28]. Consistently, the fluorescence of GFP-AtRbohF was observed at the cell periphery in

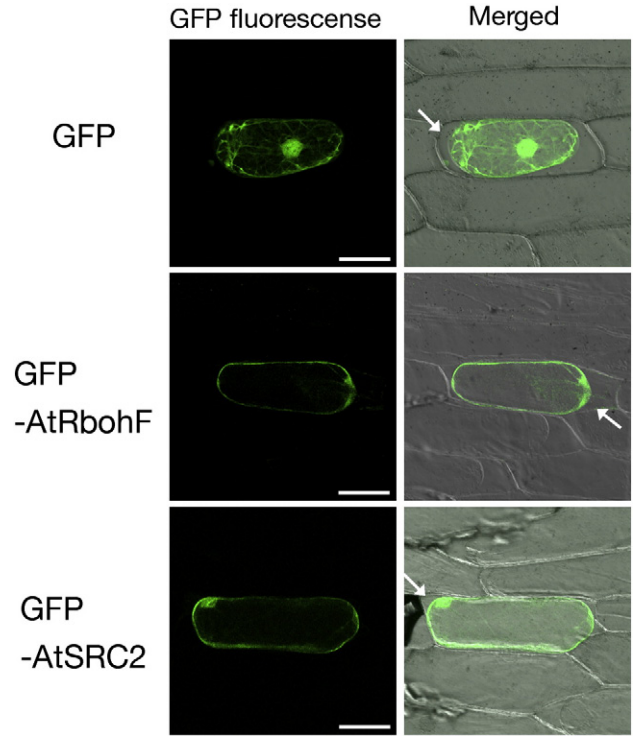

Fig. 3. Subcellular localization of GFP-AtRbohF and GFP-AtSRC2 in onion epidermal cells. Each GFP fusion protein was transiently expressed in the cells by biolistic bombardment. Plasmolysis was induced by incubation in 0.5 M mannitol for 10–30 min. Fluorescence images are single confocal sections. Bright field (differential interference contrast) and fluorescence images are merged. The arrow indicates the location of a cell wall. Scale bar = 100 μm.

plasmolyzed cells (Fig. 3 middle), supporting the notion that AtRbohF is localized to the plasma membrane. The fluorescence of GFP-AtSRC2 was observed at the cell periphery as well as in the cytoplasm of plasmolyzed cells (Fig. 3 bottom). AtSRC2 possesses a transmembrane domain in the C-terminal region (Fig. 1A), and has been detected in the highly-purified plasma membrane fraction of Arabidopsis [29]. These results support the idea that some of AtSRC2 could interact and function with AtRbohF in plant cells.

3.3. AtSRC2 enhances the Ca^{2+}-dependent ROS-producing activity of AtRbohF

To examine if AtSRC2 affects the ROS-producing activity of AtRbohF, we performed co-expression analyses in HEK293T cells. Ionomycin induced rapid and transient ROS production in the cells expressing *FLAG-tagged* full length *AtRbohF* (*FLAG-AtRbohF*), whereas no ROS production was detected in the cells transfected only with *Myc-AtSRC2*, *Myc-GFP* or the empty vector (Fig. 4A). Similarly, CA induced slow and continuous ROS production in the cells transfected with *FLAG-AtRbohF*, but not in those transfected with *Myc-AtSRC2, Myc-GFP* or the empty vector (Fig. 4B). All tagged proteins were detected in HEK293T cells by Western blotting (Fig. 4C). These results indicate that AtSRC2 does not possess any ROS-producing activity in HEK293T cells.

Then *FLAG-AtRbohF* was co-transfected with either *Myc-AtSRC2* or *Myc-GFP* into HEK293T cells. Ionomycin-induced ROS production was markedly enhanced in the cells co-expressing AtSRC2 than the control co-expressing Myc-GFP (Fig. 4D). Meanwhile, CA-induced ROS production by AtRbohF was not affected (Fig. 4E). Western blot analysis showed that co-expression of Myc-AtSRC2 did not affect the level of AtRbohF protein (Fig. 4F). These results indicate that AtSRC2 enhances

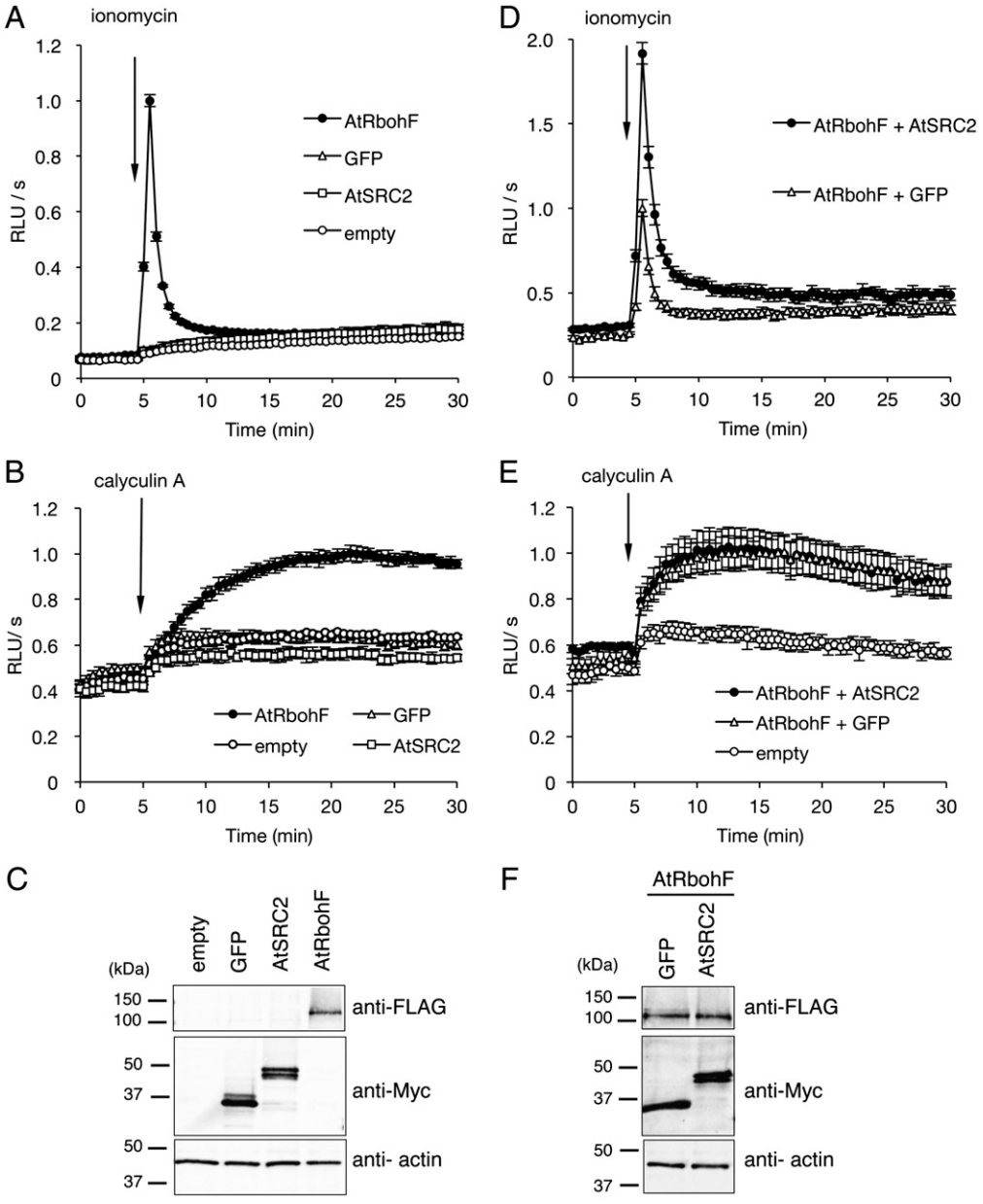

Fig. 4. Effect of AtSRC2 on the AtRbohF-mediated ROS production in HEK293T cells. (A and D) Ionomycin-induced ROS production. (B and E) Calyculin A-induced ROS production. After 5 min of baseline measurement, 1 μM ionomycin or 0.1 μM CA was added to medium. All quantified data are means ± S.E. (n = 3). (C and F) Expressed proteins were detected by Western blotting with anti-FLAG and anti-Myc antibodies. As a loading control, β-actin was used. Protein molecular masses are FLAG-AtRbohF (111 kDa), Myc-AtSRC2 (38 kDa), Myc-GFP (31 kDa) and β-actin (42 kDa).

the Ca^{2+}-dependent ROS-producing activity of AtRbohF, but does not affect its phosphorylation-induced activation in HEK293T cells.

3.4. ROS production and gene expression under cold stress

Expression of the soybean *SRC2* gene is induced by low temperature treatment [21]. Induction of *AtSRC2* expression by cold treatment within 2 h in Arabidopsis seedling leaf tissue has also been shown by serial analysis of gene expression (SAGE), a sequence-based technology developed to generate a transcript expression profile in a high throughput, accurate and non-biased manner [30]. Indeed, Northern blot analysis showed that expression of the *AtSRC2* gene was markedly induced at 4 °C within 5 h in Arabidopsis roots (Fig. 5A).

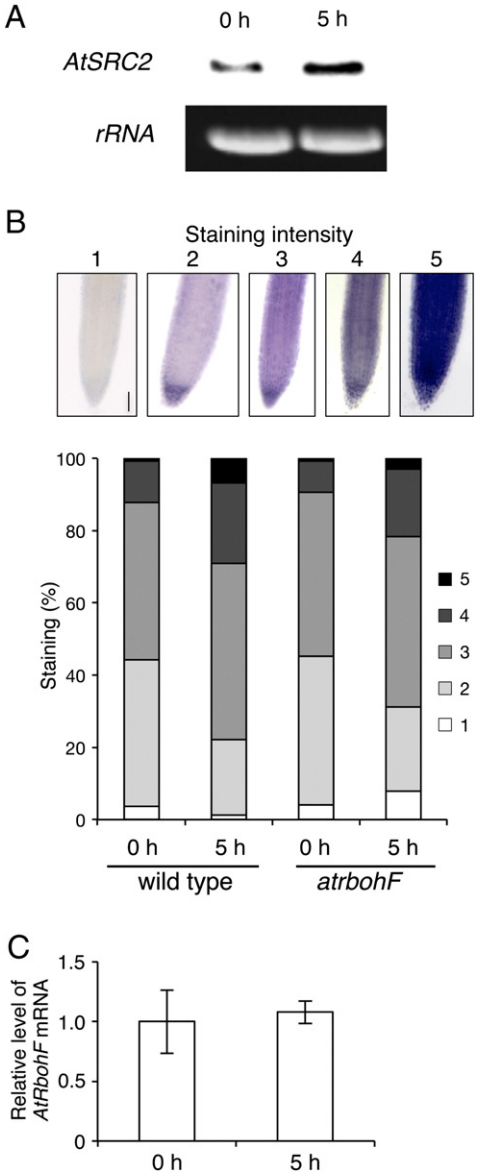

Fig. 5. Effect of low temperature treatment on the expression of *AtSRC2* and *AtRbohF*, and ROS generation in Arabidopsis roots. (A) The effect of low temperature treatment on the expression of *AtSRC2* gene was analyzed by Northern blotting. Ethidium bromide-stained rRNA (lower panel) is shown as a loading control. (B) Detection of ROS in roots using NBT. Roots were stained with NBT and categorized into five groups based on the staining intensity. Percentages of the stained roots of each category are shown for the wild-type and *atrbohF* mutant plants. n = 140 (wild-type 0 h), 162 (wild-type 5 h), 148 (*atrbohF* 0 h) or 167 (*atrbohF* 5 h). Scale bar = 50 μm. (C) Effect of low temperature treatment on the expression of the *AtRbohF* gene was detected by real-time RT-PCR. Average values and standard errors of three independent experiments are shown.

Based on our findings that AtSRC2 binds to the N-terminal region of AtRbohF in plant cells (Fig. 2) and up-regulates Ca^{2+}-dependent ROS-producing activity of AtRbohF in HEK293T cells (Fig. 4), we hypothesized that AtSRC2 may play a role in regulating the ROS production during the cold stress response. To test this hypothesis, we assayed the ROS accumulation in roots using nitroblue tetrazolium (NBT) staining and analyzed the effect of low-temperature treatment.

The intensity of NBT staining was semi-quantitatively categorized into five groups (Fig. 5B). In the wild-type plants, the number of roots categorized at the levels 4 and 5 increased under low-temperature treatment for 5 h in the dark, indicating that low-temperature treatment induced ROS production in roots. In the *atrbohF* mutant, the low temperature-induced ROS accumulation was slightly mitigated in comparison with the wild-type (Fig. 5B). The low temperature-induced ROS production in Arabidopsis is abolished by diphenylene iodonium (DPI), an NADPH oxidase inhibitor [31]. These results suggest that AtRbohF participates, at least partially, in the low temperature-induced ROS production.

Next, we examined the expression level of the *AtRbohF* gene in wild-type low temperature-treated roots by real-time RT-PCR analysis. The expression of *AtRbohF* was not affected by the cold treatment (Fig. 5C). Taken together, these results suggest that the low temperature-induced AtRbohF-dependent ROS accumulation cannot be explained by the induction of *AtRbohF* expression but is correlated with the induction of *AtSRC2* gene. This result implies that the low temperature-induced ROS production by AtRbohF may be enhanced by binding of AtSRC2 to AtRbohF.

4. Conclusions

Our findings suggest that the cold-inducible protein AtSRC2 is a novel activator of the Ca^{2+}-dependent activation of AtRbohF that enhances deliberate ROS production. This result implies a novel mechanism for regulating AtRbohF-mediated ROS production under cold stress. Low temperature induces expression of the *AtSRC2* gene (Fig. 5A) and cytosolic Ca^{2+} increase [32]. The translated AtSRC2 binds to AtRbohF to enhance the Ca^{2+}-dependent ROS-producing activity of AtRbohF. Regulated ROS production may play a role in regulating the expression of cold responsive transcription factor genes or activating MAPK cascade.

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