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Interaction between a plasma membrane-localized ankyrin-repeat protein ITN1 and a nuclear protein RTV1

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ABSTRACT

The increased tolerance to NaCl 1 (ITN1) protein is a plasma membrane (PM)-localized protein involved in responses to NaCl stress in *Arabidopsis*. The predicted structure of ITN1 is composed of multiple transmembrane regions and an ankyrin-repeat domain that is known to mediate protein–protein interactions. To elucidate the molecular functions of ITN1, we searched for interacting partners using a yeast two-hybrid assay, and a nuclear-localized DNA-binding protein, RTV1, was identified as a candidate. Bimolecular fluorescence complementation analysis revealed that RTV1 interacted with ITN1 at the PM and nuclei *in vivo*. RTV1 tagged with red fluorescent protein localized to nuclei and ITN1 tagged with green fluorescent protein localized to PM; however, both proteins localized to both nuclei and the PM when co-expressed. These findings suggest that RTV1 and ITN1 regulate the subcellular localization of each other.

1. Introduction

Ankyrin-repeat domains are present in a great variety of proteins in prokaryotes, eukaryotes and some viruses and often mediate protein–protein interactions [1]. Proteins containing ankyrin repeats are involved in diverse cellular functions including cell cycle regulation, cytoskeleton interactions, signal transduction and ion transport.

In plants, ankyrin-repeat proteins are involved in a number of important processes concerned with plant development, for example, leaf morphogenesis [2], chloroplast biogenesis [3], and embryogenesis [4]. These proteins were also found to play important roles in plant responses to biotic and abiotic stresses [5–7]. Because of the importance of ankyrin-repeat proteins in plants, genome-wide localization, phylogenetic relationships and expression profiles have been analyzed in *Arabidopsis* [8] and rice [9].

In the *Arabidopsis* genome, 105 genes encoding ankyrin-repeat proteins have been identified. Becerra et al. [8] classified these genes in 16 groups based on their structural similarity. The most abundant group contains 37 genes encoding proteins with ankyrin repeats and transmembrane domains (named the AtANKTM family), and two of these genes, *ACD6* and *ITN1* have been functionally characterized as mediators of stress responses so far. ACD6 protein is proposed to act as a plasma membrane (PM)-localized positive regulator of salicylic acid (SA) signaling that controls defense responses against pathogens [5]. We previously demonstrated that ITN1 protein was also localized to the PM and that the lack of this protein led to increased tolerance to NaCl stress in an *Arabidopsis* mutant [6]. In the *itn1-1* mutant, the NaCl- and abscisic acid (ABA)-responsive expression of genes encoding reactive oxygen species (ROS)-producing NADPH oxidases was suppressed, resulting in a corresponding reduction in ROS accumulation. ITN1 presumably acts as a positive regulator of ABA-mediated production of ROS under NaCl-stress conditions; however, it remains unclear how these ankyrin-repeats proteins transduce SA or ABA signals in response to environmental changes. That is, interacting partners of AtANKTM proteins have not been identified primarily because multiple transmembrane structures of AtANKTM proteins complicate the post-translational analysis of these proteins.

There are some reports about interactions between transcription factors and ankyrin-repeat proteins belonging to groups distinct from AtANKTM. NPR1, an ankyrin-repeats protein with no transmembrane domains, is known to interact with TGA transcription factors [10,11], and regulates systemic acquired resistance in *Arabidopsis* [12]. This interaction stimulates the DNA binding activity of TGA factors [11,13]. An *Arabidopsis* PM-localized ankyrin-repeat protein, ACBP2, is known to interact with a nuclear-localized transcription factor AtEBP [14]. It is possible that ACBP2 regulates the nuclear localization of AtEBP by interacting with it.

Here, in order to elucidate the molecular functions of ITN1, we searched for interacting partners using a yeast two-hybrid assay.

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A nuclear protein, RTV1, was identified as a candidate. To our knowledge, this is the first report identifying an AtANKTM interacting partner.

2. Materials and methods

2.1. Plant materials and growth conditions

All lines of *Arabidopsis thaliana* described here were derived from the Columbia wild type. The SALK_131754 (*rtv1-KD*) line is available from the Arabidopsis Biological Resource Center at Ohio State University (ABRC). The mutant *itn1-1* line was isolated as described previously [6]. Plants were routinely grown at 22 °C under continuous white light on solid MS medium [15] containing 1 % (w/v) sucrose and 0.5 % (w/v) gellan gum. Seedlings were used for experiments at 7 days after germination. For treatment with exogenous SA or NaCl, seedlings were transferred onto fresh media supplemented with 10, 100 μM SA or 200 mM NaCl.

2.2. Plasmid construction

Primers used for PCR are listed in Supplementary Table 1. To construct a bait plasmid used in the yeast two-hybrid screening, a transmembrane helices-lacking region encoding the first 419 amino acids of ITN1 (TAIR ID: AT3G12360), generated by PCR using primers P1 and P2, was digested with *NcoI*/*Ecl136II* and subcloned into the *NcoI*/*SmaI* sites of the pGBKT7 vector (Clontech). The resulting plasmid was named pGBK-ITN1(419).

To construct the bimolecular fluorescence complementation (BiFC) plasmid [16,17], a full-length *ITN1* cDNA, generated by PCR using primers P3 and P4, was digested with *SalI*/*SacI* and subcloned into the *SalI*/*SacI* sites of the pSY735 vector. Full-length *RTV1* (TAIR ID: AT1G49480) and *VRN1* (TAIR ID: AT3G18990) cDNAs, generated by PCR using primers P5 and P6 for *RTV1*, P7 and P8 for *VRN1*, were digested by *SacI* or *SalI*/*BamHI* and subcloned into *SacI* or *SalI*/*BamHI* sites of the pSY736 vector.

To construct the RTV1-red fluorescent protein (RFP) or RTV-yellow fluorescent protein (YFP) plasmids, a full-length *RTV1* cDNA, generated by PCR using primers P9 and P10, was digested and subcloned into the *SalI*/*NotI* sites of the pENTR1A vector (Invitrogen), followed by integration into the destination vector pH7RWG2 or pH7YWG2 [18] using Gateway LR clonase (Invitrogen). The resulting plasmids were named p35S-RTV1RFP or p35S-RTV1YFP, respectively.

The ITN1-green fluorescent protein (GFP) and cyan fluorescent protein (CFP) plasmids used in this study were described previously [6,17].

2.3. Yeast two-hybrid screening

A yeast two-hybrid screening was carried out according to the manufacturer's protocol (Clontech). Briefly, the bait plasmid, pGBK-ITN1(419) and total cDNAs of *Arabidopsis* obtained from ABRC (catalog number CD4-10) in which the cDNAs were fused to the pACT-AD, were used sequentially to transform *Saccharomyces cerevisiae* strain AH109. The resulting transformants were then plated on SD medium lacking adenine (Ade-), leucine (Leu-), tryptophan (Trp-) and histidine (His-) and including X-α-gal. The clones of cDNAs encoding candidate ITN1 interacting proteins were selected and isolated based on their growth as blue colonies. The cDNAs were isolated, sequenced and then analyzed with BLAST using TAIR databases.

2.4. Fluorescent microscopy

Plasmid DNAs for analysis of GFP, RFP and BiFC fluorescence were introduced into onion epidermal cell layers as described previously [19]. Confocal images were collected using a Zeiss LSM510 microscope for BiFC analysis, and using a Leica SP5 microscope for GFP and RFP analyses.

2.5. Plant transformation

To generate transgenic plants overexpressing *RTV1*, *Arabidopsis* plants were transformed using p35S-RTV1YFP by the floral dipping method [20].

2.6. Quantitative reverse transcription-PCR (qRT-PCR) analysis

RNA isolation and qRT-PCR were carried out as previously described [21]. The sequences of gene-specific primers used for qRT-PCR reactions are listed in Supplementary Table 2.

2.7. Endogenous SA levels

Arabidopsis seedlings (about 100 mg) were harvested at 12 days after germination. Extraction of SA was carried out as described by Forcat et al. [22]. A high resolution LC system connected to a tandem quadrupole mass spectrometer equipped with an electrospray interface (LC-ESI-qMS/qMS) [23] was used to measure endogenous SA levels.

3. Results and discussion

3.1. Identification of a possible interacting partner with ITN1

An *Arabidopsis* mutant deficient in ITN1 protein showed a NaCl-tolerant phenotype, suggesting that ITN1 is involved in plant responses to NaCl stress [6]. The deduced structure of ITN1 protein contains two characteristic regions. The N-terminal region contains seven ankyrin repeats, based on the SMART protein domain prediction program (http://smart.embl-heidelberg.de). The C-terminal region contains four predicted transmembrane helices. Ankyrin repeat domains are implicated in protein–protein interactions, suggesting that ITN1 plays a role in NaCl stress signaling together with its interacting partner.

To search for an ITN1-interacting partner, we screened an *Arabidopsis* cDNA library using a yeast two-hybrid assay with ITN1, in which the C-terminal transmembrane helices were deleted, as the bait. A nuclear protein encoded by AT1G49480 was identified as a candidate (Fig. 1A). The deduced amino acid sequence of this protein is highly homologous to *Arabidopsis* VRN1 (85.3% identity) (Fig. 1B), a transcription factor that functions in both flowering-time control and vernalization [24,25]. Therefore, the AT1G49480 locus was designated *RTV1* (related to *VRN1* 1). RTV1 is predicted to have a B3 DNA binding domain at its C terminus by Pfam (http://pfam.sanger.ac.uk/), whereas VRN1 has two B3 domains at its N and C termini. Recently, RTV1 was shown to localize at the nucleus and bound to DNA *in vitro* in a non-sequence-specific manner [26]. The authors demonstrated that RTV1 interacted with another nuclear protein EXTRA-LARGE G-PROTEIN2 (XLG2, TAIR ID: AT4G034390) and that XLG2 promoted the DNA binding activity of RTV1, leading to early flowering in *Arabidopsis*.

If the interaction between RTV1 and ITN1 occurs in *vivo*, these proteins should be expressed simultaneously in the same tissues and organelles. Reporter-GUS analyses revealed that tissue-specific expression patterns of *RTV1* and *ITN1* are consistent with their interaction [6,26]. On the other hand, we previously reported that

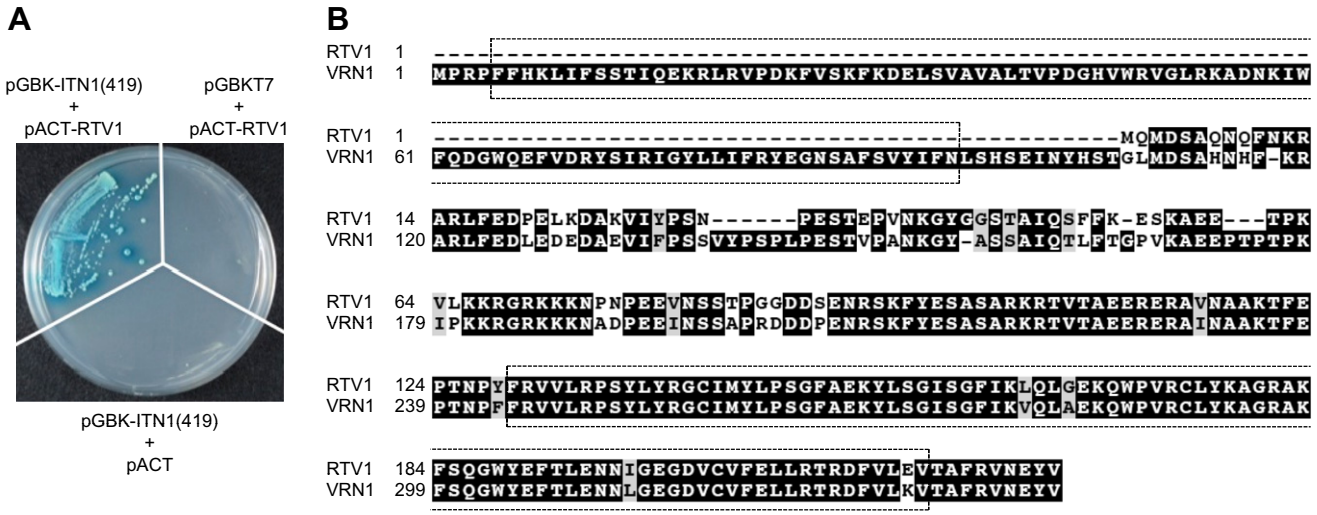

Fig. 1. Yeast two-hybrid screening for an interacting partner of ITN1. (A) Yeast two-hybrid analysis. Yeast co-transformed with plasmids harboring *ITN1* in which the C-terminal transmembrane helices coding sequence was deleted (pGBK-ITN1(419)) and *RTV1* (pACT-RTV1) was cultured on a SD (Ade-/Trp-/Leu-/His-) plate containing X-α-gal. The pGBKT7 and pACT constructs are empty vector controls. Growth of blue colonies indicates interaction between proteins encoded in the plasmids. (B) Alignment of deduced amino acid sequences of RTV1 and VRN1. The dashed boxes indicate B3 DNA binding domains. The amino acid sequences were aligned using ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) and then analyzed with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Black boxes denote conserved invariant amino acids, and gray boxes indicate conserved substitutions.

ITN1 localized at the plasma membrane (PM), whereas RTV1 is a nuclear protein. Considering that we used the transmembrane-helices-deleted form of ITN1 as the bait in our yeast two-hybrid assay, it is possible that RTV1 interacts with ITN1 only in our yeast two-hybrid system. To elucidate this possibility, it was necessary to examine whether full-length ITN1 and RTV1 could interact in plant cells.

3.2. Interaction between RTV1 and ITN1 in vivo

To verify the *in vivo* interaction between ITN1 and RTV1, we used BiFC method [16,17]. BiFC is a technique to analyze protein–protein interaction *in vivo*, wherein YFP is split into two non-overlapping N-terminal (YN) and C-terminal (YC) fragments, fused to the protein of interest, and YFP fluorescence is reconstituted only when these two proteins interact. *ITN1* and *RTV1* full-length cDNAs were subcloned downstream of the YC and YN coding sequences as described in Fig. 2 (*YC-ITN1* and *YN-RTV1*, respectively). These genes were co-introduced by particle bombardment into onion epidermal cells together with a reference plasmid encoding CFP as the introduction control. In the onion cells, YFP fluorescence was observed in nuclei and the PM, suggesting that YC-ITN1 and YN-RTV1 might localize and interact in these subcellular localizations, and that YFP was reconstituted in the resulting protein complex (Fig. 2A). On the other hand, interaction between ITN1 and VRN1, which is a homolog of RTV1, was not observed in BiFC analysis (Fig. 2A). This result suggests that ITN1 and RTV1 might specifically interact *in vivo*.

In a previous paper, we reported that ITN1 localized only at the PM in onion cells transiently expressing GFP fused to ITN1 (ITN1-GFP) [6]. Moreover, the localization of full-length RFP fused to RTV1 (RTV1-RFP) was restricted to nuclei (Fig. 2B); however, the BiFC result indicates that ITN1 and RTV1 can localize to both nuclei and the PM when *YN-RTV1* and *YC-ITN1* are co-expressed (Fig. 2A). This result raised the possibility that ITN1 and RTV1 might each affect the subcellular localization of the other protein in cells expressing both proteins simultaneously. To confirm this possibility, *RTV1-RFP* and *ITN1-GFP* were co-introduced into onion cells. In such cells, RTV1-RFP and ITN1-GFP localized to both nuclei and the PM (Fig. 2B). This result suggests that ITN1 functions as a PM anchor of RTV1 and partially inhibits the nuclear transport of RTV1 and that RTV1 can promote the nuclear localization of ITN1.

In roots of transgenic *Arabidopsis* stably expressing *ITN1-GFP*, GFP fluorescence localized to both nuclei and the PM. The nuclear localization of ITN1-GFP is possibly caused by the interaction of ITN1-GFP with endogenous RTV1 (Supplementary Fig.1). On the other hand, in cotyledons, the nuclear localization of ITN1-GFP was not detected. Taken together, the tissue- or cell-specific expression pattern of *ITN1* and *RTV1* may affect the ratio of the nuclear-type to PM-type forms of proteins.

3.3. Phenotypes of RTV1-overexpressing and RTV1-suppressing Arabidopsis lines

In a previous paper, we reported that a loss-of-function mutation of ITN1 (*itn1-1*) conferred a NaCl-tolerant phenotype [6]. To investigate whether its interacting partner RTV1 is also involved in plant NaCl tolerance, we isolated *RTV1*-overexpressing *Arabidopsis* lines (*RTV1-OX*) and a T-DNA insertion line of *RTV1* (SALK_131754, *rtv1-KD*) and examined their NaCl tolerance. We confirmed that expression levels of *RTV1* were higher in the *RTV1-OX* line and lower in the *rtv1-KD* than wild-type plants (Fig. 3A). There were no obvious phenotypic differences between these lines and wild-type plants in their responses to 200 mM NaCl, whereas *itn1-1* showed a NaCl-tolerant phenotype under the same conditions (Supplementary Fig.2). Therefore, the involvement of *RTV1* in plant NaCl tolerance remains unclear.

In the present study, we focused on characterizing phenotypes of *RTV1-OX*, *rtv1-KD* and *itn1-1* in response to exogenous phytohormone treatments in order to reveal possible functions of the interaction between RTV1 and ITN1. Recently, both *RTV1-OX* and *itn1-1* lines were found to become dwarfed and chlorotic in response to salicylic acid (SA) (Fig. 3B). These phenotypes are also observed in SA-hypersensitive mutants in some cases [27]. SA plays an important role in the defense response in many plant species to pathogen attack and also in plant NaCl responses. SA increases the oxidative damage generated by NaCl in *Arabidopsis*, which in turn is critical for seedling lethality from NaCl stress [28]. In contrast, the NaCl tolerance-promoting effects of SA have also been reported [29]. These contradictory findings reflect the current

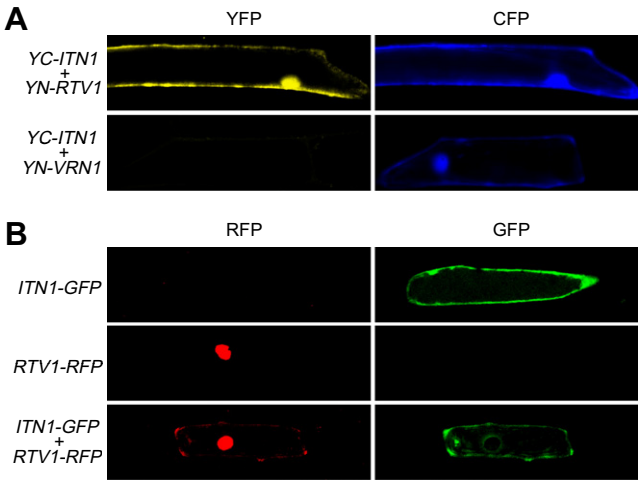

Fig. 2. Interaction of ITN1 and RTV1 in onion epidermal cells. (A) BiFC analysis. Onion epidermal cells were transiently co-transformed with plasmids harboring *YC-ITN1*, *YN-RTV1* and *CFP* (upper panel) or *YC-ITN1*, *YN-VRN1* and *CFP* (lower panel) by particle bombardment. YFP fluorescence indicates interaction between proteins encoded in the plasmids and reconstitution of entire YFP. CFP fluorescence indicates the successful introduction of plasmids in onion cells. (B) Localization of ITN1 and RTV1. GFP and RFP fluorescence indicates the subcellular localization of ITN1-GFP and RTV1-RFP, respectively. Onion cells were transiently transformed with plasmids harboring *ITN1-GFP* (upper panel) or *RTV1-RFP* (middle panel) and co-transformed with these plasmids (lower panel).

difficulties in understanding and modeling the relationships between SA signaling and plant NaCl tolerance.

To evaluate the SA-sensitivities of these lines, we quantified expression levels of three SA-inducible genes, *PR1*, *PR2* [30] and *EDS1* [31] by qRT-PCR analysis (Fig. 3C). SA-responsive expression of *PR1* and *PR2* was elevated in the *RTV1-OX* and *itn1-1* lines compared with wild-type plants. In the *RTV1-OX* line, expression levels of these two genes were higher than wild-type plants even under control conditions. Since endogenous SA level in the *RTV1-OX* line (405.69 ± 6.31 pmol/g FW) was comparable with that in the wild-type plants (449.11 ± 43.01 pmol/g FW) (mean ± SE, $n = 3$), the elevated levels of *PR1* and *PR2* expression in the *RTV1-OX* line would be directly caused by exogenous SA. These results suggest that both the *RTV1-OX* and *itn1-1* lines showed SA-hypersensitive-like phenotypes. Considering the inhibitory effect of ITN1 on the nuclear transport of RTV1 described above, both *RTV1-OX* and *itn1-1* lines probably accumulate higher levels of RTV1 protein in their nuclei than wild-type plants. SA-hypersensitivities of these two lines may be involved in the nuclear accumulation levels of RTV1.

As previously reported [26], the interacting partner of RTV1 is not only ITN1. An alternative explanation for the SA-hypersensitive-like phenotypes of the *RTV1-OX* and *itn1-1* lines could be that RTV1 positively regulates SA signaling via interaction with other proteins. In this scenario, ITN1 could act as a competitive inhibitor of the interaction in wild-type plants. Because ACD6, another PM-localized AtANKTM protein, acts as a positive regulator of SA [5], it is valuable to investigate whether RTV1 interacts with ACD6. Interaction between RTV1 and XLG2 was reported to be involved in flowering [26], and SA also regulates flowering time [32]. However, the direct involvement of the interaction between RTV1 and XLG2 in SA signaling has not been demonstrated yet.

With respect to *EDS1*, its expression was suppressed in the *itn1-1* mutant but was not in the *RTV1-OX* line (Fig. 3C). This observation suggests that ITN1 may be required in a branch of the SA signal transduction pathway regulating the expression of *EDS1*, independently of *RTV1*. Ankyrin-repeat proteins were reported to interact with multiple partners in some cases [10,14,33]. The complicated SA-sensitivity in the *itn1-1* line is probably due to impaired interaction between ITN1 and unidentified proteins in the mutant. *EDS1* is necessary for pathogen-induced SA accumulation and to be induced by SA in a positive feedback manner [34], whereas *PR1* and *PR2* are well-known downstream genes of SA.

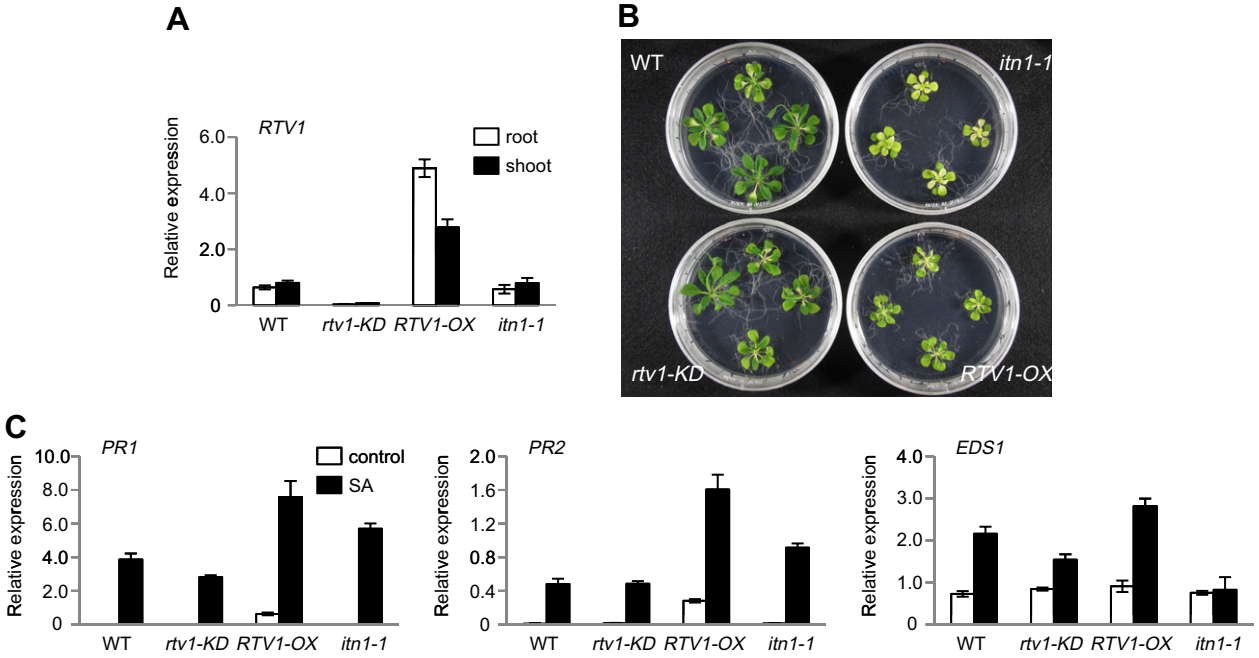

Fig. 3. Phenotypical analysis of *rtv1-KD*, *RTV1-OX* and *itn1-1* lines. (A) qRT-PCR analysis of *RTV1* transcripts. The expression of *RTV1* in roots (white bars) and shoots (black bars) was normalized to the expression of *UBQ10*. Means and SD were calculated from three independent experiments. (B) Phenotypes of WT, *rtv1-KD*, *RTV1-OX* and *itn1-1* seedlings transferred onto media supplemented with 10 μM SA and grown for 11 days. (C) qRT-PCR analysis of *PR1*, *PR2* and *EDS1* transcripts. Relative transcript levels of RNAs isolated from seedlings treated with 100 μM SA for 5 days (black bars) or mock-treated (white bars). The expression of *PR1*, *PR2* and *EDS1* were normalized to the expression of *UBQ10*. Means and SD were calculated from three independent experiments.

ROS have emerged as important signals in plant responses to environmental stresses, and SA is known to promote conversion of $O_2^{\cdot -}$ generated by stress to H_2O_2. Straus et al. [35] hypothesized that EDS1 seems to function as a master regulator of the SA-promoted ROS conversion, and that the balance between these distinct ROS may regulate initiation and spread of cell death, enabling plants to respond flexibly to different biotic and abiotic stresses. The suppression of SA-responsive *EDS1* expression in the *itn1-1* mutant may be involved in the salt tolerant phenotypes (delay of chlorosis development and low levels of H_2O_2) of the mutant [6].

In this paper, we show that PM-localized ITN1 and nuclear-localized RTV1 each affect the localization of the other protein via an ankyrin-mediated interaction. This is the first report identifying an interacting partner of the AtANKTM family [8]. The physiological functions of the interaction between ITN1 and RTV1 remain unclear. Our preliminary data raises the possibility that the interaction is involved in SA sensitivity. SA-mediated signaling controls defense responses against pathogens and SA signaling acts antagonistically with ABA signaling involved in responses to abiotic stresses [36]. The SA-hypersensitive-like phenotypes of the *itn1-1* line are consistent with a previous result indicating that this mutant is partially impaired in ABA signaling in *Arabidopsis* [6].

Following the identification of an interacting partner of ITN1, a detailed study is required on the modes of action of ITN1 in NaCl stress or SA signal transduction processes. Dissecting the biochemistry of the interaction between RTV1 and ITN1 will provide valuable information regarding the precise roles of AtANKTM family proteins in the fundamentally important adaptations of plants to biotic and abiotic stresses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.136.

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