A VAMP-associated protein, PVA31 is involved in leaf senescence in *Arabidopsis*

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VAMP-associated proteins (VAPs) are highly conserved among eukaryotes. Here, we report a functional analysis of one of the VAPs, PVA31, and demonstrate its novel function on leaf senescence in *Arabidopsis*. The expression of *PVA31* is highly induced in senescence leaves, and localizes to the plasma membrane as well as the ARA7-positive endosomes. Yeast two-hybrid analysis demonstrates that PVA31 is interacted with the plasma membrane localized-VAMP proteins, VAMP721/722/724 but not with the endosome-localized VAMPs, VAMP711 and VAMP727, indicating that PVA31 is associated with VAMP721/722/724 on the plasma membrane. Strong constitutive expression of *PVA31* under the control of the Cauliflower mosaic virus 35S promoter induces the typical symptom of leaf senescence earlier than WT in normal growth and an artificially induced senescence conditions. In addition, the marker genes for the SA-mediated signaling pathways, *PR-1*, is promptly expressed with elicitor application. These data indicate that *PVA31*-overexpressing plants exhibit the early senescence phenotype in their leaves, and suggest that PVA31 is involved in the SA-mediated programmed cell death process during leaf senescence and PR-protein secretion during pathogen infection in *Arabidopsis*.

Introduction

In the final stage of leaf development, leaf tissue undergoes an age-dependent deterioration process that leads to cell death. This process is called leaf senescence. Leaf senescence is a highly regulated degradative process that involves age-dependent programmed cell death (PCD), which is important for plant reproduction as it relocates nutrients from leaves to growing seeds.[1] PCD also plays a critical role in various developmental and physiological processes, including xylogenesis, embryogenesis, seed development, and seed germination.[2,3]

VAMPs (also referred as synaptobrevins) are the transport vesicle–residing soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that are involved in the membrane fusion process between transport vesicles and target membranes.[4] VAMP-associated proteins (VAPs) are highly conserved type-II integral membrane proteins in eukaryotes, which are involved in diverse cellular functions, including regulation of lipid transport, membrane trafficking, neurotransmitter release, stabilization of presynaptic microtubules, and unfolded protein response.[5] First VAP protein, VAP-33 was isolated as a VAMP-associated protein with a yeast 2-hybrid screen of a sea hare, *Aplysia californica*; it is involved in the VAMP-mediated neurotransmitter release at the plasma membrane (PM).[6] A mammalian VAP-related protein, VAP-A has been reported to bind not only VAMP protein but also other SNAREs and SNARE-binding proteins, including syntaxin 1A, rbet1, rsec22, αSNAP, and NSF.[7] A rat VAP-33-related protein, ERG-30, has been reported to function in COPI-mediated ER-Golgi trafficking, although no direct interaction between ERG-30 and SNARE proteins was detected.[8]

An alternate function of VAPs that has been reported is lipid transfer from ER to a wide range of intracellular membranes via membrane contact sites (MCSs) coordinated with the FFAT (double phenylalanine in an acidic tract) motif containing lipid transfer proteins.[5] The FFAT motif consists of the consensus EFFDAxE motif, which binds to the major sperm protein (MSP) domain of VAPs forming a 2:2 complex.[9] VAPs have been reported to interact with various FFAT motif-containing lipid binding proteins, including oxysterol-binding proteins (OSBPs),[10-12] ceramide transport proteins (CERTs),[13] a glycolipid transfer protein (GLTP),[14] and a PI/PC-transfer protein.[15] In *Arabidopsis*, 10 VAP-33 family proteins, which are referred to as plant VAP homologs (PVAs), are found in the genome.[16] Although several VAPs have been reported to interact with various proteins,[17-20] the physiological functions of VAPs in plants remain largely unknown.

In this study, we found that one of the PVA-family proteins, PVA31, was highly expressed in senescent leaves in *Arabidopsis*.
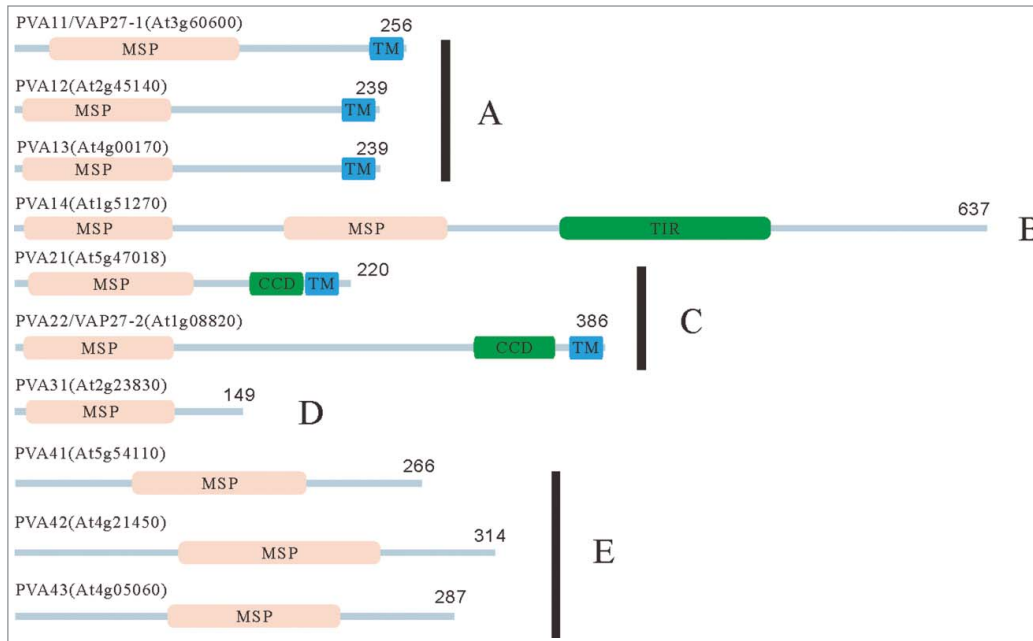
Figure 1. The domain structures of *Arabidopsis* VAP proteins. Domain structures of *Arabidopsis* VAP (PVA) proteins were predicted with the SMART program (http://smart.embl-heidelberg.de/). On the basis of the molecular structures and similarities of VAP proteins, we categorized 10 *Arabidopsis* VAP proteins into 5 subgroups. The abbreviations of domains used in this figure are as follows: CCD: coiled-coil domain, MSP: major sperm protein domain, TIR: Toll/interleukin-1 receptor homology domain TM: transmembrane domain. The numbers indicate the length of molecules in amino acid.

GFP-PVA31 localized to the PM and was interacted with the PM-resided VAMP proteins. The strong constitutive expression of *PVA31* under the control of the Cauliflower mosaic virus 35S promoter induced the early senescence phenotypes in rosette leaves. In *PVA31* overexpressing plants, the SA-mediated signaling pathway was promptly activated with elicitor application. These findings suggest that PVA31 may be involved in the PCD during leaf senescence and PR-protein secretion during pathogen infection to regulate the SNARE-mediated membrane trafficking to the PM in *Arabidopsis*.

Results

Identification of one of the VAP-family proteins, PVA31, as a senescence-induced VAP in *Arabidopsis*

It has been reported that 10 VAP33-related molecules designated as PVAs (plant VAPs) were found in the *Arabidopsis* genome.[16] Typical VAP homologues among eukaryotes consist of an N-terminal major sperm protein (MSP) and a C-terminal transmembrane (TM) domains.[5] According to the molecular structures and similarities of VAP proteins, we categorized 10 *Arabidopsis* PVA proteins into 5 subgroups; 3 PVAs in group-A (PVA11, PVA12 and PVA13) have typical N-terminal MSP and a C-terminal TM domains, group-B PVA consists of only one group protein, PVA14, which has a unique protein structure with 2 conserved MSP domains and a Toll/interleukin-1 receptor homology (TIR) domain in the middle of the molecule with no

C-terminal TM domain. Two Group-C PVAs (PVA21 and PVA22) possess typical VAP protein structure with the N-terminal MSP, the C-terminal TM domains, and the coiled-coil domain adjacent to the TM. PVA31, which belongs to group D, has no TMD in its C-terminus, with the smallest molecular weight (17-kDa) among *Arabidopsis* PVAs, and 3 group-E (PVA41, PVA42 and PVA43) PVAs have an N-terminal extension sequence with no C-terminal TMD (**Fig. 1**).

To identify VAP proteins that might be involved in the leaf senescence process, we searched genes encoding the VAP proteins in the publicly available *Arabidopsis* microarray database on the various developmental stages of leaves by using the *Arabidopsis* eFP Browser,[21] and found that *PVA31* is specifically expressed in the senescent leaves (**Fig. S1**).

PVA31 localizes to the plasma membrane and the endosomes

Next, we transiently expressed GFP-fused PVA31 (GFP-PVA31) under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter by *Agrobacterium*-mediated transient transformation, and observed the subcellular localization of GFP-PVA31 with confocal laser scanning microscopy. The GFP-PVA31 protein predominantly localized to the plasma membrane (PM) of the epidermal cells of hypocotyl (**Fig. 2A and B**), leaf mesophyll cells as well as leaf epidermal cells (**Fig. 2C-E**). The GFP-fluorescence was also observed on the intracellular mobile punctate structures near the PM (arrowheads in **Figure 2C and E; Movie S1**). To determine the subcellular localization of the punctate structures of GFP-PVA31, we co-expressed GFP-PVA31 with a PM-localized VAMP protein, mRFP-VAMP722[22] and an endosomal RAB protein, mRFP-ARA7.[23] The fluorescence of PVA31 and VAMP722 was completely merged on the PM (**Fig. 2F-H**), and the fluorescent punctate structures of GFP-PVA31 were merged with mRFP-ARA7 (arrowheads in **Figure 2I-K**), indicating that PVA31 localizes to the PM and the ARA7-positive endosomes.

PVA31 is interacted with the plasma membrane localized-VAMPs but not with endosome-localized VAMPs

VAP-33 was firstly isolated as a VAMP-associated protein in a yeast 2-hybrid screen of a sea hare, *Aplysia californica*, and is
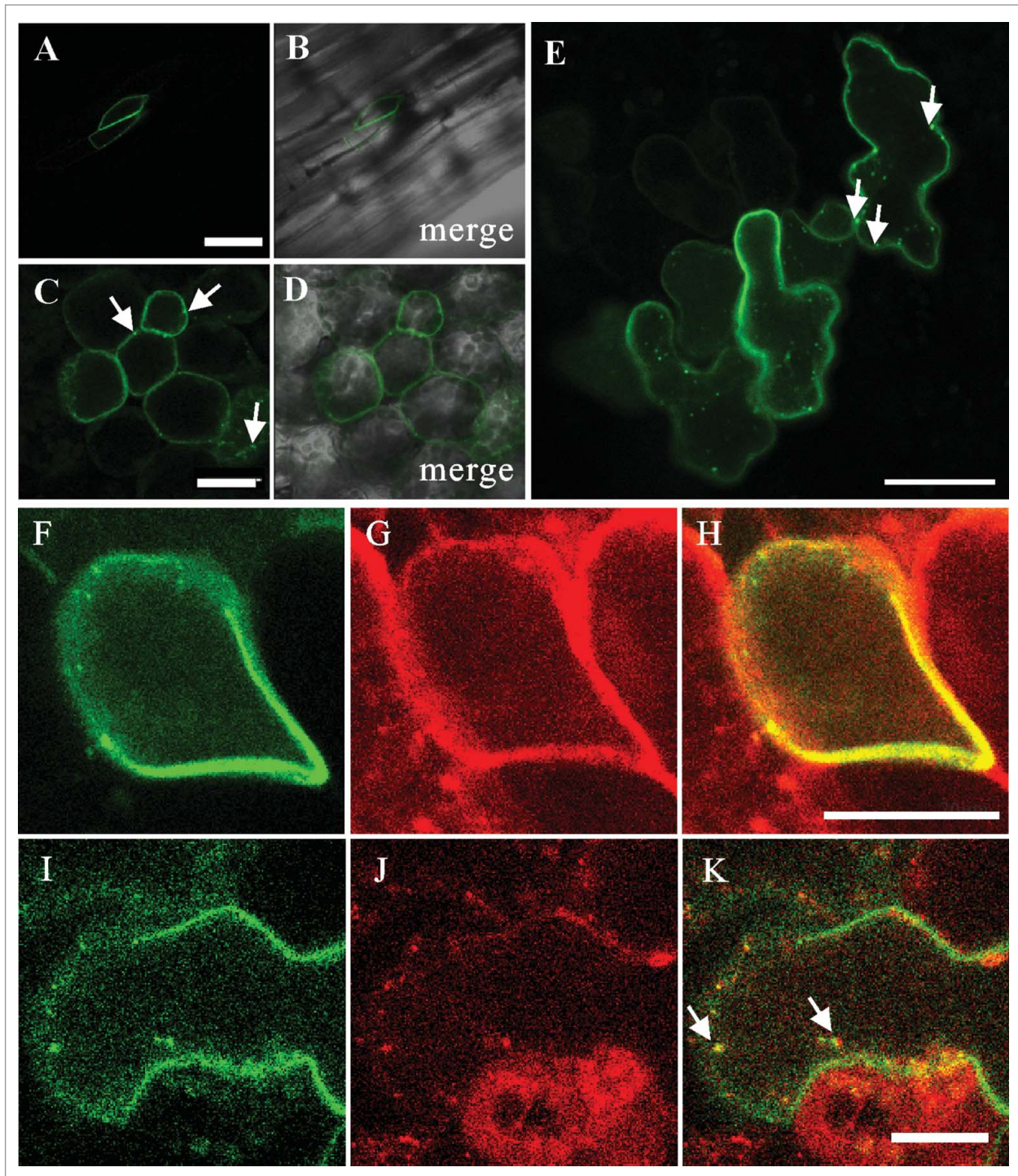
Figure 2. Localization of GFP-PVA31 in *Arabidopsis*. The confocal images of GFP fluorescence (**A, C**) and merged DIC and GFP-fluorescence (**B, D**) were captured in epidermal cells of hypocotyls (**A, B**) and mesophyll cells of cotyledon (**C, D**) of *Arabidopsis* plants transiently expressing GFP-PVA31. The images of GFP fluorescence in epidermal cells (**E**). The confocal images of GFP-PVA31 (**F**), mRFP-VAMP722 (**G**) and merged image of F and G (**H**) in epidermal cells of hypocotyl. Arrows indicate the mobile punctate structures of GFP-PVA31. Bars = 50 μm (**A**) , 20 μm (**C**) and 10 μm (**H**, **K**).

involved in the VAMP-mediated neurotransmitter release at the PM,[17] suggesting that PVA31 is also interacted with particular VAMP proteins in *Arabidopsis*. To investigate whether PVA31 is interacted with particular *Arabidopsis* VAMP proteins, we tested an interaction of PVA31 with several *Arabidopsis* VAMPs by using a yeast 2-hybrid interaction analysis. As shown in **Figure 3**, PVA31 is specifically interacted with VAMP721/722/724 but not with VAMP711 and VAMP727. VAMP721/722/724 reportedly localized to the PM in *Arabidopsis* cells, while VAMP711 and VAMP727 localized to the vacuolar membrane
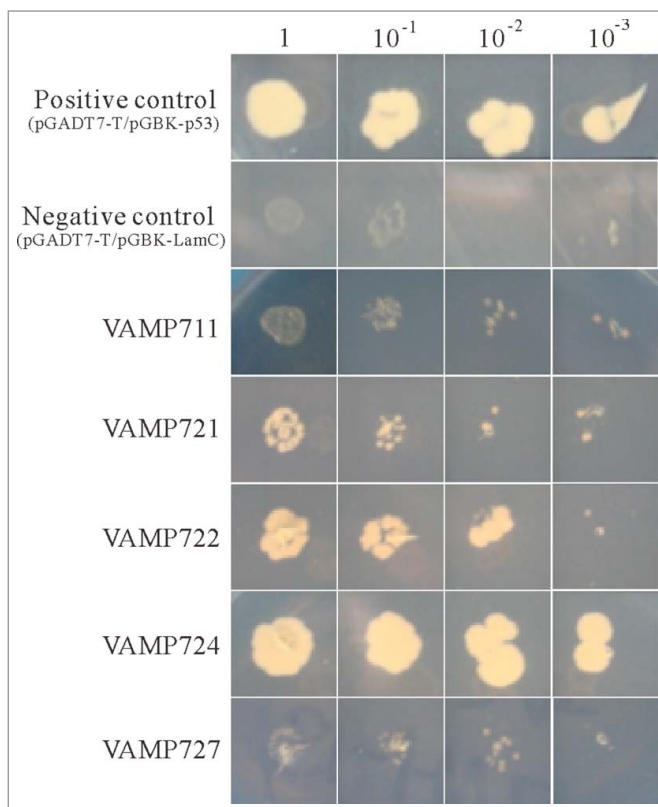
Figure 3. Interaction of PVA31 and VAMP proteins. Yeast two-hybrid assay were performed to confirm between PVA31 and *Arabidopsis* VAMP proteins. PVA31 and VAMP/711/721/722/724/727 were fused with activation domain (AD) and DNA binding domain (BD) of the transcription factor GAL4, respectively. The yeast strains co-transformed with PVA31 and indicated VAMPs plasmids were grown on selective media lacking leucine, tryptophan and histidine and adding 3-AT. Positive control: pGADT7-T-antigen and pGBKT7-p53. Negative control: pGADT7-T-antigen and pGBKT7-Lamin C.

and the endosomes, respectively.[27,28] These results suggest that PVA31 is specifically associated with VAMP721/722/724 at the PM.

Overexpression of *PVA31* confers the early senescence phenotype in *Arabidopsis*

Next, we attempted to generate transgenic *Arabidopsis* plants overexpressing *PVA31* under the control of the CaMV 35S promoter to investigate the physiological role of PVA31 on leaf senescence. From several hygromycin-resistant lines of the transgenic plants, we selected 2 overexpressing (OE) lines with about fold8- (line #7) and fold2- (line #13) higher expression levels compared with that in WT. The growth of root and rosette leaf was inhibited in the OE lines, especially in line #7 (**Fig. S2 A-C**). The relative expression levels of a senescent marker, *SAG12* of rosette leaves of 4-weeks old plants was enhanced in the overexpression lines (**Fig. 4A**), suggesting that leaf senescence is enhanced dependent on the *PVA31* expression level. Both OE-lines exhibited the early senescence phenotype in the rosette leaves of 8-week-old plants (**Fig. 4B**). To measure the rate of senescent rosette leaves in 8-week-old plants, we counted the

numbers of leaves revealing the feature of leaf senescence in which the edge to leaves have yellowish color, and found that about half of the rosette leaves of the both *VAP31*-OE lines (58% in #7 and 59% in #13) exhibited typical feature of senescence, whereas the rate of senescent leaves was 12% in WT (**Fig. 4C and D**; **Fig. S2D**; **Table S1**). We also characterized the leaf senescence phenotype by the artificially induced senescence experiment. After incubated the detached leaves on 3 mM MES under the dark condition for 6 d, the rosette leaves of the *PVA31*-OE lines, #7 and #13, became more yellowish than WT (**Fig. S3**). Especially, the chlorophyll contents of rosette leaves in the dark condition was significantly lower in lines #7 than that in WT (**Fig. 4E**), suggesting that the degree of the senescence is consistent with the expression dosage of *PVA31*. Taken together, these results suggest that PVA31 was involved in the leaf senescence process in *Arabidopsis*.

Salicylic acid–dependent biotic stress responsive pathway is highly activated in *PVA31* overexpressing plant

A search of the publicly available *Arabidopsis* microarray expression data of *PVA31* for various stress treatments revealed that the expression of *PVA31* is strongly induced by *Pseudomonas syringae* infection (**Fig. S4**). We therefore measured the expression level of *#7* line and the expression of marker genes, *PR-1* for the SA-mediated defense pathway after a bacterial elicitor, flg22 treatment. As depicted in **Fig. 5**, the expression of *PR-1* was gradually induced over 24 h after the flg22 treatment in WT, whereas in *PVA31*-OE plants, the *PR-1 g*ene was expressed earlier than WT after the flg22 treatment. These data indicate that the constitutive overexpression of *PVA31* promptly induced the SA-mediated signaling pathway with the elicitor stimulation in *Arabidopsis*.

Discussion

Leaf senescence includes the PCD, which is a sequential deterioration process that results in the massive degradation of subcellular components, and finally leads to the cell death. During the PCD process in leaf senescence, a highly dynamic disintegration of host cell organelles is induced; the grana structure of chloroplast is disrupted and chlorophyll and chloroplast proteins are massively degraded. Concomitantly, chromatin condensation and DNA laddering are detected.[25-27] In the final stage of PCD, loss of the integrity of the plasma and vacuolar membranes appears to disrupt cellular homeostasis, leading to cell death.[1,28] PCD is also induced in the defense response to pathogens in plants. At the site of infection, localized PCD known as a hypersensitive response (HR) occurs in order to prevent the spread of biotrophic pathogens.[9,10] HR is known to be regulated by the salicylic acid (SA)-mediated defense signaling pathway.[11,12] Intriguingly, SA also plays a critical role in age-dependent leaf senescence.[13,14] The concentration of endogenous SA is considerably higher in senescent leaves of *Arabidopsis*, and age-dependent leaf senescence is delayed in NahG overexpressing transgenic plants with reduced levels of endogenous SA.[13] Thus, part of the
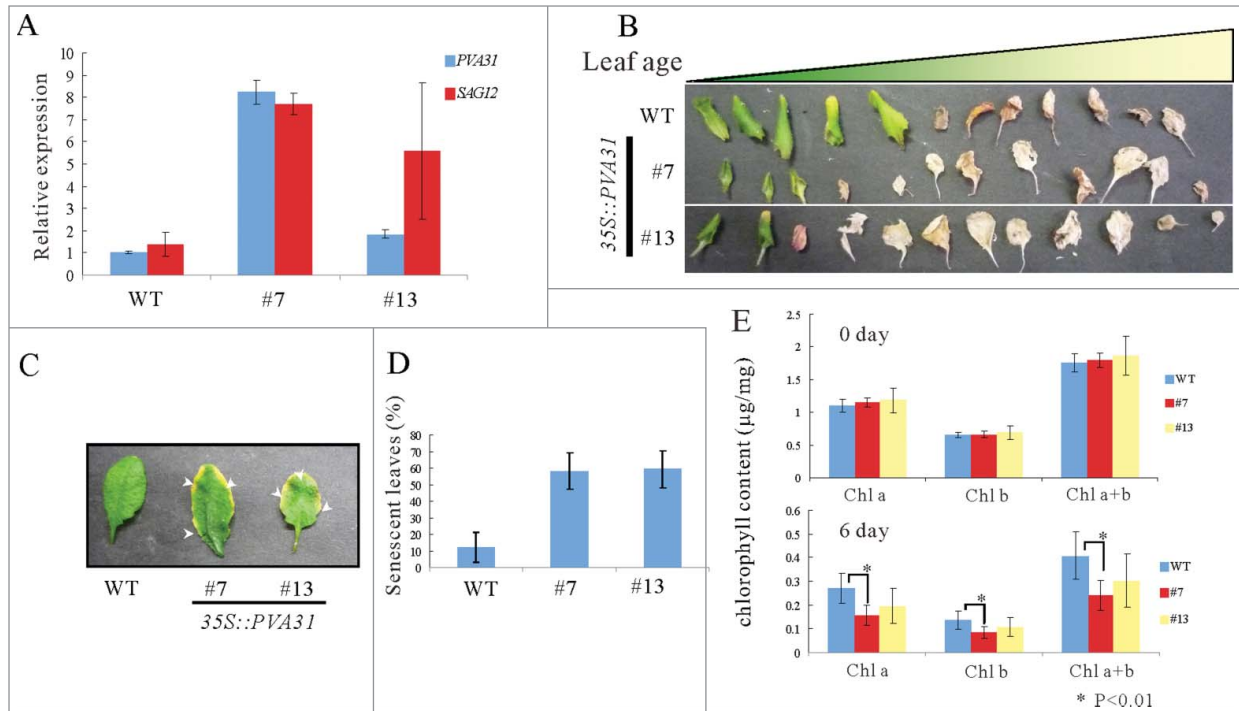
Figure 4. Age-dependent senescence symptoms in PVA31-overexpressing plants. A quantitative RT-PCR analysis of *SAG12* transcripts isolated green rosette leaves of WT and *PVA31*-overexpressing plants growing for 4-weeks. *UBQ10* was used as an internal control. Each vertical bars represented the mean ± standard error (3 leaves from each plant were used for the analysis) (**A**). Senescence phenotype of the rosette leaves of WT and PVA31-overexpressing plants (lines #7 and #13) grown for 8 weeks. The whole rosette leaves of 8-weeks-old plants were detached and aligned in leaf age order (**B**). The senescence symptom of rosette leaves. Arrowheads indicate the senescence symptom in representative leaves (**C**). The percentages of senescent leaves of each plant (WT or *PVA31*-overexpressing lines #7 and #13) with respect to total leaf numbers are presented. Error bars indicate standard deviations. Ten plants from each line were evaluated according to the senescence symptom shown in C (**D**). The chlorophyll content was measured before and after dark treatment in detached leaves. The difference between WT and *PVA31*-overexpressing line was indicated by an asterisk ($P < 0.01$) (**E**).

regulatory pathway of PCD is shared among leaf senescence and HR.

In this study, we found that one of the *Arabidopsis* VAP orthologs, *PVA31*, was highly expressed during leaf senescence and
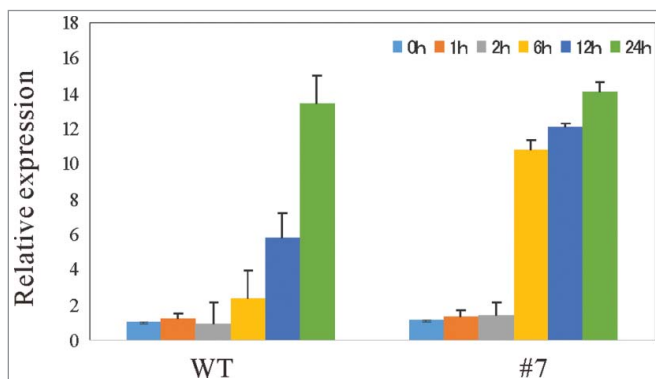

Figure 5. Expression of marker genes for SA-mediated biotic stress responsive pathway are upregulated in *PVA31*-overexpressing plants with elicitor application A quantitative RT-PCR analysis of *PR-1* gene was performed with total RNA isolated from a whole 14-day-old WT plant at various time points (0, 1, 6, 12, and 24 h) following flg22 treatment. *UBQ10* was used as an internal control. Each vertical bars represented the mean ± standard error (3 leaves from each plant were used for the analysis).

that the constitutive overexpression of *PVA31* induced the early senescence phenotype in rosette leaves (**Fig. 4; S2D**). The results indicate that PVA31 might be involved in the senescence-associated PCD process in *Arabidopsis*. Although PVA31 protein lacks the C-terminal transmembrane domain and coiled-coil domain typically found in the conventional VAP proteins,[5] PVA31 localizes to the PM and the ARA7-resided endosomes (**Fig. 2**). Additionally, the yeast-2 hybrid assay showed that PVA31 is interacted with the PM-VAMPs, VAMP721/722/724 (**Fig. 3**). These data suggest that PVA31 is associated with the PM and the endosome membranes via the VAMP protein interaction, and is involved in the senescence-associated PCD process in *Arabidopsis*.

Although a wide variety of function of VAP proteins have been reported in various eukaryotes, one possible fundamental function of the VAP proteins is thought to be an ER-resident tethering factor that forms a complex with lipid transfer proteins and SNAREs to facilitate lipids transport and protein secretion from the ER to various membranous compartments, thereby mediating the lipid transfer and the membrane trafficking in eukaryotes.[5] Taking into the fundamental function of VAP proteins, what kinds of function of PVA31 might be considered in the leaf senescence process? One of the *Arabidopsis* VAP proteins, PVA11/VAP27–1, was isolated as one of the interactors of a shingosine transfer protein, ACD11, in a yeast 2-hybrid screen.[19]

The loss-of-function *acd11* mutant is highly expressed in several genes related to the SA-mediated disease resistance process, HR,[29] and exhibited an accelerated cell death phenotype even under normal growth conditions.[30] Given that similar PCD phenotype between *acd11* mutant and *PVA31* overexpressing plants, the overexpression of *PVA31* may interfere the ACD11 function during leaf senescence in a dose dependent manner. To improve this hypothesis, a confirmation of the interaction between PVA31 and ACD11 is needed in the future study.

Avirulent and virulent *P. syringae* infection induced *PVA31* expression within 24 h (**Fig. S4**). Furthermore, the expression of the pathogenesis-related (PR) gene was promptly activated with the elicitor application in the *PVA31*-overexpressing plants (**Fig. 5**). These data suggest that increased expression of *PVA31* strongly induces PR gene expression. Intriguingly, Not only the expression of PR genes, but also the expression of the genes of the ER-resident secretory proteins is also up-regulated by the control of NPR1-regulated SA-signaling pathway, and the mutations in the secretory proteins result in diminishing in the secretion of PR proteins, thereby reducing the resistance to pathogen, suggesting that the enhanced expression of secretory proteins is necessary for the secretion of PR proteins for the defense reaction.[31] We found that PVA31 resides on the PM and the ARA7-positive endosomes, and interacts with the PM-resident VAMPs, VAMP721/722/724. VAMP721/722 is proposed to be involved in the defense pathway against a wide variety of pathogens including a virulent oomycete *Hyaloperonospora arabidopsidis*, non-adapted *Blumeria graminis* f. sp. hordei, *Erysiphe pisi* and host-adapted *Golovinomyces orontii* powdery mildew fungi.[32,33] Recently, it was reported that VAMP721/722 is stabilized by the application of flg22 and forms SNARE complex with the plasma membrane syntaxin SYP132 that is required for plant growth and immunity.[34] Since SYP132 is reported to be involved in the secretion of PR proteins, the SYP132-VAMP721/722 SNARE complex might mediate the PR protein secretion.[35] Although VAMP721/722 is known to form SNARE complexes with distinct PM syntaxins including PEN1, SYP122 and SYP132 to perform multiple functions in plant growth and defense reaction,[33,35,36] how VAMP721/722 could execute various functions to form SNARE complexes with distinct syntaxins is largely unknown. Therefore, it is likely that the specific vesicle fusion is mediated not only by SNARE molecules but also requiring additional regulatory proteins including Rab, SM and synaptotagmin proteins.[37] For instance, changes in the VAMP727 interaction with PEN1 and SYP22 is regulated by the plant-specific Rab protein Ara6 for balancing membrane fusion of intracellular compartments.[22] If this is the case, PVA31 might be involved in the secretion process of PR proteins and the changes in the membrane integrity of the PM to regulate the formation of the PM-localized SNARE complex composed of VAMP721/722 and syntaxins as a SNARE accessory protein in response to pathogen infection or leaf senescence process. Of course, we cannot exclude the possibility that PVA31 could be interacted with the PM and endosomal proteins other than VAMP721/722. Future studies need to identify the interacting proteins of PVA31 in *Arabidopsis*.

Materials and Methods

Plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments in this study. Plants were grown on soil or 1/2 Murashige and Skoog (MS) salts at 22°C under a 16-h light, 8-h dark photoperiod. Transgenic *Arabidopsis* plants expressing the full-length *PVA31* cDNA were constructed as described as follows. The full-length cDNA of PVA31 was amplified from total RNA of rosette leaves of 5-weeks-old WT *Arabidopsis* by RT-PCR. The amplified cDNA was subcloned into pENTR/D-TOPO vector (Invitrogen), and then the sequence of the clone was confirmed. The DNA construct was introduced into pGWB502[38] by the LR reaction. Transgenic lines were screened on 1/2 MS plates containing 50μg/ml hygromycin. The candidate plants were selected by reverse transcription polymerase chain reaction (RT-PCR) and 2 independent homozygous lines expressing *PVA31* under the control of the 35S CaMV promoter were used for further analysis.

Generation of PVA31-overexpressing plants

Full-length cDNAs corresponding to *PVA31* were amplified by PCR, cloned into pENTR/D-TOPO vectors (Invitrogen), and subcloned into Gateway binary vectors by LR recombination (Invitrogen). For analysis of subcellular localization of PVA31, *PVA31* cDNA was subcloned into pGWB402 destination vectors to generate N-terminal fusions with GFP. For gain-of-function analysis, *PVA31* full-length cDNA was inserted into pGWB502 destination vectors. The constructs were transformed into *Arabidopsis* plants by *Agrobacterium*-mediated transformation using the floral dip method.[39] Screening of transgenic plants was performed on 1/2MS plates containing 50 μg/ml hygromycin. Homozygous T2 transgenic lines were used for further experiments.

Transient expression of GFP-PVA31 by agrobacterium-based transformation

To transiently express GFP-PVA31 in *Arabidopsis* seedlings, we employed the fast Agro-mediated seedling transformation (FAST) method.[39] Briefly, WT and mRFP-ARA7[39] and mRFP-VAMP722 expressing transgenic *Arabidopsis* seedlings were grown in 1/2 MS plate at 22°C under a 16-h light, 8-h dark photoperiod for 5 d and transformed by co-cultivation with *A. tumefaciens* GV3100 harboring the GFP-PVA31 construct in the dark for 36 hr.

Confocal microscopy

GFP fluorescence signals and differential interference contrast (DIC) images were obtained using a Nikon ECLIPSE E600 laser scanning microscope equipped with a C1 si-ready confocal system (Nikon, Tokyo, Japan) and excited with a blue argon ion laser (488 nm) for GFP-PVA31 and with a Green HeNe laser (543 nm) for mRFP-ARA7. The collected images were processed using Nikon EZ-C1 software.

Yeast two-hybrid assay

The full-length cDNAs of *PVA31* and VAMPs (VAMP721/722/724/727/711) were introduced into pGADT7 and pGBKT7 vectors, respectively. The pGADT7 and pGBKT7 constructs were co-transformed into yeast AH109 strain as described in the Yeast Protocols Handbook (Clonetech). The yeasts were incubated on the solid SD medium lacking leucine and tryptophan at 30 °C for 5 d For each 2-hybrid interaction tested, each colony was suspended in 100 μl of DW then diluted 10-, 100- and 1000-fold. Each dilution series was spotted on a solid SD medium lacking leucine, tryptophan and histidine but adding 5 mM 3-amino-1, 2, 4-triazole (3-AT). Plates were incubated for 7 d at 30 °C.

RT-PCR and quantitative RT-PCR analyzes

For qRT-PCR, total RNA was isolated WT and *PVA31*-overexpressing plant with the Nucleo Spin RNA kit (TaKaRa). cDNAs were synthesized from 500 ng (for amplifying *SAG12*) and 100 ng (for amplifying *PR-1*) of total RNA of each plant using ReverTra Ace qPCR RT Master Mix (TOYOBO). Quantitative real-time PCR was performed using the THUNDERBIRD SYBR qPCR mix (TOYOBO) and Eco Real-Time PCR System (illumine) according to the manual. The expression level was normalized to an internal control gene (*UBQ10*, At4g05320). The primers used are listed in Supplementary **Table S2**.

Artificial induction of leaf senescence

Detached rosette leaves of 4-weeks-old plants were floated adaxial side up on 2 ml of 3 mM MES (pH 5.8) in 12-well Petri dishes. Leaves were incubated at 22 °C in the dark for 6 d. Rosette leaves were collected from WT and *PVA31*-overexpressing plants and detached rosette leaves. The rosette leaves were extracted with 95% (v/v) EtOH at 80°C. The absorbance was measured at 649 nm, 665 nm and 750 nm. Total chlorophyll and each chlorophyll (a and b) concentrations were calculated according to.[41]

Author Contributions

M. H. S. conceived the study. M. I., Y. N., K.A. and S. N. carried out most of the genetic, biochemical, and physiological experiments. T. H. performed the quantitative RT-PCR analysis. M. I. performed the confocal laser scanning microscopy experiment. M. H. S. supervised the study and wrote the paper.

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