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Overexpression of a LAM Domain Containing RNA-Binding Protein LARP1c Induces Precocious Leaf Senescence in *Arabidopsis*

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Leaf senescence is the final stage of leaf life history, and it can be regulated by multiple internal and external cues. La-related proteins (LARPs), which contain a well-conserved La motif (LAM) domain and normally a canonical RNA recognition motif (RRM) or noncanonical RRM-like motif, are widely present in eukaryotes. Six *LARP* genes (*LARP1a-1c* and *LARP6a-6c*) are present in *Arabidopsis,* but their biological functions have not been studied previously. In this study, we investigated the biological roles of LARP1c from the LARP1 family. Constitutive or inducible overexpression of *LARP1c* caused premature leaf senescence. Expression levels of several senescence-associated genes and defense-related genes were elevated upon overexpression of *LARP1c*. The *LARP1c* null mutant *1c-1* impaired ABA-, SA-, and MeJA-induced leaf senescence in detached leaves. Gene expression profiles of *LARP1c* showed age-dependent expression in rosette leaves. Taken together, our results suggest LARP1c is involved in regulation of leaf senescence.

INTRODUCTION

Leaf senescence, which is the final stage of leaf life history, is a self-regulatory developmental process influenced by growth and environmental factors. Dramatic changes in cell structure and metabolism occur during leaf senescence, including the degradation of chloroplasts, mitochondria, and nuclei, and simultaneous catabolism of chlorophyll pigments, nucleic acids, proteins, and lipids. The nutrients released from senescing leaves are transported to newly-developing leaves, fruits, or seeds (Buchanan-Wollaston et al., 2005; Lim et al., 2007a; Munné-Bosch and Alegre, 2004).

Leaf senescence can be induced by biotic or abiotic stresses in the environment, including pathogen infection, nutrient deficiency, and osmotic, light, and temperature stresses (Hopkins et al., 2007; Lim et al., 2007a; Zhou et al., 2009; 2011). Leaf senescence can also be initiated by internal signals, such as developmental cues and phytohormone balance. The internal or environmental cues that induce aging are perceived by plants, which then express senescence-associated genes (*SAG*s), resulting in dysfunctional developmental processes and, ultimately, the appearance of a senescence phenotype (Guo and Gan, 2005; Lim et al., 2007a). A large number of genes show enhanced or reduced expression during leaf senescence. Transcriptome analysis of senescent leaves indicated that approximately 2,500 genes (approximately 10%) in the *Arabidopsis* genome were expressed in senescent leaves (He et al., 2001). Microarray analysis showed that more than 800 *SAG*s were distinctively up-regulated during leaf senescence (Buchanan-Wollaston et al., 2005; Van Der Graaff et al., 2006).

Post-transcriptional regulation plays an essential role in the regulation of gene expression during plant growth, development, and response to external stresses. RNA-binding proteins are involved in multiple steps of post-transcriptional regulation, including pre-mRNA splicing, mRNA transport, localization, translation, and stability (Dreyfuss et al., 2002). Although many RNA-binding proteins are up- or down-regulated during leaf senescence, the biological functions of these genes are largely unknown (Buchanan-Wollaston et al., 2005; Van Der Graaff et al., 2006). Overexpression of three *UBA2* genes (*UBA2a*, *UBA2b*, and *UBA2c*), which encode heterogeneous nuclear ribonucleoprotein (hnRNP)-type RNA-binding proteins, induce leaf senescence and hypersensitive-like cell death (Kim et al., 2008).

La-related proteins (LARPs), which contain a conserved La motif (LAM) domain and are normally immediately followed by a canonical RNA recognition motif (RRM) or noncanonical RRM-like (RRM-L) motif, are widely present in eukaryotes (Bayfield et al., 2010; Bousquet-Antonelli and Deragon, 2009). The co-evolving LAM-RRM/RRM-L regions are suggested to cooperate functionally for RNA (e.g., SnRNA and mRNA) binding activity (Bousquet-Antonelli and Deragon, 2009). Genuine La proteins specifically recognize and bind the 3′-UUU-OH of RNA polymerase III primary transcripts (Wolin and Cedervall, 2002), but knowledge of the function of the four LARP subfamilies (LARP1, 4, 6 and 7) is still limited (Bousquet-Antonelli and Deragon, 2009). The LARP1 subfamily members contain a LAM immediately followed by a noncanonical RRM-L5, with the exception of some members that lack this motif (Bousquet-Antonelli and Deragon, 2009). A DM15 box of unknown function is also found

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in the C-terminal region of some members of this subfamily (Bousquet-Antonelli and Deragon, 2009; Nykamp et al., 2008).

The yeast *Saccharomyces cerevisiae* LARP1 proteins Sro9p and Slf1p, which have a LAM but lack an adjacent RRM or DM15 box, are mainly cytoplasmic proteins able to bind translating mRNAs (Sobel and Wolin, 1999). Sro9p inhibits the activity of the transcript activator Hap1p by assembling a repressed protein complex, and this inhibition can be released by an increase in the intracellular heme concentration (Lan et al., 2004). The *Drosophila melanogaster* LARP1 was reported to be associated with the poly (A)-binding protein (PABP) and was important for spermatogenesis (Blagden et al., 2009; Chauvet et al., 2000; Ichihara et al., 2007). In *Caenorhabditis elegans, Ce-LARP1* is required for normal oogenesis but does not affect the viability of the worm (Nykamp et al., 2008). CeLARP1 is localized in the procession bodies (P-bodies) where mRNA decay occurs, and null-mutant worms accumulated a high level of mRNAs in the Ras-MAPK pathway, indicating that CeLARP1 functions to attenuate specific mRNA levels by promoting mRNA decay (Nykamp et al., 2008). More recently, human LARP1 was shown to form a complex with PABP and the cap-binding protein eukaryote initiation factor 4E (eIF4E) (Burrows et al., 2010). Reduced expression of *LARP1* in HeLa cells led to decreased global protein synthesis rates, and resulted in cell cycle arrest and an inhibition of cell migration (Burrows et al., 2010). *Drosophila* LARP1 appears to participate in a RNA-binding complex in which LARP1 is associated with 7SK and Bin3 (Bicoid interacting protein 3), a RNA methyltransferase that methylates the 5′ end of 7SK RNA, to serve as a scaffold for PABP and Ago2, which bind directly to mRNAs and negatively regulate initiation of translation (Singh et al., 2011).

Bioinformatic analyses of the LAM region showed that the *Arabidopsis* genome harbors six LARP genes (*LARP1a-1c* and *LARP6a-6c*) (Bayfield et al., 2010; Bousquet-Antonelli and Deragon, 2009), but their biological functions have not been studied previously. Here, we report that overexpression of *LARP1c* led to premature leaf senescence. A number of *SAG*s and defense-related genes were elevated upon overexpression of *LARP1c*. The *LARP1c* null mutant *1c-1* retarded abscisic acid (ABA)-, salicylic acid (SA)-, and methyl jasmonate (MeJA)-induced leaf senescence in detached leaves. Gene expression profiles of *LARP1c* showed age-dependent expression in rosette leaves. These data suggest that the *Arabidopsis* LARP1c is involved in regulation of leaf senescence.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis plants were grown in an environmentally controlled culture room at 22°C with a relative humidity of ~60% under long-day conditions (16 h light/8 h dark photoperiod). The T-DNA insertional mutant *1c-1* (FLAG_372B03, Wassilewskija [WS] background) was obtained from the French National Institute for Agricultural Research. Gene-specific primers 1c-F, 1c-R, and LB4 (T-DNA left border primer for *1c-1*) were used to isolate the T-DNA insertional homozygous lines. The sequences of primers used for T-DNA insertional analysis and gene expression in the mutant lines are shown in Supplementary Table 2. All *Arabidopsis* transgenic plants, including *1c-GUS*, *1a-OE*, *1b-OE*, *1c-OE*, *1c-DEX*, and *1c-GFP*, were generated by the floral dip method in the Col-0 background (Clough and Bent, 1998). Transgenic plants were selected on plates containing 25 mg L^{-1} hygromycin. Homozygous T3/T4 lines were used in the study unless otherwise specified. To induce *LARP1c* expression, 4-week-old *1c-DEX* transgenic plants were once sprayed with 15 μM dexamethasone (~1 mL per plant).

Plasmid constructs

For histological assays, the promoter region (1,453 bp) of *LARP1c* was amplified from *Arabidopsis* DNA and cloned into the pCAMBIA 1391 vector. To generate *LARP1a*, *1b,* or *1c* overexpression plasmids (*1a-OE*, *1b-OE* or *1c-OE*), the individual full-length coding sequence (CDs) was cloned into Super Promoter 1300 vector (pSuper 1300), a binary vector carrying the *Cauliflower mosaic virus* (CaMV) 35S promoter for overexpression. For DEX-inducible expression of *LARP1c*, the full-length *LARP1c* CDs without stop codon was cloned into the pTA7002 vector, a dexamethasone (DEX)-inducible expression vector. To generate the *1c-GFP* construct, the full-length *LARP1c* CDs without stop codon was fused to the 5′-terminal of the pSuper 1300-GFP vector, a binary vector generated from pSuper 1300. All of the PCR amplifications, enzyme digestions, and plasmid transformations used in plasmid constructs were performed using standard protocols. The sequences of primers and restriction enzymes used are presented in Supplementary Table 2.

Histological assays and subcellular localization assay

For histochemical analysis of β-glucuronidase staining (GUS) activity, the *1c-GUS* transgenic materials were submerged in stain solution [50 mM sodium phosphate, pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Sigma-Aldrich, China)], and subsequently incubated at 37°C for ~3 h. The materials were then transferred to ethanol:acetic acid (3:1, v/v) solution to remove background pigmentation. The stained tissues were examined with an Olympus SZ16 stereomicroscope and Olympus SZX16-DP72 microscope (Olympus, Japan) and digital images recorded with a Canon G12 camera (Canon, Japan).

For subcellular localization assays, live roots of 5-day-old seedlings grown on Murashige and Skoog medium were used for GFP analysis. All tissues were examined with a Zeiss 510 Meta laser scanning confocal microscope (Zeiss, Germany).

Chlorophyll content and membrane ion leakage analysis

Chlorophyll was extracted from leaves and measured according to the protocol of Grbi and Bleecker (1995). The leaves used at each sampling time point were excised from at least three separate plants. Membrane ion leakage was measured as described by Guo and Gan (2006).

Trypan blue staining and hormone treatments

To visualize dying cells in senescing leaves, Trypan blue (TB) staining was performed similar to the method of Koch et al. (1990). Leaves were submerged in lactophenol-TB solution (0.1% TB, 50% methanol, 16.6% glycerol, 16.6% lactic acid, and 16.7% water-saturated phenol) and stained in a boiling water bath for 2 min. The samples were then transferred to 2.5 g mL^{-1} chloral hydrate solution to remove background pigmentation.

For hormone treatments, the 5th rosette leaves were collected from 4-week-old WS and *1c-1* mutant plants and placed adaxial side up on treatment solutions containing 3 mM 2-(4-morpholino) ethanesulfonic acid (MES) and either 20 μM ABA, 200 μM MeJA, 300 μM SA, or MES only (control). The leaves were incubated at 22°C under continuous illumination. The leaves were incubated in ABA or SA for 6 days and in MeJA for 5 days.

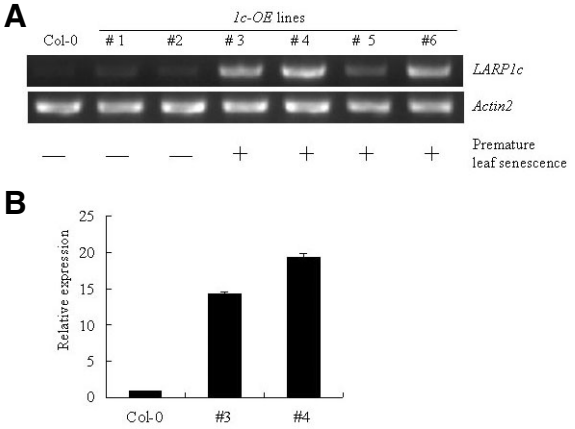

Fig. 1. Screening of *LARP1c* overexpression (*1c-OE*) lines. (A) Seven-day-old seedlings of Col-0 and each of six homozygous lines were used for RNA extraction. The yellowing phenotype was observed by 5 weeks after germination. –, yellowing phenotype absent; +, yellowing phenotype present. (B) Relative expression of *LARP1c* in Col-0 and *1c-OE* transgenic lines 3 and 4. Data represent the mean ± SE of three replicates.

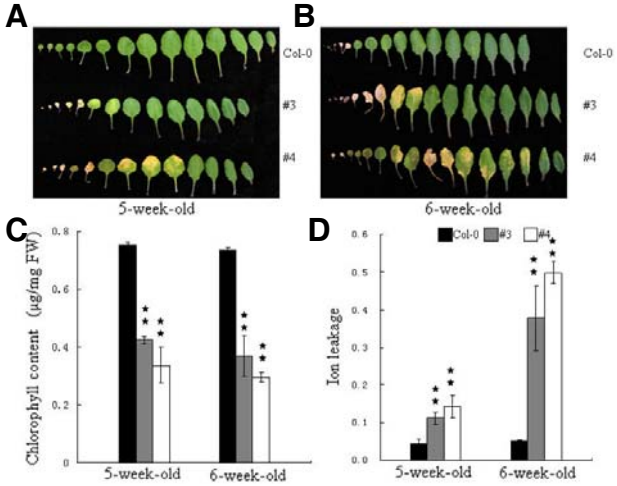

Fig. 2. Accelerated leaf senescence in *LARP1c* overexpression (*1c-OE*) lines. Rosette leaves and cotyledons detached from (A) 5- and (B) 6-week-old Col-0 and *1c-OE* transgenic lines #3 and #4. (C, D) Chlorophyll content (C) and membrane ion leakage (D) in 5th to 8th rosette leaves from plants in (A, B). Data represent mean ± SE of three replicates. Asterisks indicate significant differences between Col-0 and the *1c-OE* lines (Student's *t*-test, $P < 0.01$).

RNA isolation, reverse-transcription PCR and quantitative real-time PCR analysis

Total RNA was isolated from plant samples following a previously described protocol (Oñate-Sánchez, L., and Vicente-Carbajosa, J., 2008). First-strand cDNA was synthesized from RNA using the Promega M-MLV Reverse Transcription system according to the manufacturer's protocol (Promega, China). The sample cDNA was used for subsequent analyses by reverse-transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR). For qRT-PCR, the ABI7500 Fast Real-Time PCR system (Applied Biosystems, USA) using the SYBR Green I Master Mix (Takara, Beijing) in a volume of 20 μL was employed. *AT4G34270* was used as an internal control gene. The qRT-PCR reaction was performed following the protocol recommended by the manufacturer. For RT-PCR, the first-strand cDNA was used and PCR products were detected with GoldView (Newprobe, China), a novel dye for staining of nucleic acids. *Actin2* was used as an internal control. All qRT-PCR and RT-PCR primers can be found in Supplementary Table 2. All RT-PCR reactions were performed for 25-28 cycles.

RESULTS

Overexpression of *LARP1c* causes premature leaf senescence

To investigate the functions of LARP1c, we first used the T-DNA insertional mutant *1c-1* (FLAG_372B03, WS background), which has a T-DNA insertion in the second exon (Supplementary Fig. 1A). RT-PCR analysis showed that *1c-1* is a T-DNA knockout line (Supplementary Fig. 1B). Under the experimental growth conditions, the *1c-1* mutant plants showed normal growth and development almost identical to that of WS (Supplementary Fig. 1C). Because the *LARP1c* knockout line showed no obvious developmental defects, we employed gain-of-function approaches to investigate the roles of LARP1c. We obtained CaMV 35S promoter-driven overexpression lines of *LARP1c*. Fifty-seven of 85 T_1 transformants with *LARP1c* overexpression (*1c-OE*) exhibited precocious leaf yellowing.

Six homozygous *1c-OE* transgenic lines were generated, and their *LARP1c* transcript levels were detected by RT-PCR (Fig. 1A). Among them, four lines with higher expression levels of *LARP1c* displayed a leaf-yellowing phenotype 5 weeks after germination; the remaining two lines with expression levels similar to Col-0 plants showed no obvious leaf yellowing. The relative expression levels of *LARP1c* in lines 3 and 4 were examined by qRT-PCR (Fig. 1B), and these two lines were selected for further analysis.

Rosette leaves and cotyledons from 5- and 6-week-old Col-0 and *1c-OE* plants are shown in Figs. 2A and 2B. The *1c-OE* plants displayed premature leaf senescence compared with Col-0. The senescence phenotype was not observed in rosette leaves of 5-week-old Col-0 plants, but appeared in 2nd to 7th rosette leaves of *1c-OE* transgenic plants (Fig. 2A); a more severe senescence phenotype was observed in 6-week-old *1c-OE* plants (Fig. 2B). Decreased chlorophyll content (Fig. 2C) and increased membrane ion leakage (Fig. 2D) were also detected in *1c-OE* plants.

Cell death in the 4th rosette leaves of 5-week-old Col-0 and *1c-OE* plants was examined by TB staining. Leaves of *1c-OE* plants contained more dying cells than the wild type (Fig. 3A). *RBCS1A*, which encodes the small subunit of ribulose bisphosphate carboxylase, a key enzyme for CO_2 assimilation, had decreased expression in *1c-OE* plants (Fig. 3B), indicating that the leaves of transgenic lines had reduced photosynthetic activity.

Because the disruption of housekeeping or homeostatic genes could lead to early senescence symptoms, the premature leaf senescence phenotype observed in *1c-OE* transgenic plants may be due to indirect disturbances of developmental processes. Inducible overexpression experiments could avoid potential complications in interpreting the phenotype (Woo et al., 2010). We applied a glucocorticoid-mediated transcriptional induction system (Aoyama and Chua, 1997) to generate conditional *LARP1c* transgenic plants. *LARP1c* was cloned into the pTA7002 vector, which harbors a DEX (a synthetic glucocorticoid)-inducible promoter. After DEX induction for 15 h, the relative expression of *LARP1c* in 4-week-old T2 transgenic lines

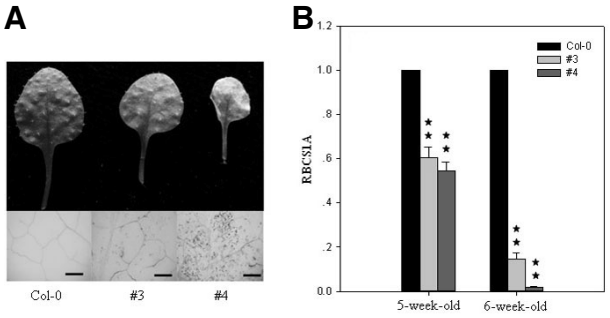

Fig. 3. Cell death and expression of *RBCS1A* in leaves of Col-0 and *LARP1c* overexpression (*1c-OE*) transgenic lines 3 and 4. (A) Cell death of 4th rosette leaves from 5-week-old plants was examined by Trypan blue staining. Bar = 0.5 mm. (B) QRT-PCR analysis of *RBCS1A* expression levels. Relative transcript levels were normalized to *AT4G34270*. Data represent mean ± SE of three replicates. Asterisks indicate significant differences between Col-0 and the *1c-OE* lines (Student's *t*-test, P < 0.01).

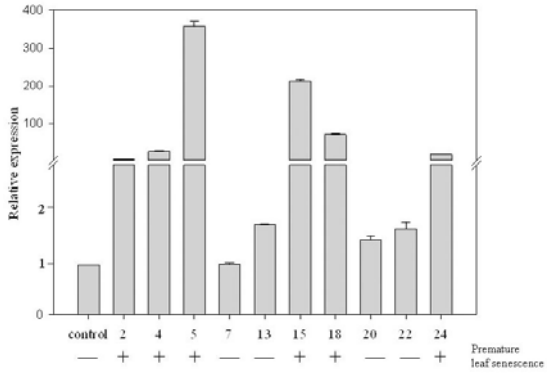

Fig. 4. Quantitative RT-PCR analysis of *LARP1c* expression in dexamethasone (DEX)-inducible lines revealed a correlation between *LARP1c* overexpression and leaf yellowing. Four-week-old transgenic plants (T2 generation) were sprayed with DEX (15 μM), and aerial parts were collected 15 h later. The leaf yellowing phenotype was observed 4 days after application of DEX. –, yellowing phenotype absent; +, yellowing phenotype present. Data represent mean ± SE of three replicates.

was examined by qRT-PCR, and the leaf yellowing phenotype was recorded at later stages (Fig. 4). Only the inducible-lines with higher *LARP1c* expression levels displayed subsequent leaf yellowing phenotypes (Fig. 4), suggesting that overexpression of *LARP1c* was the cause of the phenotype.

Several *1c-DEX* homozygous lines were generated for further analysis. After 3-4 days of DEX treatment, the 4-week-old *1c-DEX* transgenic lines showed leaf yellowing in rosette leaves, but the transgenic plants harboring the empty pTA7002 vector displayed no yellowing after DEX induction (Fig. 5A). Gene expression analysis showed that *LARP1c* was induced in *1c-DEX* transgenic plants (Fig. 5B). Consistent with the yellowing phenotype in the leaves of *1c-DEX* transgenic lines (Fig. 5A), the chlorophyll content decreased rapidly (Fig. 5D) and the frequency of cell death increased markedly (Fig. 5C) after DEX treatment for 4 days. Based on these results and the senescence phenotype of *1c-OE* lines, we conclude that *LARP1c* overexpression was responsible for the premature leaf senescence.

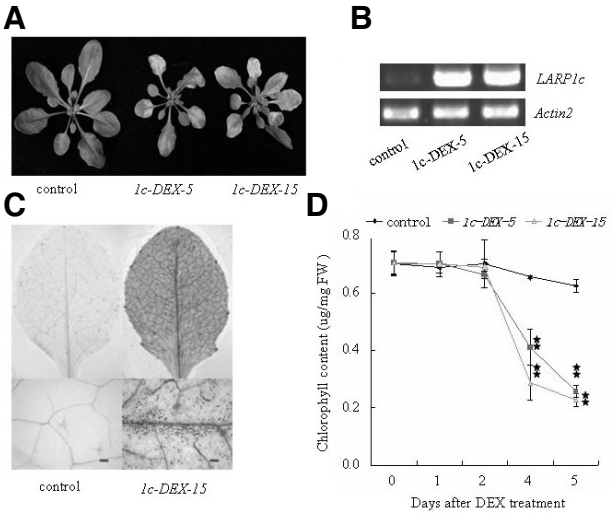

Fig. 5. Inducible overexpression of *LARP1c* led to precocious leaf senescence. (A) Phenotypes of transgenic lines after dexamethasone (DEX) induction. The image was taken 4 days after 4-week-old plants were sprayed with 15 μM DEX. (B) RT-PCR analysis of the expression of *LARP1c* in 5th to 8th rosette leaves of plants pictured in (A). (C) Cell death in 8th rosette leaves of plants pictured in (A). Bar = 0.1 mm. (D) Chlorophyll content of 5th to 8th rosette leaves after DEX treatment. Data represent mean ± SE of three replicates. Asterisks indicate significant differences between Col-0 and the *1c-DEX* transgenic lines (Student's *t*-test, P < 0.01).

Inducible overexpression of *LARP1c* elevates transcript levels of *SAG*s and defense-related genes

To confirm that *LARP1c* plays an important role in regulating leaf senescence, we analyzed the expression of several *SAG*s in the *1c-DEX* transgenic lines after DEX induction for 24 and 48 h, before the transgenic plants showed signs of chlorosis. In parallel with *LARP1c* overexpression, increased expression of several *SAG*s, including *SAG13* (Lohman et al., 1994), *SEN4* (Park et al., 1998), and *WRKY6* (Robatzek and Somssich, 2001), were observed (Fig. 6). *SAG12* (cysteine proteinase), a highly senescence-specific marker gene (Noh and Amasino, 1999; Pontier et al., 1999), was also detected 72 h after treatment, when the transgenic plants showed signs of yellowing (data not shown). The regulatory pathway of leaf senescence has substantial cross-talk with plant defense signaling pathways (Quirino et al., 2000). Thus, we examined the expression levels of several defense-related genes by RT-PCR. Pathogenesis-related (*PR*) genes *PR1* and *PR2* are SA pathway defense genes (Mang et al., 2009). *Phytoalexin deficient 4* (*PAD4*) and *Enhanced Disease Resistance 1* (*EDS1*) act upstream from SA biosynthesis but are also induced by SA (Rustérucci et al., 2001). *Plant defensin gene 1.2* (*PDF1.2*) is a marker gene frequently used to monitor jasmonate response (Pieterse et al., 2009). *Arabidopsis aldehyde oxidase 3* (*AAO3*) and *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) encode key enzymes in the biosynthesis of ABA (Seo and Koshiba, 2002). All of these defense-related genes examined were upregulated in *1c-DEX* transgenic lines after DEX treatment for 24 and 48 h (Fig. 6).

The *LARP1c* mutant *1c-1* impairs ABA-, SA- and MeJA-induced leaf senescence

We showed above that inducible overexpression of *LARP1c*

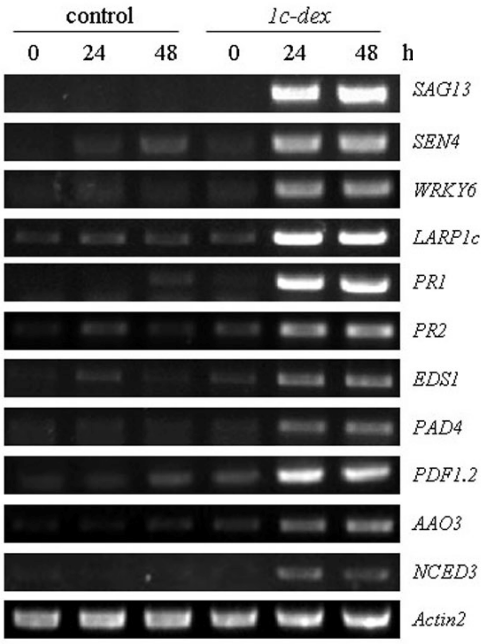

Fig. 6. Expression of *SAGs* and defense-related genes in control and *1c-DEX* transgenic plants. Four-week-old *Arabidopsis* plants were sprayed with 15 μM dexamethasone (DEX), and 5th to 8th rosette leaves were collected at 0, 24, and 48 h after treatment. RT-PCR was done for 25-28 cycles using gene-specific primers. *Actin2* served as an internal control.

elevated transcript levels of *SAG*s and defense-related genes (Fig. 6). Thus, we tested the effects of senescence- and defense-related plant hormones ABA, SA, and MeJA on mutant *1c-1*. Fifth rosette leaves were detached from 4-week-old plants and treated with ABA, SA, or MeJA. As shown in Fig. 7, ABA, SA and MeJA prompted the senescence of detached leaves in WS and *1c-1*. However, compared with WS, the leaves from *1c-1* mutant displayed delayed yellowing (Fig. 7A) and retained more chlorophyll content (Fig. 7B) after ABA, SA and MeJA treatment for 6 or 5 days. These results suggested that LARP1c mediated ABA-, SA-, and MeJA-induced senescence of detached leaves.

LARP1c expression in rosette leaves is age-dependent

To investigate the expression pattern of *LARP1c* during leaf development, qRT-PCR analyses at different leaf developmental stages were carried out. As shown in Fig. 8, transcript levels of *LARP1c* increased as leaves developed and senesced. The relative expression level of *LARP1c* in early-senescent leaves was double that in young leaves, and more was detected in late senescent leaves (Fig. 8).

To further confirm the expression pattern of *LARP1c*, a ~1.5 kb promoter region upstream of the start codon was cloned and inserted into the pCAMBIA-1391 vector. Transgenic plants were generated and the GUS activity was analyzed in 4- to 7-week-old plants. As shown in Fig. 9, GUS activity was detected in the older rosette leaves of these plants, such as the 1st-5th rosette leaves of 5-week-old plants. Older plants had more rosette leaves with GUS activity, increasing from four in 4-week-old plants to eight in 7-week-old plants. Interestingly, some blue spots were also detectable at the leaf margins of

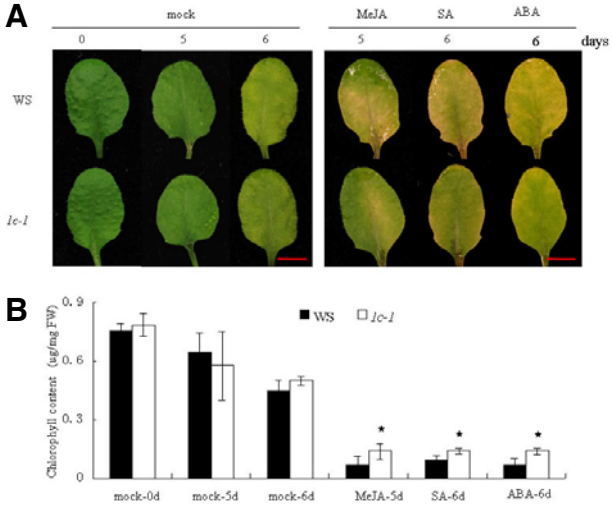

Fig. 7. Leaf senescence responses of the WS and *1c-1* mutant to methyl jasmonate (MeJA), salicylic acid (SA), and abscisic acid (ABA) treatments. The 5th rosette leaves from 4-week-old plants were detached and incubated under continuous light in 2-(4-morpholino) ethanesulfonic acid (MES) buffer containing hormone or MES only as mock. Chlorophyll content was examined 0, 5, and 6 days after treatments. Data represent mean ± SE of four replicates. Asterisks indicate significant differences between WS and the *1c-1* mutant (Student's *t*-test, $0.01 \leq P < 0.05$). Bar = 5 mm.

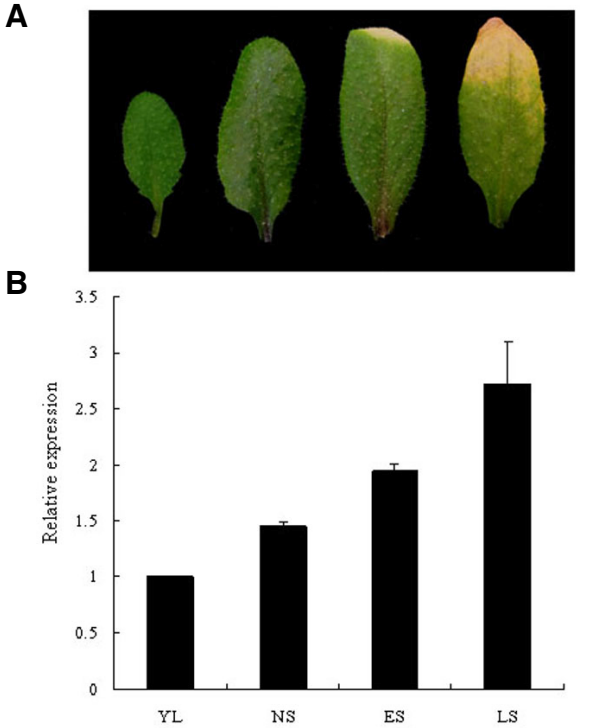

Fig. 8. Quantitative RT-PCR analysis of *LARP1c* expression during leaf development in *Arabidopsis*. YL, young leaves, half the size of fully-expanded leaves; NS, non-senescent, fully-expanded leaves; ES, early-senescent leaves with < 25% leaf-area yellowing; LS, late-senescent leaves, with > 50% leaf-area yellowing. Relative transcript levels were normalized to *AT4G34270*. Data represent mean ± SE of three replicates.

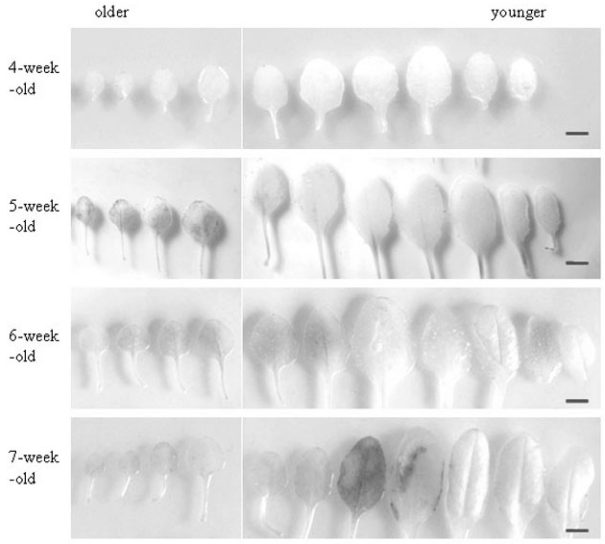

Fig. 9. *LARP1c* promoter: GUS activity in rosette leaves of 4- to 7-week-old transgenic plants. The rosette leaves are pictured in order. Bar = 5 mm.

younger rosette leaves, such as the 8th rosette leaves of 5-week-old plants (Supplementary Fig. 2A), which might be the initial sites of *LARP1c* expression. We also searched for *LARP1c* for gene expression information in AtGenExpress (http://jsp.weigelworld.org/expviz/expviz.jsp). As shown in Supplementary Table 1, *LARP1c* expression increased in senescing leaves compared with other rosette leaves examined, which is consistent with our expression data. These results indicated that *LARP1c* expression in rosette leaves was age-dependent. GUS activity was also detected in other tissues, such as seedlings and inflorescences (Supplementary Figs. 2B-2E), implying that *LARP1c* plays diverse roles in *Arabidopsis*.

LARP1c is a cytoplasmic protein

To determine the subcellular localization of LARP1c in *Arabidopsis*, we generated the *1c-GFP* construct that carried the CaMV 35S promoter for constitutive expression and obtained *1c-GFP* transformants. The *1c-GFP* transgenic lines displayed a premature leaf yellowing phenotype (Fig. 10A), which implied that GFP fusion did not interfere with the biological function of LARP1c. Thus, the 1c-GFP localization pattern was biologically relevant. Subcellular localization of the GFP fusion protein in the transgenic lines was analyzed by confocal laser scanning microscopy. In roots of 5-day-old seedlings, 1c-GFP fluorescence was largely localized to distinct cytoplasmic spots (Fig. 10B), in contrast with the cytoplasmic localization of 35S:GFP (Fig. 10C). This suggests that LARP1c is a cytoplasmic protein with foci distribution.

Overexpression of *LARP1b* also causes a premature leaf yellowing phenotype

Do LARP1 members have similar functions in leaf senescence? To investigate this possibility, we also generated *LARP1a* and *LARP1b* overexpression lines. None of 12 transgenic T_1 plants for *LARP1a* overexpression (*1a-OE*), regardless of increased *LARP1a* transcript levels, displayed premature leaf yellowing compared with wild type (data not shown). Fourteen of 74 transgenic T_1 plants for *LARP1b* overexpression (*1b-OE*) displayed premature leaf yellowing (Fig. 11A) 6 weeks after germination. As expected, the transcript levels of *SAG*s and *LARP1b* were induced in *1b-OE* transgenic lines (Fig. 11B). These findings suggested that LARP1b, similar to LARP1c, is involved in regulation of leaf senescence.

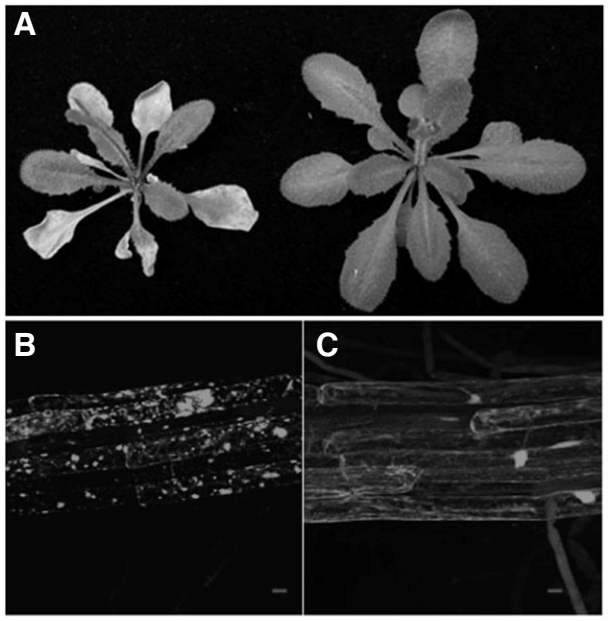

Fig. 10. Localization of LARP1c in the cytoplasm. (A) Phenotype of 30-day-old *LARP1c-GFP* transgenic (left) and *35S:GFP* control (right) plants. The *LARP1c-GFP* transgenic line displayed a premature leaf senescence phenotype. (B) Confocal micrographs of *1c-GFP* transgenic plant; GFP signal formed aggregates within the cytoplasm. (C) Confocal micrographs of *35S:GFP* transgenic plant; GFP signal was evenly dispersed in the cytoplasm. Both GFP images were taken from mature root zones of 5-day-old transgenic plants. Bar = 10 μm.

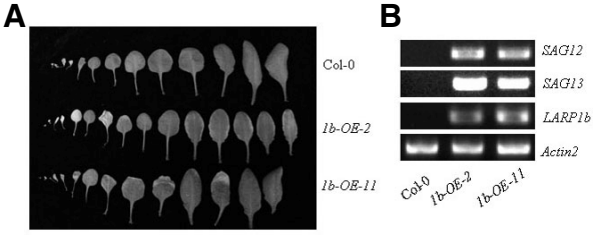

Fig. 11. Overexpression of *LARP1b* caused premature leaf senescence. (A) Leaf senescence phenotypes of Col-0 and *LARP1b* overexpression (*1b-OE*) transgenic lines. Compared with Col-0, *1b-OE* transgenic lines showed premature leaf senescence. Rosette leaves and cotyledons were excised from soil-grown 7-week-old plants. Fourteen of 74 transgenic T1 plants displayed premature leaf senescence. (B) RT-PCR analysis of gene expression in leaves of Col-0 and *1b-OE* lines. The 7th and 8th rosette leaves from 7-week-old plants of *1b-OE* lines and Col-0 were used for RNA extraction. For all genes, 25 PCR cycles were employed. *Actin2* was used as an internal control. The sequences of primers used for *1b-OE* construct and RT-PCR analysis are presented in Supplementary Table 2.

DISCUSSION

Leaf senescence is accompanied by the activation of a subset of genes (e.g., *SAG*s) and the inactivation of another subset that represses the senescence program. Microarray data showed that expression of many genes that encode RNA-binding proteins is altered during leaf senescence (Buchanan-Wollaston et al., 2005), but functional information for RNA-binding proteins involved in leaf senescence is very limited. Kim et al. (2008) reported that overexpression of three hnRNP-type RNA-binding proteins (UBA2a, UBA2b, and UBA2c) induced leaf senescence and hypersensitive-like cell death. In the present study, we obtained evidence that a RNA-binding protein, LARP1c, in *Arabidopsis* is involved in regulating leaf senescence.

In animals, LARP1s are mainly cytoplasmic proteins that function in metabolism of specific mRNAs. LARP1 formed a complex with PABP and eIF4E and was localized to P-bodies, where mRNA decay occurs (Blagden et al., 2009; Burrows et al., 2010; Chauvet et al., 2000; Ichihara et al., 2007; Nykamp et al., 2008; Singh et al., 2011). Furthermore, LARP1 is involved in cellular remodeling, migration, and apoptosis (Burrows et al., 2010). Deletion of *LARP1* leads to developmental defects during spermatogenesis in *D. melanogaster* (Blagden et al., 2009; Ichihara et al., 2007) and oogenesis in *C. elegans* (Nykamp et al., 2008). Thus, LARP1 members appear to bind to and regulate the stability and translational activities of target mRNAs.

In *Arabidopsis*, three members are classified into LARP1 subfamily (Bousquet-Antonelli and Deragon, 2009). No functional data for these LARP1 proteins has been reported previously. In this study, we employed gain-of-function approaches to investigate the function of LARP1s. Our results suggested that LARP1c has an important role in regulating leaf senescence. The first evidence came from constitutive overexpression of *LARP1c* under the control of the 35S promoter; plants with high *LARP1c* transcript levels displayed precocious leaf senescence (Fig. 1). The second line of evidence came from experiments in which *LARP1c* overexpression was induced. The inducible accumulation of *LARP1c* transcripts also caused premature leaf senescence (Fig. 4), as well as elevated transcript levels of *SAG*s, such as *SAG13*, *SEN4*, *WRKY6* (Fig. 6), and *SAG12*. Furthermore, both RT-PCR and *LARP1c* promoter: GUS analyses confirmed that *LARP1c* expression was age-dependent (Figs. 8 and 9). More importantly, although the *1c-1* mutant showed no obvious developmental defects under normal condition, detached leaves of *1c-1* impaired ABA-, SA- and MeJA-mediated leaf senescence (Fig. 7). These observations suggested that LARP1c positively regulates leaf senescence.

The signaling pathway governing leaf senescence has substantial overlap with plant defense signaling pathways (Kim et al., 2008; Quirino et al., 2000). A number of defense-related genes are expressed during leaf senescence, and defense-related mutants also showed altered *SAG* expression (Quirino et al., 2000). Furthermore, ABA, SA, and MeJA have been shown to play important roles in both leaf senescence and defense signaling pathways (Adie et al., 2007; He et al., 2001; 2002; Lim et al., 2007b). We showed that several defense-related genes were upregulated upon overexpression of *LARP1c* (Fig. 6). The *1c-1* mutant impaired ABA-, SA- and MeJA-mediated leaf senescence (Fig. 7). These results suggest that LARP1c functioned not only in leaf senescence but also in defense signaling pathways.

Subcellular localization of 1c–GFP showed that LARP1c is a cytoplasmic protein with foci distribution (Fig. 10B). In *C. elegans*, CeLARP1 was reported to be a cytoplasmic protein that localizes to processing bodies (P-bodies) (Nykamp et al., 2008), which are sites of mRNA decay and storage. Interestingly, we found that 1c-GFP foci were sensitive to cycloheximide treatment, an inhibitor that can disrupt P-body formation (Goeres et al., 2007; Sheth and Parker, 2003) (data not shown). Does LARP1c localize to P-bodies? Colocalization analysis of LARP1c and P-bodies marker protein (e.g., DCP1 or DCP2) will be required to answer this question. However, one may reasonably assume that LARP1c assembles into P-bodies and regulates the dynamics of certain mRNAs that are required for initiation or facilitation of senescence in leaves.

In addition to LARP1c, there are two other LARP1 members, LARP1a and LARP1b, in *Arabidopsis*. Overexpression of *LARP1b* but not *LARP1a* caused premature leaf yellowing (Figs. 11A and 11B), but with minor effects compared with *LARP1c* overexpression. Thus, we inferred that LARP1b may have a similar role to LARP1c in regulating leaf senescence and that functional redundancy between LARP1b and LARP1c may exist. Further investigations of the effects of LARP1b on leaf senescence and on the phenotype of *larp1b larp1c* double mutants will reveal more about the roles of LARP1b and LARP1c in leaf senescence. For LARP1a, which might have a divergent role from other LARP1s during leaf senescence, carefully testing the *LARP1a* mutant may yield more information.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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