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RESEARCH PAPER

The RAV1 transcription factor positively regulates leaf senescence in *Arabidopsis*

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Abstract

Leaf senescence is a developmentally programmed cell death process that constitutes the final step of leaf development and involves the extensive reprogramming of gene expression. Despite the importance of senescence in plants, the underlying regulatory mechanisms are not well understood. This study reports the isolation and functional analysis of *RAV1*, which encodes a RAV family transcription factor. Expression of *RAV1* and its homologues is closely associated with leaf maturation and senescence. *RAV1* mRNA increased at a later stage of leaf maturation and reached a maximal level early in senescence, but decreased again during late senescence. This profile indicates that RAV1 could play an important regulatory role in the early events of leaf senescence. Furthermore, constitutive and inducible overexpression of *RAV1* caused premature leaf senescence. These data strongly suggest that RAV1 is sufficient to cause leaf senescence and it functions as a positive regulator in this process.

Key words: *Arabidopsis*, leaf senescence, RAV1, senescence regulator, transcription factor.

Introduction

Senescence, the final developmental phase of leaves, entails the co-ordinated degradation of macromolecules and the subsequent mobilization of the resulting products to other parts of the plant (Noodén, 1988). It is a highly complex but ordered process that is basically governed by developmental age: when a leaf cell reaches a certain developmental age, it undergoes senescence. However, leaf senescence can be affected by other internal factors such as plant growth regulators, reproduction status, and cellular differentiation. It can also occur prematurely if triggered by external factors such as biotic and abiotic stresses (Quirino *et al.*, 2000; Lim *et al.*, 2007).

Leaf senescence is elaborately regulated to maximize plant fitness by remobilizing nutrients from senescing leaves, so that its onset, progression, and completion should be finely controlled by the differential expression of many genes. The application of recent genomics technology has enabled the isolation of a category of genes, so-called senescence-associated genes (*SAG*s), which show increased expression in senescing leaves (Buchanan-Wollaston *et al.*, 2003; Andersson *et al.*, 2004; Guo *et al.*, 2004; Zentgraf *et al.*, 2004). Transcriptome analyses in *Arabidopsis thaliana* revealed that approximately 800 among 2491 genes were specifically up-regulated during developmentally controlled

senescence (Buchanan-Wollaston *et al.*, 2005). Although the *SAG* spectrum is mostly consistent with known biochemical and physiological symptoms during leaf senescence, it also provides many new insights into the complex mechanisms that regulate the process. Nevertheless, there are only a few *SAG*s whose *in vivo* functions in leaf senescence have been investigated.

Given that leaf senescence is an active process involving the differential expression of hundreds of genes, it is presumed that numerous transcription factors are involved as central elements of the regulatory network. Genome-wide analyses of changes in gene expression have allowed the identification of many *Arabidopsis* genes encoding transcription factors that show at least a 3-fold up-regulation in senescing leaves (Chen *et al.*, 2002; Guo *et al.*, 2004; Lin and Wu, 2004; Buchanan-Wollaston *et al.*, 2005; Balazadeh *et al.*, 2008). The encoded proteins belong to 20 different families of transcription factors, with the largest groups being NAC, WRKY, C2H2-type zinc finger, AP2/EREBP, and MYB proteins. Only a few transcription factors among these have been demonstrated to be involved in regulating leaf senescence by analysing the leaf senescence phenotypes of knockout mutants or transgenic overexpressor lines, or by identifying downstream target genes. For example, the T-DNA knockout mutation of *AtNAP*, a gene encoding a NAC family transcription factor, significantly delays leaf senescence, and induced overexpression causes precocious senescence, suggesting that AtNAP functions as a positive element in leaf senescence (Guo and Gan, 2006). Recently, it was also reported that ORE1, another NAC family transcription factor, functions positively in leaf senescence and associated cell death. ORE1 is one of the components in the trifurcate feed-forward pathway that includes EIN2 and *miR164*, and the expression of *ORE1* is up-regulated in an age-dependent manner by EIN2 but is negatively regulated by *miR164*. As leaves age, *miR164* expression gradually decreases through negative regulation by *EIN2*, leading to the up-regulation of *ORE1*. However, *EIN2* still contributes to age-induced cell death in the absence of *ORE1* (Kim *et al.*, 2009). The *WRKY53* gene is up-regulated at a very early stage of leaf senescence, and a *wrky53* knockout line undergoes delayed leaf senescence (Hinderhofer and Zentgraf, 2001; Miao *et al.*, 2004). Another WRKY transcription factor gene, *WRKY6*, is strongly up-regulated during leaf senescence as well as during pathogen infection, but the *wrky6* knockout mutation does not have any apparent effect on leaf senescence (Robatzek and Somssich, 2004). Differential display analysis of the *wrky6* mutant revealed that a senescence-induced receptor kinase gene, *SIRK*, might be a WRKY6 target (Ülker and Somssich, 2004). However, the potential *in vivo* functions of most leaf senescence-associated transcription factors remain to be elucidated.

Suppression subtractive hybridization (SSH) is a powerful tool to amplify differentially expressed sequences selectively, thus enriching a library in rare and conditionally expressed transcripts (Gepstein *et al.*, 2003). An SSH strategy was adopted to isolate non-abundant novel *SAG*s in *Arabidopsis*. In this study, the isolation and functional analysis of *RAV1*, a *RAV* (*Related to ABI3/VP1*) transcription factor family gene is reported. Expression analysis of the *RAV1* gene during various developmental stages revealed that its expression level increased at a later stage of leaf maturation, reached a maximum level at an early stage of leaf senescence, but decreased again at a later stage. The *RAV1* transcript was also induced when leaf senescence was accelerated by phytohormones such as ethylene or methyl jasmonate (MJ). A similar expression pattern was also observed in other *RAV* family genes examined. Constitutive overexpression of *RAV1* conferred an early senescence phenotype by accelerating the onset of various senescence symptoms during age-dependent senescence as well as during darkness- or hormone-induced senescence. However, no obvious senescence phenotype was observed in *rav* T-DNA single or double mutant lines, implying that there may be functional redundancy among the RAV transcription factors. The early senescence phenotype was further investigated in lines in which *RAV1* overexpression could be chemically induced. In these lines, *RAV1* induction caused precocious leaf senescence during both age-dependent senescence and darkness-induced senescence. These data support the conclusion that RAV1 acts as a positive regulator of leaf senescence in *Arabidopsis*.

Materials and methods

Suppression subtractive hybridization (SSH)

Following 2–3 d of cold stratification, *Arabidopsis* Col-0 seeds were germinated and grown in a temperature-regulated growth room at 23 °C with a 16/8 h day/night cycle. Two μg of senescent leaf mRNA (tester) and 2 μg of fully expanded mature green leaf mRNA (driver) were used (Fig. 1A). SSH was performed with the PCR-Select cDNA subtraction kit (Clontech, USA) as described by the manufacturer. The PCR products generated by SSH were cloned into the vector pGEM T-easy (Promega, USA).

Assay of age-dependent leaf senescence

Plants for physiological experiments were grown in an environmentally controlled growth room (Korea Instruments, Korea) with a 16/8 h day/night cycle at 23 °C. For age-dependent leaf senescence, the third and fourth rosette leaves of each plant were harvested just before the emergence of the inflorescence stem and were designated as fully expanded mature leaves. Leaves representing various developmental ages were harvested and are presented in Fig. 1B.

Chlorophyll was extracted from individual leaves by heating in 95% ethanol at 80 °C. The chlorophyll concentration per fresh weight of leaf tissue was calculated as described by Lichtenthaler (1987). The photochemical efficiency of Photosystem II (PSII) was deduced from chlorophyll fluorescence characteristics (Oh *et al.*, 1997) using a portable plant efficiency analyser (Hansatech Instruments, England). Membrane ion leakage was determined by measuring electrolytes released by leaves (Woo *et al.*, 2001). Conductivity was expressed as the percentage of initial conductivity versus total conductivity.

Assay of artificially induced leaf senescence

For dark incubation, leaves were detached at 12 d after emergence (DAE) and floated on 3 mM MES buffer (pH 5.8) for the

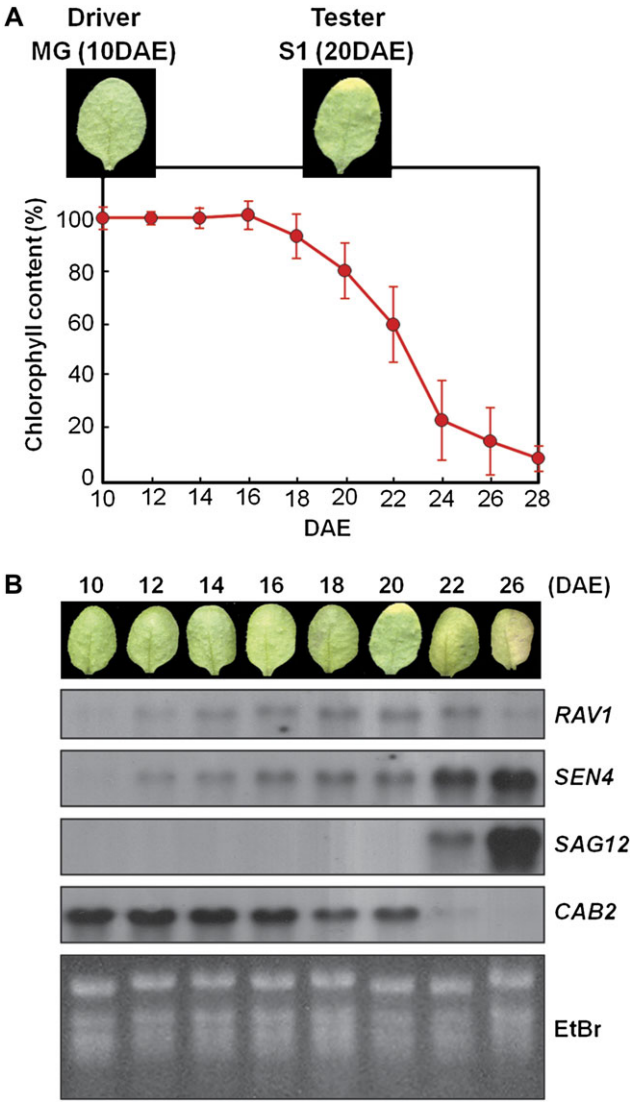

Fig. 1. Differential expression of the *RAV1* gene during maturation and leaf senescence. (A) For SSH, leaves at 10 DAE and 20 DAE were used as materials for driver and tester cDNA, respectively. Ten-DAE leaves are at the mature green stage (MG), and 20-DAE leaves are at the early senescence stage (S1), when the chlorophyll content is approximately 80% of initial values. Error bars indicate standard deviation (SD, n=25). (B) Age-dependent changes in *RAV1* gene expression. Total RNA was extracted at 10, 12, 14, 18, 20, 22, and 26 DAE from the third and fourth rosette leaves of Col plants. Samples were subjected to RNA gel blot analysis using *RAV1*, *CAB2*, *SAG12*, and *SEN4* cDNAs as hybridization probes. EtBr staining was used as a loading control.

designated days. For hormone treatment, detached leaves were floated in the same buffer in the presence or absence of 50 μM abscisic acid (ABA; Sigma, USA), 100 μM methyl jasmonate (MJ; Sigma, USA), or 100 μM 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma, USA). All hormone treatments were performed at 23 °C under continuous light. Detached leaves were floated abaxial side up in a buffer in induced leaf senescence experiments, and the 24-well plate that contained the samples was tightly sealed with parafilm to prevent leaves from being dehydrated. Chlorophyll content, photochemical efficiency, and ion leakage were measured as described above.

T-DNA insertion lines

T-DNA insertion lines were obtained from the SALK collection at Ohio State University. SALK_021865 has a T-DNA insertion in the exon of *RAV1* (*At1g13260*). SALK_097513 and SALK_139591 have a T-DNA insertion in the exon of *TEM1* (*At1g25560*) and the exon of *RAV3* (*At3g25730*), respectively. The detailed information on each T-DNA insertion line is shown in Supplementary Fig. S1 at *JXB* online. All primers used for genotyping each mutant line are listed in Supplementary Table S1 at *JXB* online.

Subcellular localization of the RAV1-GFP fusion protein

The full-length *RAV1* open reading frame (ORF) was amplified by PCR with primers containing appropriate restriction sites and then cloned upstream of the *GFP* coding region in the vector p326GFP-3G, which created a *RAV1–GFP* fusion driven by the *CaMV 35S* promoter. For transient expression in *Arabidopsis*, mesophyll cell protoplasts were transfected with the construct as previously described by Kim *et al.* (2008). Fusion protein expression was observed by Zeiss LSM 510 Meta confocal microscopy (Carl Zeiss, Germany).

Construction of plant expression vectors and generation of transgenic plants

For constitutive overexpression of *RAV1*, the *RAV1* ORF was PCR amplified with primers RAV1OX-F and -R (see Supplementary Table S1 at *JXB* online) and cloned into pCAMBIA3301. For inducible overexpression of *RAV1*, the *RAV1* ORF was PCR amplified with primers RAV1-GVG-F and -R (see Supplementary Table S1 at *JXB* online) and cloned into the binary vector pTA7001. These constructs were transformed into *Agrobacterium tumefaciens* AGL1 and introduced into Col plants by *in planta* transformation. The effect of inducible overexpression of the *RAV1* gene on promoting leaf senescence was assessed by spraying whole plants at 20 d after germination with 15 μM dexamethasone (DEX, Sigma, USA). In parallel experiments, detached mature green rosette leaves (10-d-old) were floated in MES buffer containing 15 μM DEX at 23 °C for 7 d in darkness. Control plants transformed with an empty vector exhibited normal development after MES or DEX treatment (see Supplementary Fig. S2 at *JXB* online).

RNA isolation and gel blot analysis

Total RNA was prepared using TRIzol reagent (Invitrogen, USA), following the manufacturer's protocol. RNA was separated on 1.2% (w/v) agarose formaldehyde gels and blotted to Hybond-N^+ nylon filters. The filters were hybridized using a radiolabelled probe at 65 °C overnight in hybridization buffer containing 1% BSA, 0.14 M NaH_2PO_4, 0.36 M Na_2HPO_4, 7% SDS, and 1 mM EDTA. Probes were prepared using a random labelling kit according to the manufacturer's instructions (Amersham, USA). After hybridization, the membranes were washed as previously described by Woo *et al.* (2001).

Synthesis of cDNA and RT-PCR

First-strand cDNA was generated from 1 μg RNA samples using ImProm-II Reverse Transcription System (Promega, USA) following the manufacturer's protocol. Transcript levels were determined by RT-PCR with the following primers: *RAV1* (RAV1-F and RAV1-R); *TEM1* (TEM1-F and TEM1-R); and *RAV3* (RAV3-F and RAV3-R) (see Supplementary Table S1 at *JXB* online). The primers for *CAB2*, *SAG12*, *ERF*, *PDF*, and *ACT2* are also listed in Supplementary Table S1 at *JXB* online. Identical results were obtained from three independent biological replicates, one of which is shown in Fig. 3.

Results

Identification of RAV1

The SSH approach was undertaken to isolate new regulatory genes that are differentially expressed at very early time points of leaf senescence, before signs of senescence become visible. The driver cDNA for the SSH library was synthesized from mRNA isolated from mature green leaves at 10 DAE (Fig. 1A). The tester cDNA was produced from mRNA isolated from leaves at the early senescence stage, where the chlorophyll level was approximately 80% of initial values (Fig. 1A). At this stage, leaves contain a heterogeneous cell population consisting of mature green cells to senescing cells; therefore, leaves at this stage should be a good resource for identifying crucial regulatory genes in leaf senescence. With the SSH screening technique, followed by reconfirming expression of the screened clones by RNA gel blot analysis, 132 cDNAs were identified whose transcripts increased during early leaf senescence. Identification of known senescence regulators including *At5g13330* (AP2 transcription factor), *At4g23150* (protein kinase), *At2g19810* (zinc finger family protein), and *At1g62300* (WRKY family transcription factor) confirmed the enrichment of senescence-specific clones by SSH (see Supplementary Table S2 at *JXB* online).

It was decided to characterize the role of the *RAV1* gene (*At1g13260*) in regulating leaf senescence further, because RAV1 belongs to a novel group of transcription factors whose expression is closely associated with leaf maturation and senescence. The *RAV1* expression pattern during leaf development was first analysed in detail in the third and fourth rosette leaves at different ages by RNA gel blot analysis. The developmental stages of leaves were determined by measuring chlorophyll contents (Fig. 1A) and by monitoring the expression patterns of a photosynthetic gene, *CAB2*, which encodes Chlorophyll A/B Binding Protein 2, and of two *SAG*s, *SEN4* and *SAG12* (Fig. 1B). The *RAV1* transcript began to increase at the late mature green stage, reaching a maximum level at the early senescence stage, but decreasing again when the *SEN4* and *SAG12* transcript levels markedly increased. The *RAV1* expression profile during leaf development indicated that RAV1 might play a role in regulating the onset of leaf senescence.

Characterization of RAV *genes*

Sequence analysis revealed that the *RAV1* gene encodes a protein of 300 amino acids containing a nuclear localization signal sequence and two DNA binding domains, supporting its proposed role as a transcriptional regulator. As shown in Fig. 2A, the N-terminal region of RAV1 contains an AP2 DNA-binding domain, which is found in a family of transcription factors represented by the APETALA2 and ethylene response factor (ERF) proteins, while a C-terminal region exhibits homology to the highly conserved B3 domain of VP1/ABI3 transcription factors. To determine the subcellular localization of RAV1, a RAV1-GFP (green fluorescent protein) fusion protein

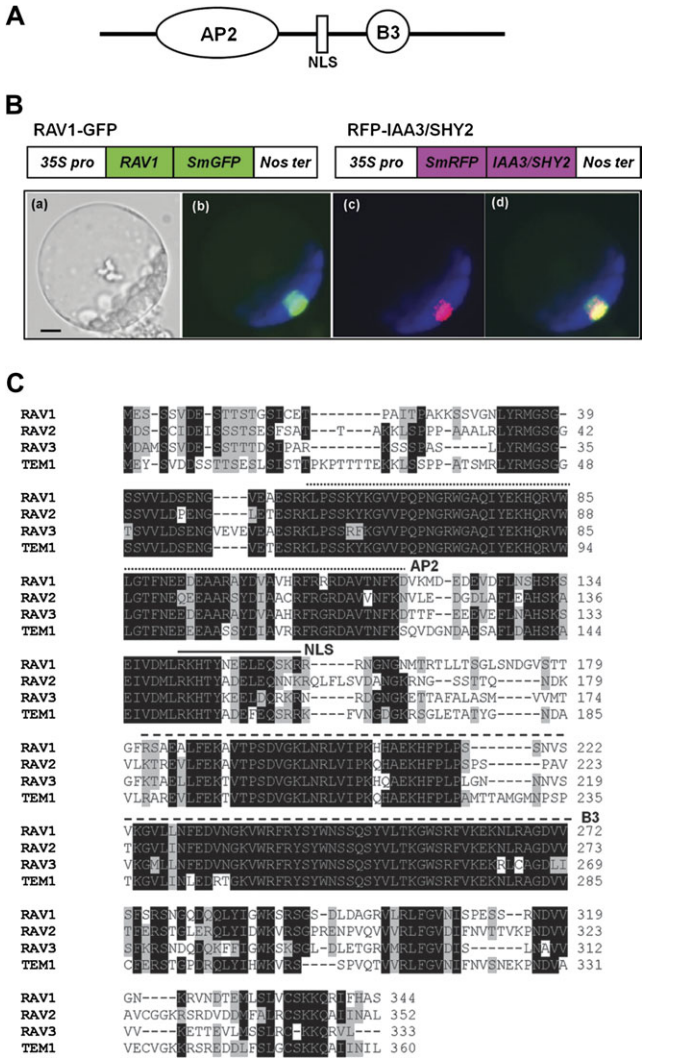

Fig. 2. Molecular analysis of RAV1. (A) Schematic of the domain organization of RAV1. (B) Nuclear localization of RAV1-GFP. Transient expression of *35S::RAV1-GFP* in *Arabidopsis* protoplasts. Shown are a bright field image (a), a GFP fluorescence image for RAV1-GFP (b), an RFP fluorescence image for RFP-IAA3/SHY2 (c), and a merging of the two fluorescence images (d) of a representative *Arabidopsis* protoplast. IAA3/SHY2 was used as a control for nuclear localization. (C) Predicted amino acid sequence of RAV1 [NP_172784] and alignment with other sequences encoded by the *RAV* gene family (TEM1 [NP_173927], RAV2 [NP_564947], and RAV3 [NP_189201]). The nuclear localization signal (NLS) and two DNA binding motifs (AP2 and B3) are indicated by lines.

was expressed under the control of the *35S* promoter in *Arabidopsis* protoplasts. Colocalization with a known nuclear protein, RFP-SHY2 (red fluorescent protein-suppressor of HY2), revealed that RAV1-GFP selectively localized to the nucleus (Fig. 2B), further implying that RAV1 functions as a transcriptional regulator.

The *Arabidopsis* genome contains five more *RAV1*-like genes, each of which encodes a protein containing two characteristic DNA binding domains, AP2/EREBP and B3, both of which are found only in vascular plants. Four of the

RAV proteins [RAV1 (At1g13260), TEMPRANILLO1 (TEM1; At1g25560), RAV2/TEM2 (At1g68840), and RAV1-like protein (designated as RAV3 in this study; At3g25730)] are highly conserved, sharing amino acid identities of 65–72% throughout their lengths (Fig. 2C; Castillejo and Pelaz, 2008). These proteins have also been reported as EDF1 (Ethylene response DNA binding Factor1) to EDF4, which redundantly function in ethylene signalling (Alonso *et al.*, 2003). The expression of *RAV1* and *RAV2* were shown to be up-regulated by various external and environmental cues, including low temperature, darkness, wounding, drought and salt stress, and pathogen attack (Fowler and Thomashow, 2002; Lee *et al.*, 2005; Sohn *et al.*, 2006).

The expression patterns of two *RAV1* homologues, *TEM1* and *RAV3*, whose encoded proteins share the highest amino acid identity with RAV1, was then investigated. Similar to that of *RAV1*, the expression of *TEM1* was triggered at a late mature green stage (16 d) but decreased at a late senescence stage (28 d) (Fig. 3A). Contrary to that of *RAV1* and *TEM1*, the expression of *RAV3* remained at a high level during late senescence.

The regulatory network governing leaf senescence has substantial cross-talk with plant defence signalling pathways (He *et al.*, 2002). In *Arabidopsis*, at least three genetically distinguishable pathways for defence signalling have been characterized: those mediated by salicylic acid, jasmonic acid, and ethylene. These signalling molecules increase during senescence and can modulate the expression of specific downstream genes (Buchanan-Wollaston *et al.*, 2005). A previous study showed that the *RAV1* and *RAV2* genes play an important role in regulating biotic and abiotic stresses (Sohn *et al.*, 2006). To gain an insight into whether the *RAV* genes are also involved in pathways common to plant defence and senescence, the expression patterns of *RAV1*, *TEM1*, and *RAV3* were examined in response to the defence-associated phytohormones, ethylene and MJ. As shown in Fig. 3, the transcript levels of these three genes were regulated in a similar manner. Two hours after treatment with ACC, the ethylene precursor, the expression of the three *RAV* genes was strongly triggered but declined again 20 h afterwards (Fig. 3B). The transcript levels for the three *RAV* genes also rapidly increased in response to MJ (Fig. 3C). Twenty hours after MJ treatment, *RAV1* transcripts remained at higher levels, but the *TEM1* and *RAV3* transcripts decreased. These results indicate that the *RAV* genes might play an important role during senescence modulated by ethylene and MJ as well as during age-dependent senescence. These data also imply that the RAV proteins may be functionally redundant, although regulation of the *RAV* genes may differ slightly.

Analysis of rav *single and double mutants*

To explore the function of the *RAV* genes in leaf senescence, senescence symptoms were analysed first during age-dependent and darkness-induced senescence in a *rav1* T-DNA insertion line (SALK_021865). This mutation did not significantly alter the senescence process, when senescence symptoms were visually observed or assayed by measuring typical senescence parameters, such as chlorophyll content and photochemical efficiency (data not shown). Lack of the senescence phenotype in the *rav1* mutant could be due to a possible functional redundancy of RAV proteins. To test this possibility, homozygous T-DNA insertion lines (SALK_097513 and SALK_139591) were identified in which the expression of *TEM1* and *RAV3* is disrupted, respectively, and *rav1 tem1* and *rav1 rav3* double mutants were generated. However, both double mutations had little effect on age-dependent and darkness-induced senescence (data not shown). Although a more detailed analysis of other *RAV* genes remains to be performed, these results imply that the *RAV* genes may not be required for regulating leaf senescence, or alternatively, they function redundantly in the leaf senescence process.

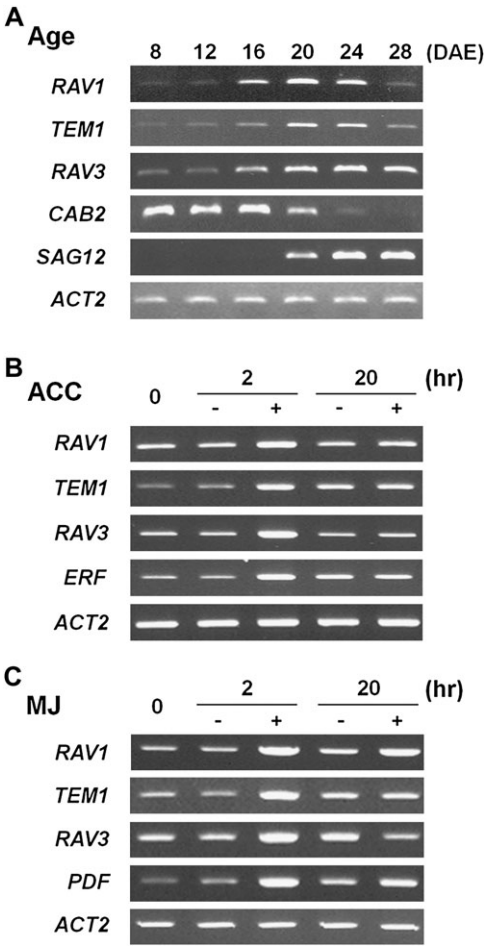

Fig. 3. Expression of *RAV* homologues in response to senescence-inducing factors. Temporal expression of *RAV* genes during leaf development (A), and in response to ACC (B) and MJ (C). Total RNA was isolated from leaves harvested at the indicated days (A) or the indicated times after treatment (B and C). Transcripts were analysed by RT-PCR using primers specific to *RAV1*, *TEM1*, or *RAV3*. *CAB2* and *SAG12*, *ERF*, and *PDF* were used as responsive genes for age-dependent senescence, ACC treatment, and MJ treatment, respectively. *ACT2* was used as an internal control for cDNA. –, without treatment; +, with treatment.

Constitutive overexpression of RAV1 *accelerates age-dependent leaf senescence*

The initiation of leaf senescence can be affected by many internal and external factors (Quirino *et al.*, 2000; Lim *et al.*, 2007). It is possible that loss-of-function mutation of a given gene, in some cases, may not alter the senescence phenotype to a detectable level, although that gene may be important for regulating senescence. Thus, a gain-of-function genetic approach was taken to investigate the role of *RAV1* in leaf senescence further. The 'gain-of-function' transgenic plants overexpressing the *RAV1* gene under the control of the constitutive *35S* promoter were generated. Although a few overexpressor lines exhibited slight dwarfism, most transgenic lines did not show any developmental or growth defects (data not shown). The senescence phenotype in *RAV1* overexpressor lines was initially analysed by visual examination of a single leaf throughout its life span. Most transgenic lines showed an early senescence phenotype, and the *RAV1* gene was highly expressed at the young green stage in these lines (Fig. 4A).

The effect of *RAV1* overexpression on leaf senescence was determined by comparing the degree of yellowing in the wild type and two *RAV1* overexpressors (*RAV1OX*2 and *RAV1OX*3) during age-dependent leaf senescence (Fig. 4B). At 16 DAE, the third and fourth leaves of the overexpressors started to turn yellow, but wild-type leaves remained green. At 22–24 DAE, the overexpressor leaves had turned completely yellow and showed signs of death with drying. By contrast, wild-type leaves retained their integrity and showed only partial yellowing (Fig. 4B). Leaf senescence symptoms were also analysed by measuring typical senescence-associated physiological markers, such as chlorophyll content and the photochemical efficiency of PSII. At 18 DAE, the chlorophyll content of wild-type leaves started to decline, whereas overexpressor leaves had already lost 24–43% of their chlorophyll (Fig. 4C). The accelerated senescence of the overexpressors was also observed, when photosynthetic activities were measured (Fig. 4D; see Supplementary Fig. S3 at *JXB* online).

The expression of the photosynthesis-related gene *CAB2* as well as of the two senescence molecular markers, *SEN4* and *SAG12* (Fig. 4E) was investigated further. In wild-type leaves, the *CAB2* transcript was highly expressed until 20 DAE. However, in *RAV1*-overexpressing lines, *CAB2* expression was greatly reduced at 18 DAE. The expression of *SEN4* was detectable at 22 DAE and 16 DAE in the wild type and overexpressors, respectively. Similarly, *SAG12* transcripts started to accumulate in leaves at 22 and 16–18 DAE in wild type and overexpressors, respectively, and their abundance continually increased with the propagation of leaf senescence. All these data indicate that the overexpression of *RAV1* causes precocious senescence along with leaf age.

RAV1 *overexpression causes an earlier onset of senescence during artificially induced leaf senescence*

Leaf senescence can be accelerated by incubation in the dark and by several plant growth regulators, such as ethylene, ABA, or MJ. It was therefore examined whether *RAV1* overexpression influences leaf senescence artificially induced by these factors. As shown in Fig. 5A, the decrease in chlorophyll content proceeded very rapidly in *RAV1* overexpressors incubated in the dark, when compared with wild-type plants. Similarly, *RAV1* overexpression accelerated the decrease in PSII activity during incubation in darkness (Fig. 5B; see Supplementary Fig. S4A at *JXB* online).

Senescence phenotypes developed faster in *RAV1* overexpressor leaves treated with senescence–accelerating hormones (Fig. 5C, D; see Supplementary Fig. S4B at *JXB* online). When detached leaves were treated with MJ, ABA, or ACC, more rapid decreases in chlorophyll content and PSII activity were observed in the *RAV1* overexpressors than in wild-type leaves. Two days after incubation in ACC-, ABA-, or MJ-containing solutions, the chlorophyll content of wild-type leaves was 93%, 69%, and 56% of the initial content, respectively. By contrast, treated *RAV1* overexpressor leaves retained only 44–39%, 29–27%, and 18–10% of their original chlorophyll contents, respectively. A similar finding was obtained for PSII activity. These data show that RAV1 plays an important role in senescence mediated by darkness or senescence-enhancing hormones as well as in age-mediated senescence.

Inducible overexpression of RAV1 *also causes premature leaf senescence*

Any perturbation in the expression of homeostatic or housekeeping genes could give apparent early senescence symptoms. Thus, the early senescence phenotypes that have been observed may be the result of an indirect effect of metabolic or physiological disturbances. To avoid potential complications in interpreting the phenotypes caused by constitutive overexpression of *RAV1*, transgenic lines were generated that express the *RAV1* gene under the control of a glucocorticoid-inducible promoter. RT-PCR analysis showed that *RAV1* expression was readily induced by treatment with dexamethasone (DEX, a synthetic glucocorticoid) (Fig. 6A). The effect of inducible overexpression of the *RAV1* gene on promoting leaf senescence was first assessed by spraying whole plants with DEX at 20 d after germination. Treatment with DEX caused precocious leaf yellowing (Fig. 6B) and significant reductions in chlorophyll content (Fig. 6C) and photochemical efficiency (Fig. 6D) in *RAV1*-inducible lines, but not in mock-treated transgenic or wild-type plants. Membrane ion leakage, which is one of the parameters measuring cell death, was also assayed. The leaves of *RAV1*-inducible lines exhibited faster increases in membrane ion leakage than did control leaves (Fig. 6E). The precocious leaf yellowing was confirmed as a senescence phenotype by RNA gel blot analysis of the senescence-specific marker genes *SAG12*, *SAG13*, and *SAG24* (Fig. 6F).

These early leaf senescence symptoms in *RAV1*-inducible lines were also observed when senescence was induced in detached leaves (12-d-old) by incubation in darkness. After 4 d of incubation in darkness, DEX-treated leaves of the

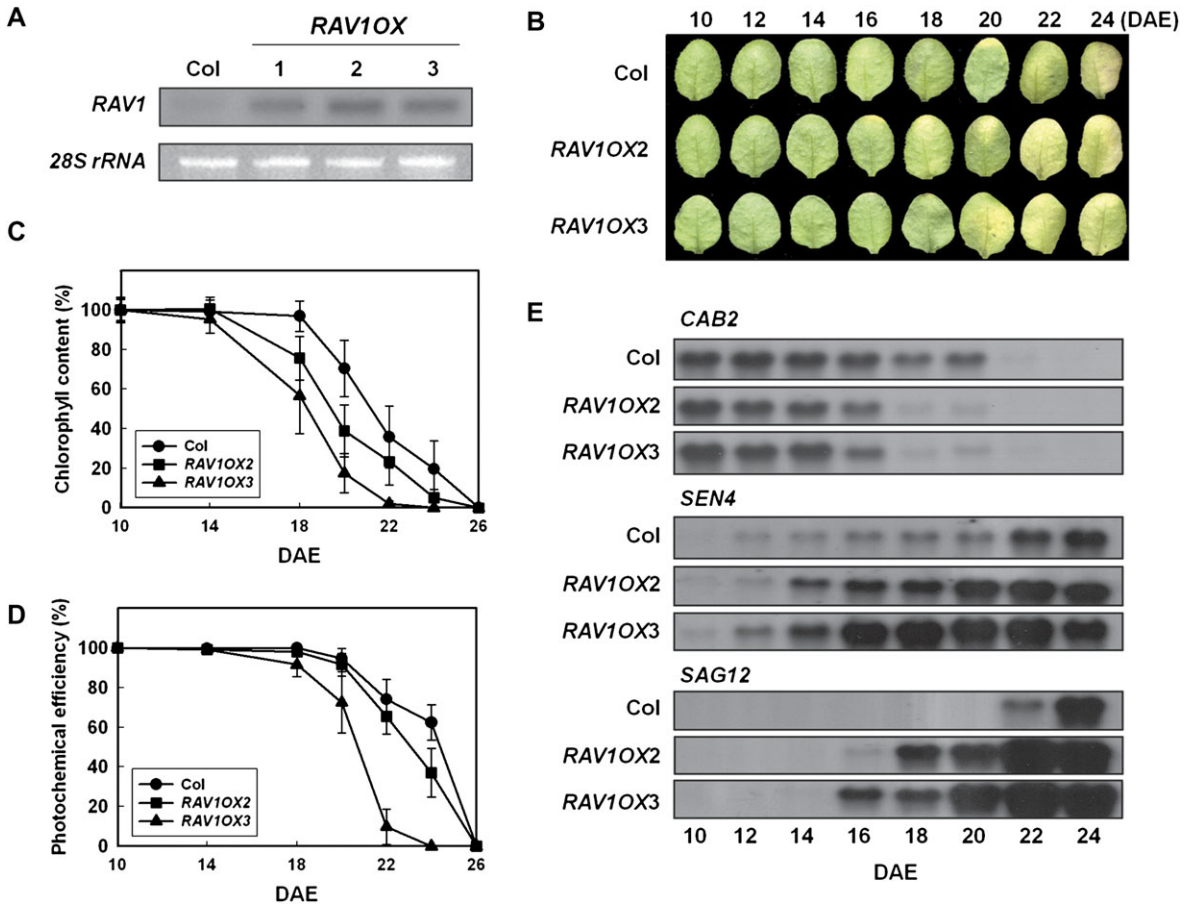

Fig. 4. Accelerated age-dependent leaf senescence phenotypes of constitutive *RAV1* overexpressor lines. (A) Expression of *RAV1* in three independent transgenic lines carrying a *35S::RAV1* construct, compared with wild-type Col plants. *RAV1* expression was analysed by RNA gel blot analysis. (B) Age-dependent senescence phenotype of the fourth rosette leaves of wild-type (Col) plants and two *RAV1* overexpressors. (C, D) Chlorophyll content (C) and photochemical efficiency of PSII (D) were examined in leaves of the indicated ages. Chlorophyll content and photochemical efficiency as compared with initial values of each line at 10 DAE are shown. F_v/F_m, maximum quantum yield of PSII electron transport (maximum variable fluorescence/maximum yield of fluorescence). Error bars, SD (n=25). (E) Age-dependent changes in gene expression. Total cellular RNA was isolated at the indicated DAE from wild-type leaves and *RAV1* overexpressors, and RNA blots were hybridized with *CAB2*, *SEN4*, and *SAG12*.

transgenic *RAV1*-inducible lines turned yellow, while those of control lines remained green (data not shown). Measurements of photochemical efficiency and chlorophyll contents revealed that the DEX-treated leaves of *RAV1* inducible lines exhibited lower values for both parameters than did control leaves (Fig. 7A, B). Membrane ion leakage also rapidly increased in DEX-treated inducible lines (Fig. 7C). These data clearly show that RAV1 is sufficient to promote leaf senescence, suggesting that it plays an important role in positively controlling this process.

Discussion

RAV1 is a transcription factor induced at the early stages of leaf senescence

Leaf senescence is a genetically controlled developmental process, eventually leading to cell death. Apparently, senescence does not occur in young leaves under normal growth conditions. Possibly, senescence repressors efficiently suppress the onset of senescence during early leaf development, and/or activators are switched on as a leaf ages. Various molecular and genomics strategies have been used to identify genes that are differentially expressed during leaf senescence, supporting the concept that re-wiring an extensive regulatory network is an important mechanism of the pre-senescence process. To re-wire such a regulatory network, transcription factors have to be activated, which will then turn on the expression of a large number of genes involved in leaf senescence. Thus, the isolation and functional analysis of transcription factors that show enhanced expression during leaf senescence helps with understanding their roles in regulating gene expression at that time. Therefore, an SSH screen was undertaken with mRNA isolated from mature green leaves and leaves at the early senescence stage in an effort to isolate genes that are involved in triggering the onset of leaf senescence. The *RAV1* gene was chosen among newly identified *SAG*s because the RAV1 protein belongs to a group of

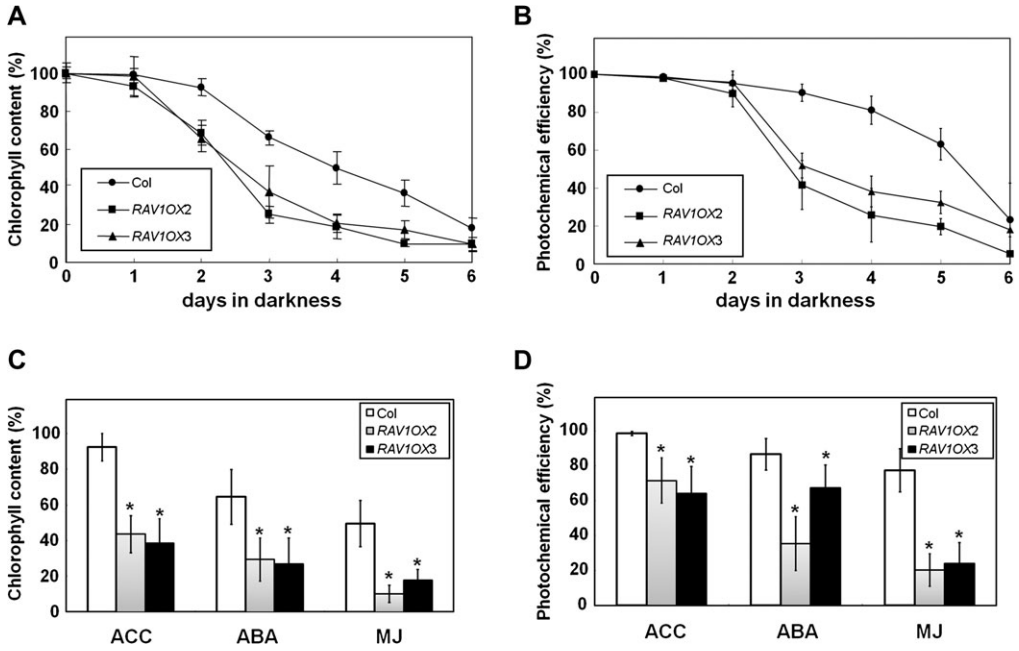

Fig. 5. Premature senescence symptoms in constitutive overexpressors of *RAV1* during senescence accelerated by darkness or plant hormones. (A, B) The darkness-induced senescence phenotype of detached leaves of wild-type Col and *RAV1*-overexpressing plants. The third and fourth rosette leaves were detached at the age of 10 DAE and incubated in darkness. Chlorophyll content (A) and the photochemical efficiency of PSII (B) were monitored during the incubation in darkness. Error bars, SD (n=25). (C, D) Senescence symptoms of the *RAV1* overexpressors during senescence induced by phytohormones (ACC, ABA, or MJ). The third and fourth rosette leaves were detached at the age of 10 DAE and incubated in continuous light with 50 μM ACC, 50 μM ABA, or 100 μM MJ for 2 d. Chlorophyll content (C) and photochemical efficiency (D) are shown as mean ±SD (n=25), relative to those of leaves incubated in light without hormones. Asterisks indicate values that are statistically different from the Col plants (Student's t test; P <0.05).

transcription factors whose expression is closely associated with leaf maturation and senescence.

Temporal expression patterns of *SAG*s may indicate the role of each gene during the various steps from the initiation signal to the terminal phase of cell death, providing valuable information concerning the sequence of events in the senescence programme. There are genes whose expression is induced at the early senescence stages, but thereafter their transcript levels drop. For example, genes encoding a Ras-related small GTP-binding protein (Gepstein *et al.*, 2003) and a putative senescence-related receptor kinase (Hajouj *et al.*, 2000) display such a temporal profile. By detailed expression analyses, it was found that the expression of *RAV1* increased at a later stage of leaf maturation and reached a maximum level at an early stage of senescence, but decreased again at a later senescence stage (Fig. 1B, 3A). This profile indicates that RAV1 could play a regulatory role during the initiation of leaf senescence and that as a transcription factor it might control senescence by the transcriptional activation and/or repression of genes involved in the execution of leaf senescence.

Plant transcription factors of the same family often have similar functions. For instance, the *NAC* and *WRKY* family genes are well-known senescence-related transcription factors (Buchanan-Wollaston *et al.*, 2005). More than 20% of 109 *NAC* family genes in *Arabidopsis* are specifically induced during developmentally triggered senescence (Buchanan-Wollaston *et al.*, 2005; Olsen *et al.*, 2005). Furthermore, *AtNAP* and *ORE1* are highly expressed in senescing leaves, and loss-of-function mutants of each gene display significantly delayed leaf senescence (Guo *et al.*, 2004; Guo and Gan, 2006; Kim *et al.*, 2009). The AtWRKY family constitutes the second largest group of transcription factors encoded by the senescence transcriptome (Guo *et al.*, 2004). Functional analyses of AtWRKY proteins have shown that *AtWRKY6*, *AtWRKY53*, and *AtWRKY70* are up-regulated during the progression of leaf senescence and that they regulate leaf senescence as transcription factors (Miao *et al.*, 2004; Robatzek and Somssich, 2001; Ülker *et al.*, 2007). The *Arabidopsis* genome contains six *RAV* family genes. The expression patterns of two other *RAV1* homologues, *TEM1* and *RAV3*, have been examined in addition to that of *RAV1* in order to investigate the possibility that other RAV transcription factors have roles in leaf senescence. Overall, the expression patterns of the three tested *RAV* genes were similar during leaf development and senescence, indicating that the RAV proteins may be functionally redundant.

RAV1 plays a role in triggering the initiation of leaf senescence

To gain a deeper insight into the function of RAV in regulating leaf senescence, senescence symptoms in *RAV1*, *TEM1*, and *RAV3* T-DNA insertion lines were examined. However, none of the *rav* single mutants or double mutants

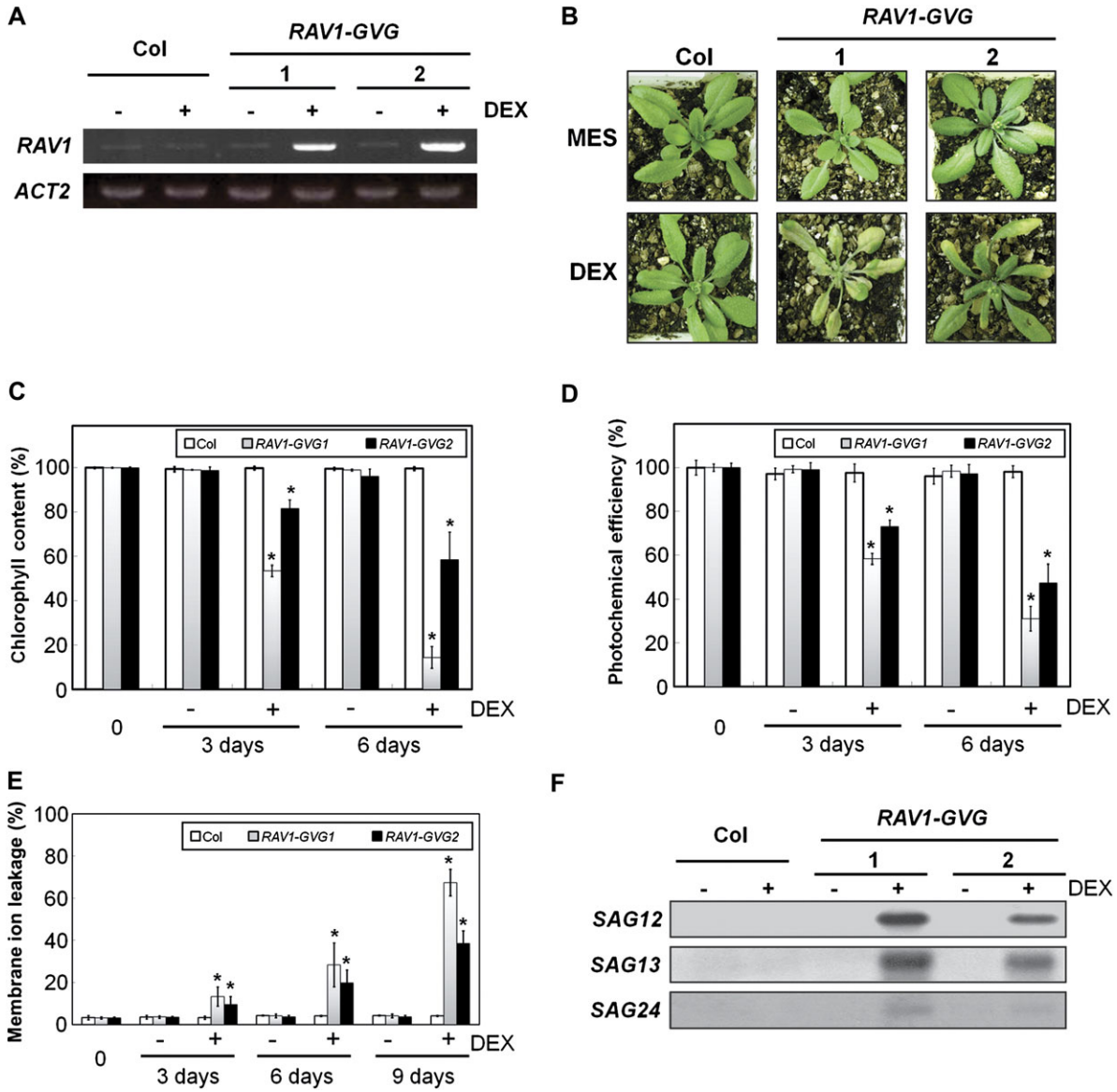

Fig. 6. Inducible overexpression of *RAV1* causes precocious age-dependent leaf senescence. (A) RT-PCR analysis with *RAV1*-specific primers of Col plants and two independent transgenic lines carrying the *GVGpro::RAV1/35S::GVG* construct 8 h after 15 μM DEX treatment. (B) Visible phenotypes of wild-type Col and transgenic plants harbouring *GVGpro::RAV1/35S::GVG* after treatment with MES or DEX. Pictures of representative plants were taken 6 d after DEX treatment. Chlorophyll content (C), photochemical efficiency (D), and ion leakage (E) were measured at the indicated time after DEX treatment. Error bars, SD (n=12). Asterisks indicate values that are statistically different from the Col plants (Student's t test; P <0.05). (F) Expression of *SAG* genes was observed 6 d after DEX treatment. –, Without DEX treatment; +, with DEX treatment.

exhibited significant alterations in senescence. This negative result implies that these *RAV* genes are not required for controlling leaf senescence, or alternatively, that they may be functionally redundant in this respect. Functional overlap among the *RAV* gene family members would not be surprising, because it was previously reported that detectable alterations in ethylene responses were not observed in any of the *edf* single mutants (Alonso *et al.*, 2003). Our data in this study support the suggestion that RAV1 has an important role in regulating leaf senescence. The first line of evidence comes from analyses with plants that constitutively overexpress *RAV1* under the control of the *35S* promoter; these plants displayed premature leaf senescence (Figs 4, 5). The second line of evidence comes from experiments in which *RAV1* was inducibly overexpressed. The inducible accumulation of *RAV1* transcripts also caused precocious leaf senescence (Figs 6, 7). These observations suggest that RAV1 is sufficient to cause leaf senescence and that it functions as a positive regulator of leaf senescence.

Although it is suggested that RAV1 is sufficient to cause leaf senescence and acts as a positive regulator of senescence, it is not conclusive whether RAV1 is necessary to regulate leaf senescence because of possible functional redundancy among the RAV proteins. Further studies that address how RAV1 regulates leaf senescence and other physiological processes, and that identify its immediate

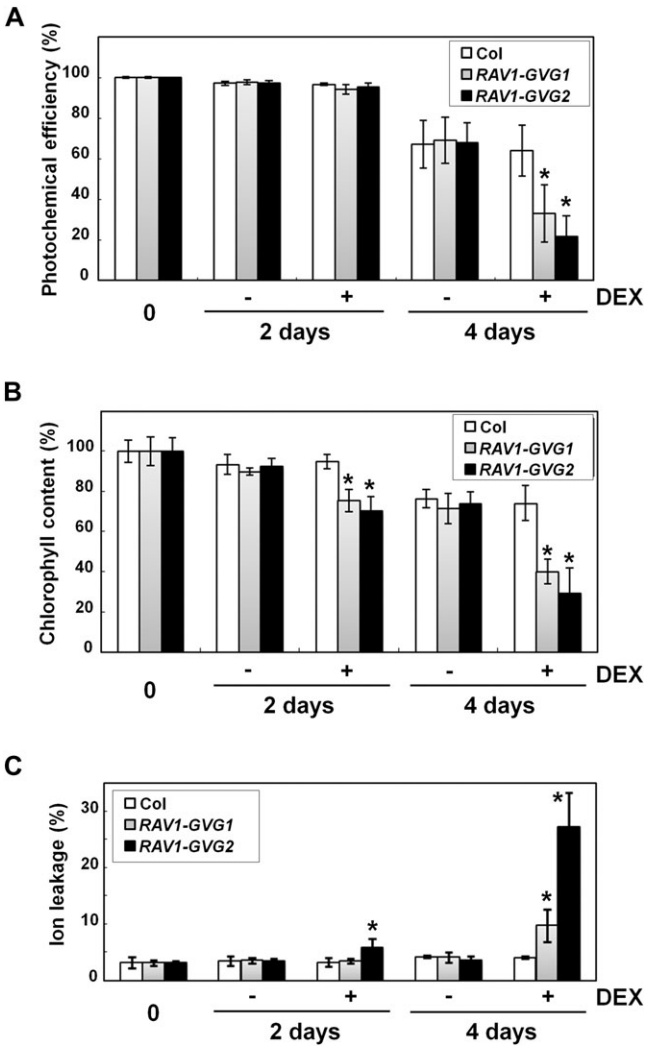

Fig. 7. Inducible *RAV1* overexpression is sufficient to accelerate darkness-induced leaf senescence symptoms. Photochemical efficiency (A), chlorophyll content (B), and ion leakage (C) were measured 2 d or 4 d after DEX treatment. –, Without DEX treatment; +, with DEX treatment. Error bars, SD (n=12). Asterisks indicate values that are statistically different from the Col plants (Student's t test; P <0.05).

target(s), will help us fully understand its biological functions and the underlying mechanisms of its activity.

RAV proteins may interconnect various developmental phenomena

As discussed earlier, it has been revealed that RAV1 plays an important part in regulating age-dependent leaf senescence. Since *RAV* family transcripts were induced when leaf senescence was accelerated by senescence-accelerating hormones such as ethylene and MJ (Fig. 3B, C), it was tested whether *RAV1* overexpression could alter artificially induced leaf senescence. Indeed, darkness- or phytohormone (ethylene, ABA, and MJ)-induced senescence of detached leaves was accelerated in *RAV1* constitutive or inducible overexpression lines (Figs 5, 7), suggesting that RAV1 possibly has a role in integrating the age-dependent aspects of leaf senescence with those that reflect environmental influences, such as darkness or phytohormone exposure.

Although several pieces of evidence have been provided that RAV1 is a positive regulator of leaf senescence, it should be noted that previous studies have implicated RAV proteins in several developmental pathways. First, it has been shown that the expression of *RAV1* and *RAV2* are induced by various external and environmental cues, including pathogen attack, low temperature, drought and salt stress, darkness, and wounding (Fowler and Thomashow, 2002; Lee *et al.*, 2005; Sohn *et al.*, 2006). Second, some RAV proteins have been shown to be involved in flowering time. At1g25560 and RAV2, also known as TEM1 and TEM2, respectively, act as direct repressors of the *FT* gene (Castillejo and Pelaz, 2008). Similar to our result, neither the *TEM1* nor *TEM2* loss-of-function mutation alone confers a distinct phenotype, but RNAi-mediated knockdown of both genes induces early flowering, while constitutive overexpression of either gene delays flowering (Castillejo and Pelaz, 2008). Third, *RAV1* is down-regulated by brassinosteroid and may function as a negative regulator during plant development (Hu *et al.*, 2003). As mentioned earlier, the RAV proteins were previously identified as EDF proteins, which are essential for the ethylene response (Alonso *et al.*, 2003). Taking together previous studies and our current findings, it is proposed that RAV proteins play an important role in regulating numerous developmental processes, including the onset of leaf senescence, by integrating a variety of internal and external stimuli.

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