Running head
Role of class II ERFs in leaf senescence

Corresponding author
Tomotsugu Koyama
e-mail: koyama@sunbor.or.jp
address: Suntory Foundation for Life Sciences, Bioorganic Research Institute,
Wakayamadai, Shimamoto, Osaka 618-8503, Japan.
fax: +81-75-962-2115

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Title
A regulatory cascade involving class II ETHYLENE RESPONSE FACTOR transcriptional repressors operates in the progression of leaf senescence.

Authors
Tomotsugu Koyama²*, Haruka Nii, Nobutaka Mitsuda, Masaru Ohta³, Sakihito Kitajima, Masaru Ohme-Takagi, Fumihiko Sato

Graduate School of Biostudies, Kyoto University, Sakyo, Kyoto, 606-8502 Japan (T.K., H.N., F.S.), Department of Applied Biology, Kyoto Institute of Technology, Sakyo, Kyoto, 606-8585 Japan (H.N., S.K.), Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, 305-8562 Japan (N.M., M.O., M.O.-T.), Institute for Environmental Science and Technology, Saitama University, Sakura, Saitama, 338-8570 Japan (M.O.-T.)

One-sentence summary
This work demonstrates the proteasome-mediated regulation of class II ERF transcriptional repressors and involvement of these factors in the progression of leaf senescence.
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2 Present address: Suntory Foundation for Life Sciences, Bioorganic Research Institute, Wakayamadai, Shimamoto, Osaka 618-8503, Japan

3 Present address: Genetically Modified Organism Research Center, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, 305-8666 Japan.

* Corresponding author (koyama@sunbor.or.jp).

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the Journal policy described in the Instructions for Authors (http://www.plantphysiol.org) is: Tomotsugu Koyama (koyama@sunbor.or.jp).
ABSTRACT

Leaf senescence is the final process of leaf development that involves the mobilization of nutrients from old leaves to newly growing tissues. Despite the identification of several transcription factors involved in the regulation of this process, the mechanisms underlying the progression of leaf senescence are largely unknown. Herein, we describe the proteasome-mediated regulation of class II ETHYLENE RESPONSE FACTOR (ERF) transcriptional repressors and involvement of these factors in the progression of leaf senescence in Arabidopsis thaliana (Arabidopsis). Based on previous results showing that the Nicotiana tabacum ERF3 (NtERF3) specifically interacts with a ubiquitin-conjugating enzyme, we examined the stability of NtERF3 in vitro and confirmed its rapid degradation by plant protein extracts. Furthermore, NtERF3 accumulated in plants treated with a proteasome inhibitor. The Arabidopsis class II ERFs AtERF4 and AtERF8 were also regulated by the proteasome and increased with plant aging. Transgenic Arabidopsis plants with enhanced expression of NtERF3, AtERF4, or AtERF8 showed precocious leaf senescence. Our gene expression and chromatin immunoprecipitation analyses suggest that AtERF4 and AtERF8 targeted EPITHIOSPECIFIER PROTEIN/EPITHIOSPECIFYING SENESCENCE REGULATOR gene and regulated the expression of many genes involved in the progression of leaf senescence. By contrast, an aterf4 aterf8 double mutant exhibited delayed leaf senescence. Our results provide insight into the important role of class II ERFs in the progression of leaf senescence.
INTRODUCTION

Leaf senescence, which is characterized by progressive yellowing, is the final stage of leaf development and involves the mobilization of nutrients from old leaves to newly growing tissues. The progression of leaf senescence requires programmed cell death combined with the cessation of photosynthesis, organelle breakdown, and protein degradation. The regulation of leaf senescence depends largely on the developmental age of plants, although it is also influenced by various external stimuli (Gan and Amasino, 1997). The detection of internal and external signals activates various processes mediated by signaling molecules such as plant hormones and reactive oxygen species (ROS; Buchanan-Wollaston et al., 2003; Lim et al., 2007).

The progression of leaf senescence is associated with the downregulation of genes involved in chlorophyll biosynthesis, carbon metabolism, and photosynthesis and the upregulation of genes involved in responses to hormones, ROS, and various stresses (Gepstein et al., 2004; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Balazadeh et al., 2008; Breeze et al., 2011). The coordinated regulation of gene expression during leaf senescence depends on the combined action of several families of transcription factors (TFs). In particular, genes coding for NAM/ATAF/CUC2 (NAC), zinc finger, WRKY, MYB, and APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) TFs are transcriptionally upregulated during leaf senescence (Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; Breeze et al., 2011). Functional analyses using Arabidopsis thaliana (Arabidopsis) have revealed that NAC and WRKY TFs including AtNAP1, ANAC092/ORESARA1 (ORE1), ORE1-SISTER1, VND-INTERACTING2, NAC WITH TRANSMEMBRANE MOTIF-LIKE4, JUNGBRUNNEN1, WRKY6, WRKY53, WRKY54, and WRKY75 regulate leaf senescence (Robatzek and Somssich, 2002; Miao et al., 2004; Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2011; Besseau et al., 2012; Lee et al., 2012; Wu et al., 2012). By contrast, the role of AP2/ERF TFs in leaf senescence remains unclear.

AP2/ERF TFs consist of 146 members in Arabidopsis and are involved in responses to various external and internal stimuli. Several AP2/ERFs modulate responses to leaf senescence-associated signaling molecules such as ROS, ethylene, jasmonic acid (JA), abscisic acid (ABA), and cytokinin (Nakano et al., 2006; Mizoi et
Gain-of-function studies suggest that RAV1 and C-REPEAT BINDING FACTOR (CBF)/DEHYDRATION RESPONSIBLE ELEMENT BINDING1 (DREB1) positively and negatively regulate leaf senescence, respectively (Sharabi-Schwager et al., 2010; Woo et al., 2010). However, knowledge of the molecular mechanisms of these AP2/ERFs in the regulation of leaf senescence is lacking.

Class II ERFs are characterized by the ERF-associated amphiphilic repression (EAR) motif (Ohta et al., 2001). Because class II ERFs repress target gene transcription in the presence of ERF activators in transient gene expression assays (Fujimoto et al., 2000; Ohta et al., 2000, 2001), understanding the regulation of class II ERFs is key to elucidating the complex mechanisms underlying AP2/ERF-mediated gene regulation. In addition to studying the stress response-associated transcriptional control of class II ERF genes, (Suzuki et al., 1998; Yamamoto et al., 1999; Fujimoto et al., 2000; Kitajima et al., 2000; Nishiuchi et al., 2003), we previously analyzed the post-translational regulation of class II ERFs and showed that a class II ERF from Nicotiana tabacum, NtERF3, physically interacts with the ubiquitin-conjugating (UBC) enzyme NtUBC2 (Koyama et al., 2003). Coexpression of the dominant-negative form of NtUBC2 enhances the repressive activity of NtERF3 in transient assays, suggesting that NtUBC2 may regulate NtERF3 activity. By contrast, NtUBC2 does not interact with NtERF2, a transcriptional activator. The function of NtUBC2 in the ubiquitin-proteasome system (Dreher and Callis, 2007; Vierstra, 2009) suggests that NtERF3 may be regulated by proteolysis.

In the present study, we demonstrate the involvement of proteasomes in the control of NtERF3 and two homologous Arabidopsis class II ERFs, AtERF4 and AtERF8. We also performed functional and gene expression analyses to clarify the role of these class II ERFs in the progression of leaf senescence.

RESULTS

Rapid degradation of NtERF3 in vitro

Because NtERF3 physically and functionally interacts with NtUBC2 (Koyama et al., 2003), we first examined the regulation of NtERF3 by proteolysis in vitro (Fig. 1). NtERF3, NtERF2, and GREEN FLUORESCENCE PROTEIN (GFP), as a stable protein model were individually produced in Escherichia coli and purified with affinity
resin (Supplemental Fig. S1). Each protein was incubated for 0, 15, 30, and 60 minutes in a cultured tobacco XD6S cell extract, and protein stability was analyzed with immunoblotting. Whereas NtERF3 was extremely unstable in the tobacco cell extract solution (Fig. 1A), NtERF2 and GFP were stable for 60 minutes (Figs. 1B and 1C). NtERF3 levels remained stable in a bovine serum albumin solution (Fig. 1D), suggesting that NtERF3 is specifically degraded by plant proteins.

To identify the amino acid residues involved in the stability of NtERF3, we produced truncated NtERF3 proteins in *E. coli* (Fig. 1E and Supplemental Fig. S1). NtERF3 contains a conserved DNA binding domain, an EAR domain, and an NtUBC2-interacting region consisting of amino acid residues 83 to 190 (NtERF3[83/190]; Fig. 1E) (Koyama et al., 2003). Incubating NtERF3 (1/225), NtERF3 (1/190), NtERF3 (26/225), NtERF3 (83/190), and NtERF3 (83/225) in the tobacco cell extract solution caused their rapid degradation, whereas NtERF3 (∆83/190), in which amino acid residues 83 to 190 were deleted, was degraded at a much slower rate. By contrast, NtERF3 (1/82) and NtERF3 (191/225) remained undegraded. These results suggested that NtERF3 (83/190) was essential and sufficient for NtERF3 degradation, whereas the repression and ERF domains were not involved in the stabilization of the protein. NtERF3 amino acid residues 83 to 95 contain a PEST motif, which is involved in the instability of proteins (Asher et al., 2006); however, deletion of the PEST motif did not affect the stability of NtERF3 in vitro (Fig. 1E).

**Low accumulation of NtERF3 in plant cells**

To examine the stability of NtERF3 in plants, we individually fused the coding sequences (CDS) for the respective regions of *NtERF3* with the nuclear localization signal (NLS)-GFP sequence under the control of the cauliflower mosaic virus 35S promoter (*Pro35S:NLS-GFP*), and the resulting fusion genes were transformed into cultured tobacco XD6S cells (Supplemental Fig. S2A). More than 100 kanamycin-resistant tobacco calluses for each construct were examined for GFP fluorescence. We detected no GFP signal in the *Pro35S:NLS-GFP-NtERF3* and *Pro35S:NLS-GFP-NtERF3* (83/190) tobacco calluses, whereas the *Pro35S:NLS-GFP-NtERF3* (1/82) and *Pro35S:NLS-GFP-NtERF3* (191/225) tobacco calluses showed strong nuclear GFP fluorescence (Fig. 2A). Consistent with these
results, immunoblot analysis of protein extracts from Pro35S:NLS-GFP-NtERF3 (1/82) and Pro35S:NLS-GFP-NtERF3 (191/225) tobacco calluses showed positive reactivity against an anti-GFP antibody, whereas extracts from Pro35S:NLS-GFP-NtERF3 and Pro35S:NLS-GFP-NtERF3 (83/190) tobacco calluses were negative (Fig. 2B).

No kanamycin-resistant Pro35S:NLS-GFP-NtERF3 (Δ83/190) tobacco calluses were recovered, suggesting that the fusion gene may confer a lethal phenotype on tobacco cells and this lethal gene may have a less severe effect in a heterologous system. As expected, 2 lines of Pro35S:NLS-GFP-NtERF3 (Δ83/190) Arabidopsis plants grew and produced small amounts of seed (Supplemental Fig. S2B; see Fig. 4). Because Pro35S:NLS-GFP-NtERF3 (Δ83/190) Arabidopsis plants exhibited a severe defective phenotype, 3-day-old seedlings were analyzed by fluorescence microscopy. The cells of Pro35S:NLS-GFP-NtERF3 (Δ83/190) Arabidopsis plants showed strong GFP fluorescence similar to that of Pro35S:NLS-GFP Arabidopsis plants; the fluorescence was absent in Pro35S:NLS-GFP-NtERF3 Arabidopsis plants (Fig. 2C). These results suggest that NtERF3 levels were low in plant cells and that the NtERF3 (83/190) region may determine protein instability.

**Proteasomal degradation of class II ERF repressors**

To characterize the mechanism underlying the proteolytic degradation of NtERF3, we treated Pro35S:NLS-GFP-NtERF3 Arabidopsis plants with MG132, a proteasome inhibitor. Fluorescence microscopy and immunoblot analyses showed that MG132 treatment enhanced the accumulation of NLS-GFP-NtERF3 (Fig. 3A).

We further investigated the role of proteasomes in the stability of the NtERF3 homologues AtERF4 and AtERF8 (Ohta et al., 2001). Hemaglutinin (HA)-tagged AtERF4 and AtERF8 under the control of the 35S promoter (Pro35S:AtERF4-HA and Pro35S:AtERF8-HA) were separately introduced into Arabidopsis plants (Supplemental Fig. S2B). These transgenic plants were grown for 3 weeks and treated with MG132. Immunoblot analysis showed that MG132 treatment increased AtERF4-HA and AtERF8-HA levels, whereas dimethyl sulfoxide (DMSO) treatment had no effect (Figs. 3B and 3C). Furthermore, an upper-shifted band corresponding to AtERF4-HA suggested post-translational modification of AtERF4 (Fig. 3C). These results provided
evidence of the involvement of proteasome in the control of NtERF3, AtERF4, and AtERF8 stabilities.

Interestingly, AtERF4-HA and AtERF8-HA accumulated in aging plants in the absence of MG132 treatment (Fig. 3D). However, unlike MG132-treated plants, no shift of AtERF4-HA was detected in older plants. These results indicated that aging also stabilized AtERF4 and AtERF8.

**Cell death and precocious leaf senescence induced by class II ERFs.**

Our study of the stability of ERF indicated that ectopic expression of class II ERF repressor genes induced a phenotype characterized by cell death and precocious leaf senescence (Fig 4). We therefore attempted to characterize the functions of class II ERF repressors in Arabidopsis plants. Two Pro35S:NLS-GFP-NtERF3 (Δ83/190) lines of Arabidopsis plants established through several independent transformation trials were fertile but severely deformed (Fig. 4A and 4B), indicating the lethality of high NtERF3 accumulation. Transformation of Pro35S:NLS-GFP-NtERF3, Pro35S:AtERF4-HA, and Pro35S:AtERF8-HA genes into Arabidopsis plants induced significant but relatively moderate phenotypes associated with cell death. A significant proportion of Pro35S:AtERF4-HA (15 of 40 lines) and Pro35S:AtERF8-HA (13 of 46 lines) Arabidopsis plants exhibited moderate phenotypes with lesions in both cotyledons and leaves, and they often died before flowering. Certain Pro35S:NLS-GFP-NtERF3 (2 lines), Pro35S:AtERF4-HA (11 lines), and Pro35S:AtERF8-HA (8 lines) Arabidopsis plants exhibited mild phenotypes with smaller leaves in the seedling stage and precocious senescence in rosette leaves (Figs. 4C to 4F).

The Pro35S:NLS-GFP-NtERF3 (Δ83/190), Pro35S:AtERF4-HA, and Pro35S:AtERF8-HA leaves with the moderate phenotypes showed strong signals of trypan blue pigment, a dead cell marker (Fig. 4G). These plants also displayed a brown precipitate owing to the reaction of hydrogen peroxide and diaminobenzidine (DAB; Fig. 4H). These results suggested that ectopic expression of NtERF3, AtERF4, and AtERF8 caused cell death and precocious leaf senescence associated with hydrogen peroxide production.
Regulation of genes involved in leaf senescence by AtERF4.

Because ectopic expression of the class II ERF genes examined had a similar effect on inducing cell death and precocious leaf senescence, these ERFs might regulate expression of a common set of downstream genes. Previous studies revealed the induction of AtERF4 expression by ethylene, ABA, and JA, which regulate the progression of leaf senescence (Fujimoto et al. 2000; McGrath et al., 2005; Yang et al., 2005), and then AtERF4 was used as a model for further characterization of downstream genes. Microarray analysis detected 929 genes transcriptionally increased more than 2-fold ($P$ value of dependent t-test < 0.05; false discovery rate [FDR] < 0.04225; see Materials and Methods) and 687 genes decreased less than half ($P$ < 0.05) in 2-week-old Pro35S:AtERF4-HA plants compared with Pro35S:NLS-GFP-HA plants, which showed normal morphology. Comparative analysis of public databases revealed that a considerable number of the genes upregulated in Pro35S:AtERF4-HA plants were also upregulated in the older leaves of the wild type (Fig. 5). The heat map included 842 genes recorded in the data sets (Breeze et al., 2011) of the 929 genes upregulated in Pro35S:AtERF4-HA plants and showed that their transcripts increased according to the age of plants (see Fig. 5). In addition, reverse transcription polymerase chain reaction (RT-PCR) analysis revealed the upregulation of senescence-associated genes (SAGs) SAG12 and SAG13 in Pro35S:AtERF4-HA plants (Supplemental Fig. S3). Whereas the phenotype of Pro35S:AtERF4-HA plants could be the result of senescence, hypersensitive reaction-like cell death, or other necrosis-related events (see Fig. 4), our results suggested that ectopic expression of AtERF4 induced the expression of genes associated with leaf senescence.

We searched for TF binding sites in the 1,000-bp region upstream of the transcription initiation site of the genes upregulated in Pro35S:AtERF4-HA plants and found that the group of genes containing a W-box, which is a binding motif of WRKY TFs, was overrepresented (Supplemental Table S1). Consistently, 17 of 71 WRKY genes were significantly upregulated in Pro35S:AtERF4-HA plants (Supplemental Tables S2 and S3). Among them, WRKY30, WRKY53, and WRKY75 were positive regulators of leaf senescence, whereas WRKY18, WRKY40, and WRKY60 were involved in the basal defense response against pathogen attack (Xu et al., 2006; Miao and Zentgraf, 2007; Besseau et al., 2012; Li et al., 2012). In our RT-PCR analysis, WRKY18,
WRKY30, WRKY40, WRKY53, WRKY60, and WRKY75 transcripts were increased in Pro35S:AtERF4-HA and Pro35S:AtERF8-HA plants (Fig. 6), indicating that AtERF4 and AtERF8 regulated the expression of these genes.

Conversely, Pro35S:AtERF4-HA plants showed reduced expression of the genes downregulated in aging plants. Our comparative analysis revealed that AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) genes, which are negative regulators of auxin responses and downregulated in senescent leaves (van der Graaf et al., 2006), were overrepresented in the genes downregulated in Pro35S:AtERF4-HA plants (Supplemental Tables S2 and S4). Moreover, EPITHIOSPECIFIER PROTEIN/EPITHIOSPECIFYING SENESCENCE REGULATOR (ESP/ESR), a negative regulator of leaf senescence via the antagonistic modulation of WRKY53 activity at the transcriptional and post-translational levels (Miao and Zentgraf, 2007), was downregulated in Pro35S:AtERF4-HA plants. RT-PCR analysis showed that transcripts for IAA3/SHORT HYPOCOTYL2 (SHY2), IAA6/SHY1, IAA29, and ESP/ESR were reduced in Pro35S:AtERF4-HA and Pro35S:AtERF8-HA plants (see Fig. 6). The gene expression profiles of these plants were consistent with the precocious leaf senescence phenotype.

**Delayed leaf senescence in an aterf4 aterf8 double mutant**

To clarify the physiological role of class II ERFs in plants, we established aterf4 and aterf8 single and aterf4 aterf8 double mutants (Figs. 7A and 7B). Whereas the single mutants had apparently normal leaves, the aterf4 aterf8 double mutant had irregular, downward curled leaves and delayed leaf yellowing (Figs. 7C and 7D, and Supplemental Fig. S4). The age-dependent reduction of chlorophyll content and activation of senescence marker gene SAG12 in wild-type leaves was delayed in the leaves of the aterf4 aterf8 mutant (Figs. 7E and 7F). A dark-induced senescence assay demonstrated that mature leaves of the wild type incubated in the dark displayed yellowing (Oh et al., 1997; Weaver and Amasino, 2001), whereas those of the aterf4 aterf8 double mutants showed delayed yellowing (Fig. 7G). DAB staining demonstrated that hydrogen peroxide accumulated significantly in dark-induced senescing leaves of wild-type plants but to a lesser degree in the aterf4 aterf8 mutant (Fig. 7H). The dark-induced decline of chlorophyll content was also delayed in aterf4
aterf8 mutant leaves, and activation of SAG12 expression was inhibited (Figs. 7I and 7J). These results indicated that aterf4 aterf8 delayed leaf senescence.

In the leaves of the aterf4 aterf8 mutant, the expression of genes involved in the positive regulation of leaf senescence was downregulated, whereas that of the negative regulator genes was increased (Fig. 8). WRKY30, WRKY53, and WRKY75 transcripts were low in the mature leaves of the wild type and subsequently increased in older leaves but were maintained at low levels in mature and old leaves of aterf4 aterf8 mutants. Conversely, IAA3/SHY2, IAA6/SHY6, and ESP/ESR transcripts in mature leaves of the aterf4 aterf8 mutant were increased compared with those of the wild type, whereas these transcripts were gradually reduced in older leaves (see Fig. 8 and Supplemental Fig. S5). Taken together, these results suggest that the delayed leaf senescence phenotype of the aterf4 aterf8 mutant was responsible for changes in the expression profile.

Delayed leaf senescence in aterf4 aterf8 plants was unlikely to have resulted from general growth retardation because flowering time in this mutant was comparable to that of wild-type plants (Supplemental Fig. S6). Furthermore, no defects in the general components of the ethylene and JA response pathways in the aterf4 aterf8 mutant was detected—it progressed at a pace similar to that of wild type in the presence of 1-aminocyclopropanecarboxylic acid, a precursor of ethylene, and methyl jasmonate (Supplemental Fig. S7).

**Binding of AtERF4 and AtERF8 to ESP/ESR**

Because AtERF4 and AtERF8 function primarily as transcriptional repressors (Ohta et al., 2001), we presumed that their target genes might be downregulated in Pro35S:AtERF4-HA and Pro35S:AtERF8-HA Arabidopsis plants and upregulated in aterf4 aterf8 mutants. As described above, the pattern of ESP/ESR expression suggested that AtERF4 and AtERF8 targeted ESP/ESR. A chromatin immunoprecipitation (ChIP) assay of Pro35S:AtERF4-HA and Pro35S:AtERF4-HA Arabidopsis plants using anti-HA antibody detected the enrichment of the promoter and intron sequences of ESP/ESR that contain CCGnC motifs, the target sequence of AtERF4 (Fig. 9; Yang et al., 2009). By contrast, the ChIP assay detected no enrichment of the ESP/ESR coding sequence or the UBIQUITIN10 (UBQ10) sequence (see Fig 9).
In addition, the ChIP assay using Pro35S:NLS-GFP-HA Arabidopsis plants did not detect enrichment of the ESP/ESR sequences (see Fig. 9). These results suggested specific binding of AtERF4 and AtERF8 to ESP/ESR in plant cells.

**Induction of AtERF4 and AtERF8 expression during leaf senescence**

We investigated the transcriptional regulation of AtERF4 and AtERF8 in leaf senescence. Because the TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) genes are reportedly involved in the progression of leaf senescence (Schommer et al., 2008), we confirmed that compared with the wild type, a tcp3 tcp4 tcp5 tcp10 tcp13 (quintuple tcp) mutant displayed delayed yellowing of leaves, decline in chlorophyll content, and SAG12 activation (Koyama et al., 2010; Figs. 10A, 10B, and 10C), and we used the quintuple tcp mutant as a control for plants with delayed senescence. Our RT-PCR analysis detected basal levels of AtERF4 and AtERF8 transcripts in leaves of 3-old-week wild-type plants (Figs. 7B, 10C; Yang et al. 2005). The increase in AtERF4 and AtERF8 transcripts began 5 weeks after germination and preceded leaf yellowing, but this increase was delayed in the quintuple tcp mutant (see Fig. 10C). These results suggested that AtERF4 and AtERF8 expression was induced during leaf senescence.

**DISCUSSION**

**Class II ERF regulatory cascade for the progression of leaf senescence**

The involvement of class II ERFs in the responses to pathogens, salt, ethylene, JA, and ABA has been reported previously (McGrath et al., 2005; Song et al., 2005; Yang et al., 2005; Nasir et al., 2005; Li et al., 2011). In particular, NbCD1, a Nicotiana benthamiana class II ERF, positively regulates cell death in the defense response of tobacco and Arabidopsis (Nasir et al., 2005). Herein, we expand these findings on the proteasome-mediated control of class II ERFs and describe a novel gene regulatory cascade involving AtERF4 and AtERF8 that progresses leaf senescence.

Figure 11A presents our working model of a regulatory cascade involving the class II ERFs that progresses leaf senescence. Our gene expression and ChIP analyses suggest ESP/ESR as a direct target of AtERF4 and AtERF8. The ectopic expression of AtERF4 and AtERF8 transcriptionally repressed ESP/ESR, whereas loss of function of these ERF genes derepressed its expression. The inhibitory effect of ESP/ESR on the
progression of leaf senescence depends largely on the activity of WRKY53, a positive regulator of leaf senescence (Miao and Zentgraf, 2007). ESP/ESR inhibits the activity of WRKY53 through physical interaction that may prevent WRKY53 DNA-binding activity, and then retards leaf senescence. In addition, ESP/ESR transcriptionally suppresses WRKY53 expression. Because the primary biochemical function of AtERF4 and AtERF8 is the repression of transcription, the repression of ESP/ESR by AtERF4 and AtERF8 may activate WRKY53 and, as a consequent, initiate the progression of leaf senescence. The JA-inducible and ecotype-dependent expression of ESP/ESR has been reported (Lambrix et al., 2001; Miao et al., 2007), but the molecular mechanisms of the regulation are unclear. Our findings of the gradual reduction of ESP/ESP expression during aging (Supplemental Fig. S5) and its regulation by class II ERFs provide novel insights into the regulation of ESP/ESP.

The ectopic expression of AtERF4 and AtERF8 induced expression of WRKY30, WRKY53, and WRKY75 genes, which positively regulated leaf senescence, whereas loss of function of these ERF genes inhibited their expression. By contrast, AtERF4 did not change the expression of WRKY6, another regulator of the progression of leaf senescence, in our microarray analysis (Supplemental table S3). These results suggest that the expression of WRKY30, WRKY53, and WRKY75 genes are positively regulated by AtERF4 and AtERF8 but not the general effects of the leaf senescence-associated phenotype. In addition to aging and ESP/ESR, salicylate, hydrogen peroxide, pathogens, a mitogen-activated protein kinase kinase kinase, and histone methylation are involved in the regulation of WRKY53 (Miao and Zentgraf, 2007; Miao et al., 2007; Ay et al., 2009). Interaction between WRKY30 and WRKY53 in yeast two-hybrid system suggests the possible involvement of these WRKYs in the same signaling pathway (Besseau et al. 2012). By contrast, the regulation of WRKY30 and WRKY75 is almost unknown. Our results characterize AtERF4 and AtERF8 as novel regulators of these WRKY genes. Involvement of ESP/ESR and WRKY53 in the regulation of expression of WRKY30 and WRKY75 remains unclear.

Moreover, AtERF4 and AtERF8 might regulate these WRKY genes directly and indirectly in uncharacterized pathways. In addition to ESP/ESR, AtERF4 and AtERF8 negatively regulate the expression of many genes, including AUX/IAA genes. Several studies have reported on both the positive and the negative roles of auxin in the
progression of leaf senescence (Ellis et al., 2005; van der Graaff et al., 2006; Hou et al., 2013). Because IAA3/SHY2 suppresses expression of auxin-inducible genes through physical interactions with AUXIN RESPONSE FACTORs (ARFs) and several ARFs act as positive regulators of leaf senescence (Ellis et al., 2005; Weijers et al., 2005), the negative effects on IAA3/SHY2 by AtERF4 and AtERF8 may be involved in the progression of leaf senescence. The repression of AUX/IAA transcriptions by AtERF4 and AtERF8 may be responsible for a specific, rather than a general, role of auxin in the progression of leaf senescence.

Because plants stimulate leaf senescence prematurely under unfavorable conditions (Buchanan-Wollaston et al., 2003; Lim et al., 2007), stress responsive genes downstream of AtERF4 and AtERF8 likely contribute to signaling pathway integration for the regulation of leaf senescence. ESP/ESR, the target of AtERF4 and AtERF8, has important roles in defense against herbivores and pathogens (Lambrix et al., 2001; Miao et al., 2007). WRKY genes are involved in leaf senescence and various stress responses (Rushton et al., 2010). WRKY53 and WRKY75 are involved in responses to biotic stress and nutrient starvation, respectively (Devaiah et al., 2007; Miao and Zentgraf, 2007). WRKY18, WRKY40, and WRKY60 enhance the basal defense system against pathogens (Xu et al., 2006). In addition, our microarray analysis suggested that AtERF4 upregulates ORE1, ERF1, JASMONATE ZIM-DOMAIN1 (JAZ1), ZINC FINGER OF ARABIDOPSIS THALIANA7 (ZAT7), ZAT10, ZAT12, MYB2, and MYB44, which regulate responses to ethylene, JA, ABA, hydrogen peroxide, and dehydration (Solano et al., 1998; Sakamoto et al., 2004; Davletova et al., 2005; Chini et al., 2007; Ciftci-Yilmaz et al., 2007; Thines et al., 2007; Jung et al., 2008; Kim et al., 2009; Guo and Gan, 2011). Because these stresses affect the progression of leaf senescence, we postulate that these class II ERFs may integrate aging and stress signaling pathways. Whereas the similar phenotype of Pro35S:NLS-GFP-NtERF3 Arabidopsis plants to those of Pro35S:AtERF4-HA and Pro35S:AtERF8-HA Arabidopsis plants suggests the involvement of NtERF3 in the progression of cell death and leaf senescence, the downstream genes of NtERF3 in tobacco plants remain to be clarified.

Proteolytic control of class II ERFs by proteasomes
We demonstrate that aging stimulates expression of *AtERF4* and *AtERF8* in leaves and increases ERF accumulation. In addition to elucidating the stress response-associated transcriptional control of class II ERFs (Suzuki et al., 1998; Yamamoto et al., 1999; Fujimoto et al., 2000; Kitajima et al., 2000; Nishiuchi et al., 2003), our study adds some insights to the developmental regulation of these ERFs. The induction of *AtERF4* and *AtERF8* expression through aging is consistent with data from several transcriptome analyses (Breeze et al., 2011), suggesting robust developmental control of *AtERF4* and *AtERF8* during leaf senescence. The delayed induction of *AtERF4* and *AtERF8* expression in quintuple *tcp* mutant leaves suggests the involvement of the *TCP* genes in the regulation of class II ERFs, although the detailed mechanisms of this induction remain unclear. The progression of leaf senescence requires the coordination of many signals, and the regulation of class II ERFs during senescence is considered to be under the control of a complex process.

Because the class II ERFs act as positive regulators of leaf senescence, our results suggest that both transcriptional activation and post-translational stabilization of *AtERF4* and *AtERF8* are important for the progression of senescence. The fine-tuning of their accumulation may set the pace of the progression of leaf senescence. In younger plants, proteolytic regulation maintains low levels of class II ERFs (see Fig. 3). The severe cell death observed in the transgenic *Arabidopsis* plants with constitutive accumulation of NtERF3 (Δ83/190) demonstrates that the enhanced and continuous accumulation of class II ERFs causes premature death. *AtERF4* and *AtERF8* transcripts are maintained at basal levels in younger leaves (Figs. 7B, 10C; Yang et al. 2005), but rapidly activated to cope with various environmental stimuli (McGrath et al., 2005; Nasir et al., 2005; Song et al., 2005; Yang et al., 2005). Therefore, proteolytic regulation may prevent their excess and prolonged accumulation to protect cells against premature death.

Whereas selective protein turnover is regulated by proteasomes during leaf senescence, bulk protein degradation is processed by autophagy (Vierstra, 2009). Prior studies have described the role of several components of the ubiquitin-proteasome system (Woo et al., 2001; Gepstein et al., 2004; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Peng et al., 2007, Raab et al., 2009; Miao and Zentgraf, 2010; Breeze et al., 2011). In particular, WRKY53 is
degraded by a HECT (homologous to the E6-AP carboxyl terminus) domain ubiquitin ligase (Miao and Zentgraf, 2010). Our present study suggests that proteolytic control of class II ERFs by proteasomes occurs and these factors are involved in the progression of leaf senescence. In addition to the characterization of NtUBC2 as an interaction partner of NtERF3 (Koyama et al., 2003), investigations for identification of components that degrade class II ERFs remain to be performed. These class II ERFs interact with the co-repressor TOPLESS to negatively regulate target gene transcription (Causier et al., 2012). The post-translational control of class II ERFs by proteasomes suggests that a sophisticated mechanism may underlie the progression of leaf senescence.

In summary, we showed the proteasomal regulation, age-dependent accumulation, and regulatory cascade of class II ERFs during leaf development. Our results provide cues of a sophisticated mechanism for the progression of leaf senescence involving class II ERFs.

MATERIALS AND METHODS

Plant materials and growth conditions

Growth conditions and the transformation of tobacco XD6S cells and Arabidopsis plants have been described previously (Yamamoto et al., 1999; Koyama et al., 2010). Arabidopsis thaliana ecotype Columbia-0 was used throughout this study unless otherwise indicated. erf4-1 (SALK_073394; Alonso et al., 2003; McGrath et al., 2005) and erf8-1 (FLAG157D10; Wassilewskija; Samson et al., 2002) were crossed 3 times to Columbia-0 plants before use. The tcp quintuple mutant (tcp3 tcp4 tcp5 tcp10 tcp13) has been described previously (Koyama et al., 2010).

Plasmids

For recombinant protein production in E. coli, the CDS of NtERF2, NtERF3, GFP, and various regions of NtERF3 were cloned into pET32 (Novagen). For the expression of GFP fusion genes in plant cells, the NLS of the SV-40 T-antigen from pGAD424 (CLONTECK) was inserted upstream of GFP, and the entire or partial coding sequences of NtERF3 were cloned downstream of GFP. The resulting fusion GFP genes were individually inserted into pBI101 for the transformation of tobacco XD6S
cells and *Arabidopsis* plants. For the overexpression of *AtERF4* and *AtERF8*, the CDS without the stop codon of *AtERF4* and *AtERF8* were respectively ligated into p35SHAG, in which the **SRDX** sequence of p35SSRDXG was replaced by the **HA** sequence and transferred into pBCKH (Mitsuda et al., 2006). The primers used for plasmid construction are listed in Supplemental Table S5.

**In vitro degradation assay**

The 6xHis-tagged NtERFs and GFP were produced in *E. coli*, purified using a nickel-chelating resin according to manufacturer instructions (Amersham Pharmacia), and dialyzed against a solution containing 20 mM Tris (pH 8.0) and 100 mM NaCl. The cell-free degradation assay was modified from previous reports as described below (Osterlund et al., 2000). For preparation of the extract solution, cultured tobacco XD6S cells were ground in liquid nitrogen, suspended in a buffer containing 50 mM Tris (pH 7.5), 10 mM NaCl, 10 mM MgCl₂, 5 mM dithiothreitol, 2 mM ATP, and COMPLETE ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Promega) and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was diluted to 4 mg/mL to make the cell extract solution. Aliquots containing 100 ng recombinant NtERFs and GFP were added into 200 µL cell extract solution, incubated at room temperature for 0, 15, 30, or 60 minutes, mixed in an equal volume of 2X sodium dodecyl sulfate (SDS) loading buffer (Tris pH 6.8, 2% SDS, 1 mM EDTA, 2-mercaptoethanol), and boiled to terminate the reaction. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto Immobilon-P membranes (MILLIPORE), and subjected to immunoblot analysis.

**Preparation of tobacco and *Arabidopsis* proteins**

For preparation of plant protein samples, tobacco XD6S callus lines at 2 weeks after the inoculation on new plates and *Arabidopsis* seedlings grown on Murashige-Skoog plates were ground in liquid nitrogen, suspended in extraction buffer (Tris pH 6.8, 8M urea, 0.5% SDS, 1 mM EDTA, 2-mercaptoethanol), incubated for 2 minutes at 95°C, and mixed with one-third volume of SDS/urea loading buffer (Tris pH 6.8, 8M urea, 2% SDS, 1 mM EDTA, 2-mercaptoethanol). Aliquots containing 10 µg and 30 µg of protein from tobacco XD6S cells and *Arabidopsis* plants, respectively,
were separated using SDS-PAGE, blotted onto Immobilon-P membranes (MILLIPORE), and subjected to immunoblot analysis.

Antibodies and immunoblot analysis

For production of anti-NtERF2 and anti-NtERF3 antibodies, the recombinant NtERF2 and NtERF3 proteins fused with maltose-binding protein were produced in *E. coli*, purified using an amylose resin (New England Laboratory) according to manufacturer instructions, and injected into rabbits. For immunoblot analysis, membranes were incubated with the appropriate primary and secondary antibodies. The antibodies used were as follows: anti-NtERF2 and NtERF3 antibodies (1:2000), anti-GFP antibody (1:5000, Medical and Biological Laboratory), peroxidase-conjugated anti-HA antibody (1:500, Roche), and peroxidase-conjugated anti-rabbit immunoglobulin G (1:10000, Medical and Biological Laboratory). Immunoreactive proteins were detected using the ECL plus western blotting kit (GE Healthcare).

Microscopy

The fluorescence images in Figs. 2A and 2C were, respectively, monitored with a BHS-RFC (Olympus) as described previously (Ohta et al., 2000) and a BZ-9000 (KEYENCE) with a GFP-Bandpass filter at a fixed one-third-second exposure. Bright field images were obtained with an MZ FL III (Leica).

Gene expression analysis

Total RNA was prepared from tobacco XD6S cells and *Arabidopsis* plants using Trizol reagent, FastRNA GREEN (Qbiogene), or RNA easy Plant Mini (Qiagen) kits. RNA blot analysis was performed as described previously (Koyama et al., 2003). For reverse transcription, templates were reverse transcribed from total RNA using SuperScript II first-strand complementary DNA synthesis (Invitrogen). Real-time PCR was performed using first-strand complementary DNA, a pair of primers (Supplemental Table S5), and iQTM CYBR Green PCR Supermix (Bio-Rad) with a CFX96 real-time PCR system (Bio-Rad). Transcript levels were detected in triplicate using a standard curve derived from the reference sample. The relative values of the transcripts were normalized to the *UBQ1* level.
Microarray analysis was performed with the two-color method using aliquots of total RNA from 4 biological replicates of 2-week-old Pro35S:AtERF4-HA (line #20) and Pro35S:NLS-GFP-HA Arabidopsis plants with an Agilent Arabidopsis V3 (4x44k) microarray. We used previously described preparation and statistical analyses (Koyama et al., 2010) with the following exceptions. Only genes with average detection values of \( \geq 1.5 \) in both “test” and “reference” samples were analyzed. The P value for each gene was calculated using a dependent t-test. To estimate the FDR, we calculated the Q value from the P value using QVALUE software with the default settings (Storey and Tibshirani, 2003) and selected up-/downregulated (>2-fold/<0.5-fold) genes with a P value of \( \leq 0.05 \) (FDR < 0.04225). In Fig. 5, the transcript level of each gene at different ages was calculated as relative to that at 19 days after sowing (Breeze et al., 2011), and a clustered heat map was prepared using Cluster3 software (Eisen et al., 1998). To evaluate over-representation of some gene lists among up-/downregulated genes, we performed a binomial test using R (http://www.r-project.org/).

ChIP

Nuclear extract was prepared from 3-week-old Arabidopsis plants, subjected to sonication, and immunoprecipitated with 500 ng anti-HA antibody (clone 3F10; Roche) as described previously (Koyama et al., 2010). The chromatin precipitated was reverse-cross-linked and purified with ethanol and served as a template for PCR (CFX96 real-time PCR system; BioRad) using an appropriate set of primers (Supplemental Table S5). The values were calculated with a standard curve generated from the input sample and normalized using a background value determined from the eIF4A sequence. Figure 10 shows a representative from 3 biological replicates with similar trends.

Chemical treatment of plants

For MG132 treatment, Arabidopsis plants were immersed in a solution containing 50 mM MES (pH 5.7), 0.05% Tween-20, and 50 µM MG132 (CALBIOCHEM) for 6 h in the light. For Trypan blue staining, Arabidopsis plants were immersed in Trypan blue solution (25% lactic acid, 23% phenol, 2.5 mg/mL Trypan blue [SIGMA]) for 1 h and rinsed several times in chloral hydrate solution (25 g chloral hydrate in 10 mL...
For DAB staining, *Arabidopsis* plants were incubated in DAB buffer (50 mM Tris-acetate [pH 5.0], 0.05% Tween-20, 1 mg/mL DAB [SERVA]) in the dark for 24 h, cleared in boiling ethanol, and rendered transparent in chloral hydrate solution.

**Dark-induced senescence assay**

The sixth leaves were detached from 5-week-old *Arabidopsis* plants, floated on water, and incubated in the dark. For chlorophyll extraction, the leaves were soaked in 80% (v/v) acetone. The chlorophyll content was determined via spectrophotometric analysis (Lichtenthaler and Buschmann, 2001) and normalized to the fresh weight of the leaves.

**Accession numbers**

The sequences appearing in this article can be found in the GenBank or Arabidopsis Genome Initiative databases under the following accession numbers: NtERF3 (D38124), AtERF4 (AT3G15210), AtERF8 (AT1G53170), WRKY18 (AT4G31800), WRKY30 (AT5G24110), WRKY40 (AT1G80840), WRKY53 (AT4G23810), WRKY60 (AT2G25000), WRKY75 (AT5G13080), ESP/ESR (AT1G54040), IAA3/SHY2 (AT1G04240), IAA6/SHY1 (AT1G52830), IAA29 (AT4G32280), SAG12 (AT5G45890), SAG13 (AT2G29350), UBQ1 (AT3G52590), UBQ10 (AT4G05320), eIF4A (AT3G13920). Microarray data in this study can be found in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE41053.

**SUPPLEMENTAL DATA**

**Figure S1.** Production and purification of NtERF proteins in *E. coli.*

**Figure S2.** Expression of *NtERF3, AtERF4, AtERF8,* and *GFP* genes in tobacco XD6S callus lines and *Arabidopsis* plants.

**Figure S3.** Expression of SAG genes in *Pro35S:AtERF4-HA Arabidopsis* plants.

**Figure S4.** Leaf senescence of *aterf4* and *aterf8* single mutants.

**Figure S5.** Expression of ESP/ESR during leaf senescence in wild-type leaves.

**Figure S6.** Timing of flowering in wild-type and *aterf4 aterf8* double mutant plants.
Figure S7. An ethylene precursor and methyl jasmonate stimulated dark-induced senescence of *aterf4 ateref8* double mutant leaves.

Table S1. *cis* elements enriched in the genes regulated in *35S:AtERF4-HA* plants.

Table S2. Families containing the genes up- and downregulated in *35S:AtERF4-HA* plants.

Table S3. Expression of *WRKY* genes in *35S:AtERF4-HA* plants.

Table S4. Expression of *AUX/IAA* genes in *35S:AtERF4-HA* plants

Table S5. Primers used in this study.

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FIGURE LEGENDS

Figure 1. Rapid degradation of *Nicotiana tabacum* ETHYLENE RESPONSE FACTOR3 (NtERF3) in vitro.

(A) to (D) Levels of recombinant NtERF3 (A and D), NtERF2 (B), and green fluorescence protein (GFP) (C) incubated in the cell extract (A to C) or bovine serum albumin (D) solutions for the indicated times. Recombinant NtERF3, NtERF2, and GFP were detected via immunoblotting using anti-NtERF3 (A and D), anti-NtERF2 (B), and anti-GFP (C) antibodies, respectively.

(E) Mapping the domain responsible for NtERF3 instability in vitro. The upper panel shows a schematic representation of NtERF3. Black and grey boxes indicate the DNA binding domain (DBD) and EAR-repression domain (RD), respectively. Numbers show the position of amino acid residues from the first methionine. The lower panels show the levels of the respective regions of NtERF3 at the time points indicated in the cell extract solution. These regions of NtERF3 were detected via immunoblotting using an anti-NtERF3 antibody. The molecular size of each protein is provided in Supplemental Fig. S1.

Figure 2. Low accumulation of NtERF3 in plant cells.

(A) GFP florescence in the nucleus of transgenic tobacco cells in which the individual fused gene was introduced (left panels). 4′,6-Diamidino-2-phenylindole staining (right panels) shows the location of the nucleus.

(B) Immunoblot analysis of transgenic tobacco cells after introduction of the fusion genes. Two independent lines per construct were subjected to immunoblot analysis using an anti-GFP antibody. The molecular size markers are shown on the left.

(C) GFP florescence of *Pro35S:NLS-GFP-NtERF3* and *Pro35S:NLS-GFP-NtERF3 (Δ83/190)* roots of 3-day-old *Arabidopsis* seedlings. Bars = 10 µm in (A) and 50 µm in (C). NLS, nuclear localization signal.

Figure 3. NtERF3, AtERF4, and AtERF8 levels in *Arabidopsis* plants increased by MG132 and aging.

(A) GFP florescence of the roots of *Pro35S:NLS-GFP-NtERF3* *Arabidopsis* seedlings treated with dimethyl sulfoxide (DMSO) and MG132. Bars = 50 µm.
(B) and (C) Accumulation of NLS-GFP-NtERF3 (B), AtERF4-HA, and AtERF8-HA (C) induced by MG132 treatment. Two independent lines of 3-week-old Pro35S:NLS-GFP-NtERF3 (35S:NtERF3), Pro35S:AtERF4-HA, and Pro35S:AtERF8-HA Arabidopsis plants treated with DMSO (-) and MG132 (+) were subjected to immunoblot analysis using anti-GFP (B) and anti-HA antibodies (C). In (B), the black and white triangles indicate NLS-GFP-NtERF3 and NLS-GFP signals, respectively. Signals due to the nonspecific cross-reactivity with an unknown protein (B; asterisk) and Coomassie brilliant blue (CBB) staining (C) serve as loading controls. Molecular size markers are shown on the right.

(D) Accumulation of AtERF4-HA and AtERF8-HA induced by plant aging. Pro35S:AtERF4-HA and Pro35S:AtERF8-HA Arabidopsis plants were grown on plates for 2, 3, and 4 weeks and subjected to immunoblot using anti-HA antibody. WT, wild type.

**Figure 4.** Ectopic expression of NtERF3, AtERF4, and AtERF8 genes induced cell death and precocious leaf senescence.

(A) and (B) Wild-type (A) and Pro35S:NLS-GFP-NtERF3 (∆83/190) (B) Arabidopsis plants grown on Murashige-Skoog plates for 3 weeks.

(C) Wild-type (left) and Pro35S:NLS-GFP-NtERF3 (right) Arabidopsis plants grown on soil for 5 weeks. Inflorescences were detached for a detailed view.

(D) to (F) Wild-type (D), Pro35S:AtERF4-HA (E), and Pro35S:AtERF8-HA (F) Arabidopsis plants grown for 4 weeks.

(G) and (H) Trypan blue (G) and diaminobenzidine (DAB) (H) staining of wild-type, Pro35S:NLS-GFP-NtERF3 (∆83/190), Pro35S:AtERF4-HA, and Pro35S:AtERF8-HA leaves of 3-week-old Arabidopsis plants (from left to right).

Bars = 1 cm in (A) to (F) and 0.5 mm in (G) and (H).

**Figure 5.** Expression of the genes regulated by AtERF4 in response to plant aging.

Genes upregulated by AtERF4 are aligned along the vertical axis, and the ages of wild-type (WT) Arabidopsis plants are given along the horizontal axis. The bar represents the transcript level of each gene and is colored according to the extent of fold change in the data set (Breeze et al., 2011).
Figure 6. Expression of the genes downstream of the class II ERFs in 35S:AtERF4-HA and 35S:AtERF8-HA Arabidopsis plants.

Transcript levels were determined with RT-PCR of aliquots of total RNAs from wild type, Pro35S:NLS-GFP-HA (vector), and 4 independent lines of Pro35S:AtERF4-HA and Pro35S:AtERF8-HA Arabidopsis plants. The WT values were set at 1. Error bars indicate standard deviation. IAA/SHY, INDOLE-3-ACETIC ACID/SHORT HYPOCHOTYL; ESP/ESR EPITHIOSPECIFIER PROTEIN/EPITHIOSPECIFYING SENESCENCE REGULATOR.

Figure 7. Delayed leaf senescence in an aterf4 aterf8 double mutant.

(A) Schematic diagrams of the AtERF4 and AtERF8 structures and the T-DNA tag insertions. The white and gray boxes represent coding and untranslated regions, respectively. The triangles show the T-DNA insertion site. Numbers indicate positions of the T-DNA tag insertion relative to the translational initiation site.

(B) AtERF4 and AtERF8 transcripts in aterf4 and aterf8 mutants and aterf4 aterf8 double mutants. The coding sequences of AtERF4 and AtERF8 were amplified by RT-PCR of aliquots of total RNAs from 3-week-old wild type (WT) and erf mutants. An extraordinarily low level of AtERF8 transcript was detected in aterf8 mutants and aterf4 aterf8 double mutants.

(C) Rosettes of 6-week-old WT (left) and aterf4 aterf8 (right) plants. Triangles indicate senescing leaves. Inflorescences were detached for detailed view.

(D) The sixth leaf of WT (upper) and aterf4 aterf8 (lower) plants of different ages.

(E) Chlorophyll content of the sixth leaf of WT and aterf4 aterf8 plants at indicated ages. The error bars and asterisks indicate standard deviation (n = 12) and significant differences at P < 0.001 by Student’s t-test.

(F) SENESCENCE-ASSOCIATED GENE12 (SAG12) transcript was determined by RT-PCR of aliquots of total RNAs from the sixth leaves of WT (black squares) and aterf4 aterf8 (white circles) plants at the indicated ages. The values of 3-week-old WT leaves were set at 1. Error bars indicate standard deviation of technical triplicates.

(G) Dark-induced senescence in WT and aterf4 aterf8 leaves. The detached leaves were photographed on days 0 (left) and 4 (right) after incubation in the dark.
(H) DAB staining of the detached leaves at 0 (left) and 2 (right) days after incubation in the dark.

(I) Chlorophyll content in WT and aterf4 aterf8 leaves at indicated days after incubation in the dark. The error bars and asterisks indicate standard deviation (n = 7) and significant differences at P < 0.001 by Student’s t-test.

(J) The SAG12 transcript was determined with RT-PCR using aliquots of total RNAs from WT (black squares) and aterf4 aterf8 (white circles) leaves incubated for the indicated days in dark. The values of WT leaves at day 0 were set at 1. Error bars indicate standard deviation of technical triplicates.

Bars = 1 cm in (C), (D), (G), and (H).

**Figure 8.** Expression of leaf senescence-related genes during the aging of wild-type and aterf4 aterf8 plants.

Transcript levels were determined with RT-PCR using aliquots of total RNAs from the sixth leaves of wild-type (black squares) and aterf4 aterf8 (white circles) plants at the indicated ages. The values of 4-week-old wild-type leaves were set at 1. Error bars indicate standard deviation of technical triplicates.

**Figure 9.** Binding of AtERF4 and AtERF8 to the genomic region of ESP/ESR.

(A) A diagram of the genomic region of ESP/ESR. Black and grey boxes indicate the coding and untranslated regions, respectively. White circles show the position of the CCGnC motif, a target sequence of AtERF4. Thick bars below the diagram indicate the regions amplified with sets of primers in the ChIP analysis in (B).

(B) Enrichment of the ESP/ESR sequences in the ChIP analysis. Chromatins were prepared from Pro35S:NLS-GFP-HA (vector) and 2 independent lines of Pro35S:AtERF4-HA (AtERF4) and Pro35S:AtERF8-HA (AtERF8) Arabidopsis plants, immunoprecipitated in the absence (-) and presence (+) of antibodies, and subjected to quantitative PCR. The fold recovery was relative to the value processed in the absence of antibodies. Error bars indicate standard deviation of technical triplicates.

**Figure 10.** Expression of AtERF4 and AtERF8 during leaf senescence of wild-type (WT) and quintuple tcp plants.
(A) The delayed yellowing of leaves in quintuple tcp mutant. The sixth leaf of WT (upper) and quintuple tcp (lower) plants of different ages was photographed.

(B) Chlorophyll content in the sixth leaf of WT and quintuple tcp mutant at indicated ages. The error bars and asterisks indicate standard deviation (n = 12) and significant differences at P < 0.001 by Student’s t-test.

(C) Expression of SAG12, AtERF4, and AtERF8 in the WT and the quintuple tcp mutant. Transcript levels were determined with RT-PCR using aliquots of total RNAs from the sixth leaves of WT (black squares) and quintuple tcp mutants (white triangles) at the indicated ages. Each value of 3-week-old leaves was set at 1. Error bars indicate standard deviation of technical triplicates.

**Figure 11.** Model showing the roles of the class II ERFs in the progression of leaf senescence.

Schematic representation of a regulatory cascade involving the class II ERFs for the progression of leaf senescence. Aging activates AtERF4 and AtERF8 at both the transcriptional and the post-translational levels. These ERFs directly repress transcription of ESP/ESR to derepress the activity of WRKY53. WRKY53 and uncharacterized factors downstream of ERFs are involved in the progression of leaf senescence, as indicated by solid and dashed arrows, respectively.

**SUPPLEMENTAL FIGURE LEGENDS**

**Figure S1.** Production and purification of *Nicotiana tabacum* ethylene response factor (NtERF) proteins in *Escherichia coli*.

The proteins indicated were produced as fusion proteins with thioredoxin and a 6xHIS tag in *E. coli* and purified with a nickel affinity column. Total (t), washed (w; for NtERF3, NtERF2, and NtERF3 [1-82]), and eluted (e) proteins were separated with by SDS-PAGE using a PhastSystem High Speed Electrophoresis System (Pharmacia) and stained with Coomassie brilliant blue. Molecular weight markers are shown on the left.

**Figure S2.** Expression of *Nicotiana tabacum* ETHYLENE RESPONSE FACTOR3 (NtERF3), *Arabidopsis thaliana* ERF4 (AtERF4), AtERF8, and GREEN
**FLUORESCENCE PROTEIN (GFP)** genes in tobacco XD6S callus lines and *Arabidopsis* plants.

(A) Aliquots of 10 µg total RNA were separated on an agarose gel, transferred to Hybond N (GE Healthcare) and hybridized with the $^{32}$P-radiolabeled GFP complementary DNA.

(B) Expression of *NtERF3*, *AtERF4*, *AtERF8*, and *GFP* genes in transgenic *Arabidopsis* plants. Reverse transcribed complementary DNA prepared from 2 or 4 independent lines of transgenic *Arabidopsis* plants were used as templates for PCR performed with the primer set described in Supplemental table S5. *ACTIN8* and *TUBULIN (TUB)* served as controls.

**Figure S3.** Expression of senescence-associated genes (*SAG*) genes in *Pro35S:AtERF4-HA* *Arabidopsis* plants.

Transcript levels were determined with RT-PCR using aliquots of total RNAs from *Pro35S:NLS-GFP-HA* (vector) and 2 independent lines of *Pro35S:AtERF4-HA* *Arabidopsis* plants. The wild-type values were set at 1. Error bars indicate standard deviation (n = 3). HA, hemaglutinin.

**Figure S4.** Leaf senescence of *aterf4* and *aterf8* single mutants.

Rosettes of 6-week-old wild-type (WT), *aterf4*, and *aterf8* mutants, and *aterf4 aterf8* double mutant plants. Triangles indicate senescing leaves. Inflorescences were detached for detailed view. *aterf4* and *aterf8* mutants and WT plants exhibited leaf senescence at a similar age.

**Figure S5.** Expression of **EPITHIOSPECIFIER PROTEIN/EPITHIOSPECIFYING SENESCENCE REGULATOR (ESP/ESR)** during leaf senescence in wild-type leaves.

The graph includes the Fig. 8 data of the expression of *ESP/ESR* in more detail.

**Figure S6.** Timing of flowering in wild-type (WT) and *aterf4 aterf8* double mutant plants.
The number of rosette leaves shows the timing of flowering under long day conditions (n = 12). Error bars indicate standard deviation.

**Figure S7.** An ethylene precursor and methyl jasmonate (MeJA) stimulated dark-induced senescence of *aterf4 aterf8* double mutant leaves.

The sixth leaves of wild type (WT) and *aterf4 aterf8* double mutant plants were detached, floated on solution without (control) or with 50 µM 1-aminocyclopropanecarboxylic acid (ACC; A and B), and 30 µM MeJA (C and D) in the dark.

(A) and (C) Leaves photographed on days 0 and 4.

(B) and (D) Chlorophyll content of WT and *aterf4 aterf8* leaves floated on solution with 50 µM ACC (B) and 30 µM MeJA (D). The error bars indicate standard deviation (n = 8).
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Koyama et al. Figure 2

A

NLS-GFP

NtERF3 (1/225)

NtERF3 (1/82)

NtERF3 (83/190)

NtERF3 (191/225)

B

| NtERF3 (1/225) | NtERF3 (83/190) | NtERF3 (1/82) | NtERF3 (191/225) | Vector | NLS-GFP |
|----------------|-----------------|---------------|-----------------|--------|---------|
| #1             | #2              | #1            | #2              | #1     | #2      |

C

NLS-GFP

NtERF3

NtERF3 (Δ83-190) #1

NtERF3 (Δ83-190) #2
Koyama et al. Figure 3

A

B

35S:NtERF3

MG132

DMSO

AB

NLS-GFP

35S:NtERF3

WT+

α-GFP

C

35S:AtERF4HA

WT

#4 #20

MG132

α-HA

CBB

D

35S:AtERF4HA

#4 #20

(week) 2 3 4 2 3 4

α-HA

CBB

35S:AtERF8HA

#2 #8

20 25

α-HA

CBB

234 234
Koyama et al. Figure 4
Koyama et al. Figure 5

WT leaves at different ages

842 genes upregulated in 35S:AtERF4 plants

fold change

- >8
- >4
- >2
- >0.5
- <0.5
- <0.25
- <0.125
Koyama et al. Figure 10

A

WT

tcp

B

WT

tcp

C

SAG12 transcript

AtERF4 transcript

AtERF8 transcript

age (weeks)

chlorophyll content

age (weeks)
Koyama et al. Figure 11

Diagram:

- Aging
- ↓
- Class II ERFs
- ↓
- ESP/ESR
- ↓
- WRKY53
- ↓
- Leaf senescence