ETHYLENE-INSENSITIVE3 Is a Senescence-Associated Gene That Accelerates Age-Dependent Leaf Senescence by Directly Repressing miR164 Transcription in Arabidopsis

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Numerous endogenous and environmental signals regulate the intricate and highly orchestrated process of plant senescence. Ethylene is a well-known inducer of senescence, including fruit ripening and flower and leaf senescence. However, the underlying molecular mechanism of ethylene-induced leaf senescence remains to be elucidated. Here, we examine ETHYLENE-INSENSITIVE3 (EIN3), a key transcription factor in ethylene signaling, and find that EIN3 is a functional senescence-associated gene. Constitutive overexpression or temporary activation of EIN3 is sufficient to accelerate leaf senescence symptoms. Conversely, loss of EIN3 and EIN3-Like1 (its close homolog) function leads to a delay in age-dependent ethylene-, jasmonic acid-, or dark-induced leaf senescence. We further found that EIN3 acts downstream of ORESARA2 (ORE2)/ORE3/EIN2 to repress miR164 transcription and upregulate the transcript levels of ORE1/NAC2, a target gene of miR164. EIN3 directly binds to the promoters of microRNA164 (miR164), and this binding activity progressively increases during leaf ageing. Genetic analysis revealed that overexpression of miR164 or knockout of ORE1/NAC2 represses EIN3-induced early-senescence phenotypes. Collectively, our study defines a continuation of the signaling pathway involving EIN2-EIN3-miR164-NAC2 in regulating leaf senescence and provides a mechanistic insight into how ethylene promotes the progression of leaf senescence in Arabidopsis thaliana.

INTRODUCTION

In plants, senescence occurs at the ultimate stage in leaf development and precedes cell death (Pennell and Lamb, 1997; Lim et al., 2007a). The senescence process is initiated by chloroplast degeneration (Gepstein, 2004), which is followed by the catabolism of macromolecules, such as nucleic acids, proteins, and lipids (Ullker et al., 2007), and degeneration of mitochondria and nuclei (Gan and Amasino, 1997; Lim et al., 2007a). The released nutrients are transferred to actively growing young leaves and developing fruits and seeds, leading to increased reproductive success (Gan and Amasino, 1997; Guo and Gan, 2005; Lim et al., 2007a).

No single gene can be credited as responsible for senescence, but the mechanisms of senescence are clearly under genetic control (Nam, 1997). Many advances in the understanding of leaf senescence at the molecular level have been achieved through the identification and characterization of dozens of senescence-related mutants and hundreds of senescence-associated genes (SAGs), which are upregulated during leaf senescence (Buchanan-Wollaston et al., 2003; Lim et al., 2007a; Li et al., 2012). The genetically identified regulatory factors include transcriptional regulators, receptors and signaling components for hormonal and stress responses, and regulators of metabolism (Lim et al., 2007a).

Microarray expression profiling in Arabidopsis thaliana revealed that more than 200 transcription factors, including WRKY, NAC, MADS, MYB, bZIP, and bHLH family members, are implicated in the regulation of leaf senescence, indicating that senescence is governed by complex transcriptional regulatory networks (Buchanan-Wollaston et al., 2003; Liu et al., 2011; Guo, 2013; Li et al., 2012). However, most mutations in SAGs do not alter leaf senescence, probably due to functional redundancy or lack of a pronounced effect on senescence (Li et al., 2012).

Leaf senescence is a developmentally programmed cell death process that can be regulated by endogenous signals, such as developmental cues and plant hormones, and by environmental factors, such as osmotic stress, temperature, nutrients, light, and pathogen attack (Lim et al., 2007a; Guo and Gan, 2012; Li et al., 2012). All major plant hormones have been reported to affect leaf senescence, with ethylene, jasmonic acid (JA), salicylic acid, abscisic acid, and brassinosteroids as inducers and with cytokinins, gibberellic acid, and auxin as inhibitors (Gan and Amasino, 1997).

Ethylene is known to be an endogenous modulator of plant ageing, including fruit ripening and flower and leaf senescence (Abeles et al., 1988). Exogenous application of ethylene accelerates leaf senescence, and application of inhibitors of ethylene biosynthesis or action delays senescence (Wang et al., 2001). However, ethylene does not directly regulate the onset of leaf senescence but acts to modulate its progression (Jing et al., 2005; Lim et al., 2007a; Liu et al., 2011; Guo, 2013).
Senescence is not readily induced by ethylene in young leaves, but ethylene can induce senescence in leaves that have reached a defined age (Jing et al., 2005). Transcriptional studies have also highlighted the effect of ethylene response pathways during leaf senescence, as the expression levels of a number of genes encoding ethylene biosynthesis and signaling components increase in senescing leaves (van der Graaff et al., 2006). Overexpression of a mutant form of the Arabidopsis ethylene receptor gene, At ETHYLENE RESPONSE1-1, not only aborts ethylene sensitivity but also delays the progression of leaf and flower senescence in transgenic tobacco (Nicotiana tabacum; Yang et al., 2008).

The role of ethylene in controlling developmental leaf senescence is further demonstrated by the identification of ETHYLENE-INSSENSITIVE2 (EIN2) as the corresponding gene of two delayed senescence mutants oresara2 (ore2) and ore3 (oresara means “long-living” in Korean) (Oh et al., 1997). EIN2 is a central signaling component required for all ethylene responses examined, as loss-of-function ein2 mutants show complete ethylene insensitivity (Alonso et al., 1999). EIN2 is shown to locate in the endoplasmic reticulum membrane (Bisson et al., 2009) and undergoes a hormone-induced cleavage and translocation event that is controlled by CONSTITUTIVE TRIPLE RESPONSE 1–directed phosphorylation of its C terminus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012; Ji and Guo, 2013). In ethylene signaling, one of the key components working downstream of EIN2 is the EIN3 transcription factor (Chao et al., 1997). In addition to EIN3, the Arabidopsis genome encodes five EIN3-Like (EIL) transcription factors (Chao et al., 1997), wherein EIN3 and EIL1, its closest homolog, seem to mediate the vast majority of ethylene-regulated processes (Alonso et al., 2003; Binder et al., 2004). Genetic and biochemical studies revealed that EIN3 and EIL1 functions overlap, and both are subject to proteasomal degradation mediated by the F-box proteins EIN3-BINDING F BOX PROTEIN1 (EBF1) and EBF2 in the absence of ethylene signal (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007; An et al., 2010).

It has been found recently that EIN2 controls leaf senescence partly through regulating the expression of a plant microRNA (miRNA), microRNA164 (miR164), and one of its target genes, ORE1/NAC2 (Kim et al., 2009). ORE1/NAC2 was genetically identified as a positive regulator of leaf senescence, as knockout of ORE1/NAC2 extends plant longevity in Arabidopsis (Kim et al., 2009). Notably, miR164 expression progressively increases during leaf ageing, but remains unchanged in ein2 loss-of-function mutants, particularly at early developmental stages, suggesting a repressive role of EIN2 on miR164 expression (Kim et al., 2009). This raises the question of how EIN2 represses miR164 during developmental leaf senescence. In this study, we report that EIN3 acts downstream of EIN2 to directly repress miR164 transcription in an age-dependent fashion. EIN3 transcript level and activity markedly increase during leaf ageing, and elevated EIN3 expression accelerates age-dependent leaf senescence by repressing miR164 expression and inducing ORE1/NAC2 accumulation. We find that EIN3 and EIL1 are also involved in dark- and JA-induced leaf senescence. Therefore, EIN3 and EIL1 integrate the response to developmental cues and environmental signals to advance the progression of leaf senescence.

RESULTS

EIN3 Transcript Levels and EIN3 Activity Increase during Leaf Senescence

Given that EIN3 and EIL1 are key transcription factors in the ethylene response pathway, we sought to investigate the role of these transcription factors in the regulation of leaf senescence. A search of microarray data revealed that EIN3 expression increases in senescing leaves (Schmid et al., 2005; van der Graaff et al., 2006). A time-course analysis of miRNA level monitored by RNA gel blot and quantitative RT-PCR confirmed that the level of EIN3 transcript increased gradually during the progression of leaf development and senescence (Figures 1A and 1B), indicating that EIN3 is a SAG. We also examined the transcript levels of EIL1 at different developmental stages and found that its expression peaked in mature leaves but gradually declined as leaves aged (see Supplemental Figure 1 online). As a positive control, SAG12, a widely used molecular marker of leaf senescence (Noh and Amasino, 1999; Pontier et al., 1999), was specifically expressed in the senescing leaves (Figures 1A and 1B). We also measured the transcript levels of NAP (for NAC-LIKE, ACTIVATED BY AP3/PI) and WRIKY3, two known positive regulators of leaf senescence (Hinderhofer and Zentgraf, 2001; Guo and Gan, 2006), and found that their transcript levels increased with leaf ageing, whereas the expression levels of RBCS1A (for RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN1A) and CAB1 (for CHLOROPHYLL A/B INDING PROTEIN1), two photosynthesis genes, decreased (Figure 1B). We also measured the expression levels of EIN3 and other SAGs in detached leaves upon dark treatment (see Supplemental Figure 2 online), a procedure that is commonly used to induce senescence (Weaver and Amasino, 2001; del Rio et al., 2003; Oh et al., 2003; Chrost et al., 2004). Upon dark treatment for 1 d, the level of EIN3 transcript evidently increased, suggesting that EIN3 may be one of the early-acting regulators to initiate leaf senescence. As previously reported, we also found that dark treatment induced the expression of NAP and SAG12, but not WRIKY3 (Zentgraf et al., 2010).

To further assess whether the increase of EIN3 mRNA is due to transcriptional regulation, we generated transgenic plants expressing a GUS (for β-glucuronidase) gene driven by the EIN3 promoter containing a 1312-bp fragment upstream of the start codon (pEIN3:GUS/Col-0) has been used to monitor the transcriptional activity of EIN3 (Stepanova et al., 2007; He et al., 2011). It has been reported that senescence symptoms usually start from the tip and outer edge of a rosette leaf at a given age (Guo and Gan, 2006). Higher intensity of GUS staining was detected in the senescing area than in the nonsenescing area of the same leaves of 5XEB:GUS/Col-0 plants (Figure 1D). SAG12, At NAP, and At MKK9 expression showed a similar pattern within a
This strongly suggests that EIN3 activity is elevated in senescing leaves (or leaf area). To gain further evidence, we measured the activity of EIN3 (indicated by GUS activity) in the rosette leaves of 5XEBS:GUS/Col-0 plants at different developmental stages and found a progressive elevation of EIN3 activity during leaf senescence (Figure 1E).

**Loss of EIN3 and EIL1 Function Delays Natural and Dark-, Ethylene-, and JA-Induced Leaf Senescence**

To further assess the role of EIN3 in leaf senescence, we examined the senescence phenotypes of the ein3 loss-of-function mutant. Because EIN3 and its close homolog EIL1 functionally overlap (Chao et al., 1997; Tieman et al., 2001; Binder et al., 2007; An et al., 2010), we studied ein3eil1 double mutants instead of ein3 single mutants, to preclude the functional redundancy and the possibly compromised phenotype of the single mutants (see below). Compared with the wild type, ein3eil1 plants exhibited a delayed-senescence phenotype (Figure 2A). In 7-week-old plants, wild-type plants already showed yellow and brownish dry leaves, while most ein3eil1 leaves remained green (Figure 2A). As previously reported (Oh et al., 1997), loss-of-function mutations in EIN2 delayed leaf senescence (Figure 2A). Consistent with the delayed senescence, we found that the chlorophyll contents in wild-type plants began to decline after 32 d, but the same chlorophyll loss took 44 d for ein3eil1 and 48 d for ein2-5 plants (Figure 2B). Furthermore, in...
7-week-old plants, the chlorophyll contents were remarkably lower in wild-type leaves than in ein3 eil1 or ein2 mutant leaves (Figure 2B).

We also examined the senescence of detached leaves upon treatment with dark or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The leaves of ein3 eil1 and ein2-5 mutants exhibited compromised dark- and ACC-induced senescence, evidenced by green leaves after treatment with water (Mock), 100 μM ACC, or 50 μM MeJA for 4 d under dark conditions. For AgNO3 plus MeJA treatment, the detached leaves were pretreated with 10 μM AgNO3 for 1 h, washed with water, and treated with 50 μM MeJA in dark for 4 d.

We also examined the senescence phenotype of ein3 and eil1 single mutants upon treatment with darkness, ACC, or MeJA. In contrast with an apparent delayed senescence phenotype of the ein3 eil1 double mutant, the ein3 single mutant exhibited a modest delayed senescence phenotype, and eil1 was almost like the wild type (see Supplemental Figure 3 online), demonstrating the functional redundancy between EIN3 and EIL1 in leaf senescence. Together with the finding that EIL1 transcript levels peaked in mature leaves but gradually declined with leaf ageing (see Supplemental Figure 1 online), these data suggest that EIN3, but not EIL1, is an authentic SAG that promotes leaf senescence, although EIL1 seems to have a minor role due to its functional similarity with EIN3.

Figure 2. ein3 eil1 Loss-of-Function Mutant Delays Natural and Dark-, Ethylene-, and JA-Induced Leaf Senescence

(A) The senescence phenotypes of 7-week-old Col-0, ein2-5, and ein3 eil1 plants.
(B) Chlorophyll content in the fourth leaves of Col-0, ein2-5, and ein3 eil1 plants at the indicated leaf age. Three biological replicates and two technical repeats were performed. Error bars represent SD.
(C) The senescence phenotypes of detached leaves of Col-0, ein2-5, and ein3 eil1 plants treated with ACC, MeJA, and/or AgNO3 in dark. Detached leaves were treated with water (Mock), 100 μM ACC, or 50 μM MeJA for 4 d under dark conditions. For AgNO3 plus MeJA treatment, the detached leaves were pretreated with 10 μM AgNO3 for 1 h, washed with water, and treated with 50 μM MeJA in dark for 4 d.
(D) Chlorophyll contents in leaves from (C) were measured. Three biological replicates were performed. Error bars represent SD.
Constitutive Overexpression of *EIN3* Accelerates Leaf Senescence

The above data demonstrated that EIN3 is necessary for the proper progression of leaf senescence under both natural and stress-induced conditions. Next, we tested whether activation of EIN3 is sufficient to accelerate leaf senescence. To this end, we examined the senescence phenotype of a transgenic line overexpressing *EIN3* under the control of the 35S promoter (35S: *EIN3/*Col-0 or *EIN3ox*) (Figure 3A), which displays an enhanced ethylene response (Chao et al., 1997; An et al., 2010). We found that *EIN3ox* plants exhibited an early-senescence phenotype compared with Col-0 (Figure 3B). Next, we examined the characteristics of single leaves at different ages. The rosette and cauline leaves of *EIN3ox* plants turned yellow faster and showed decreased longevity when compared with their wild-type counterparts (Figure 3C). For instance, the fourth rosette leaves showed a fully green appearance for at least 30 d from their emergence in Col-0 compared with 25 d in *EIN3ox* (Figure 3C). We also monitored the expression of SAG12, chlorophyll contents, and membrane ion leakage and found that the expression of SAG12 and membrane ion leakage increased in the 30-d-old rosette leaves of *EIN3ox* but not Col-0 (Figures 3D and 3F), while the chlorophyll contents decreased more quickly and evidently in *EIN3ox* plants than in Col-0 (Figure 3E). Together, these results suggest that constitutive overexpression of *EIN3* leads to accelerated leaf senescence.

Temporary Activation of *EIN3* Is Sufficient to Induce Leaf Senescence

To further investigate the regulatory role of EIN3 in leaf senescence, we examined transgenic plants expressing estradiol-inducible *EIN3-FLAG* in the *ein3 eil1 ebf1 ebf2* quadruple mutant (*iE/qm*) background (An et al., 2010). The *iE/qm* seedlings display a constitutive ethylene response phenotype in an estradiol

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**Figure 3.** Constitutive Overexpression of *EIN3* Promotes Leaf Senescence.

(A) RNA gel blot analysis of *EIN3* mRNA accumulation in 5-d-old light-grown seedlings.  
(B) The senescence phenotypes of 6-week-old Col-0 and *EIN3ox* plants. The leaves were detached and arranged according to their age.  
(C) The age-dependent senescence phenotypes of wild-type Col-0 and *EIN3ox* leaves. The fourth rosette leaf and first cauline leaf from plants grown in soil under long-day light growth conditions for up to 35 d were photographed. DAS, day after soiling.  
(D) to (F) SAG12 expression (D), chlorophyll contents (E), and membrane ion leakage (F) of Col-0 and *EIN3ox* leaves at the indicated leaf age. Three biological replicates and two technical repeats were performed. Error bars represent SD.
concentration-dependent fashion (An et al., 2010). However, iE/qm seedlings are completely unresponsive to exogenous ethylene application because of the lack of EBF1 and EBF2 functions, which are essential for ethylene signal transduction (An et al., 2010). Conversely, EIN3x plants are hyperresponsive to ethylene (An et al., 2010). Another advantage of iE/qm is the ability to induce EIN3 activity at different developmental times. Therefore, the usage of iE/qm plants could exclude any additional effect of ethylene bypassing EIN3 on leaf senescence, enabling us to dissect the effect of EIN3 at distinct developmental stages. We induced EIN3 by treating 2-week-old iE/qm plants with 20 μM estradiol, which produced no obvious effect on leaf senescence of ein3 eil1 ebf1 ebf2 plants (Figure 4B). Upon estradiol treatment for 8 d, rosette leaf yellowing, chlorophyll loss, and SAG12 gene expression in the leaves of iE/qm plants became readily visible, whereas it took at least 24 d to observe chlorophyll loss and SAG12 gene induction in the leaves of untreated plants (Figures 4A, 4C, and 4D). After 36 d, most of the estradiol-treated leaves became fully senesced or died, while untreated leaves were only partly yellowish (Figure 4A). We also found that the transgenic plants constitutively overexpressing EIN3-GFP in the ein3 eil1 ebf1 ebf2 (qm) background (He et al., 2011) also displayed early senescence phenotypes compared with the qm mutant (see Supplemental Figure 4 online).

Previous studies identified EIN2 as a key inducer of leaf senescence, as EIN2 loss-of-function mutants, originally isolated as ore2 and ore3 mutants (Oh et al., 1997), exhibited a delayed senescence phenotype. Because EIN3 acts downstream of EIN2 in the ethylene-regulated triple response (Alonso et al., 1999; An et al., 2010), we next tested whether ectopic activation of EIN3 rescues the delayed-senescence phenotype of ein2 mutants. We found that overexpression of EIN3-GFP in the ein2-5 mutant caused early leaf senescence compared with ein2-5, including augmented chlorophyll loss and accelerated SAG12 gene expression (see Supplemental Figure 5 online). Taken together, our results demonstrate that EIN3, a transcription factor acting downstream of EIN2, is sufficient to promote leaf senescence in an ethylene-insensitive background.

**EIN3 Represses miR164 Transcription and Induces ORE1/NAC2 Expression**

EIN2 negatively regulates the expression of miR164, which cleaves the transcript of its target gene ORE1/NAC2, a positive regulator of leaf senescence (Kim et al., 2009). Given our finding

![Image](image-url)
that EIN3 acts downstream of EIN2 to promote leaf senescence, we sought to determine whether EIN3 regulates miR164 and ORE1/NAC2 expression. miR164 is encoded by three genomic loci: miR164A, miR164B, and miR164C (Dugas and Bartel, 2004). We measured the expression levels of miR164A and miR164B in wild-type (Col-0), ein2-5, ein3 eil1, and EIN3ox plants at different developmental stages. Consistent with previous report (Kim et al., 2009), the expression levels of miR164A/B were significantly higher in young leaves than in old leaves of Col-0 but remained nearly the same in the ein2-5 mutant (Figures 5A and 5B). We further found that the expression of miR164A/B was elevated in the old leaves of ein3 eil1 mutants, similar to the pattern in ein2-5, suggesting that EIN3 and EIL1 are also necessary to downregulate miR164 during leaf senescence (Figures 5A and 5B). Conversely, lower levels of miR164 transcripts were detected in young leaves of EIN3ox compared with those of the wild type, supporting the sufficiency of EIN3 to repress miR164 (Figures 5A and 5B). In contrast with miR164, the ORE1/NAC2 transcript levels increased with leaf aging, and this increase was compromised in the ein2-5 and ein3 eil1 mutants (Figure 5C). We also observed higher levels of ORE1/NAC2 expression in the young leaves of EIN3ox plants, as well as miR164abc triple mutants (Kim et al., 2009), as compared with the wild type (Figure 5C). Together, our findings demonstrate that EIN3 represses miR164 expression and induces ORE1/NAC2 expression.

To further investigate the regulation of miR164 and ORE1/NAC2 expression by EIN3, we used the transgenic iE/qm plants to temporarily activate EIN3 by estradiol treatment (An et al., 2010). Upon estradiol treatment, we found that the levels of miR164A and miR164B expression progressively decreased with time (Figures 5D and 5E), which was accompanied by a gradual increase of ORE1/NAC2 mRNA levels (Figure 5F). These results further support the idea that activation of EIN3 is sufficient to downregulate miR164 expression and upregulate ORE1/NAC2 expression.

To determine whether the EIN3-induced reduction of miR164 occurs by repressing miR164 transcription, we generated transgenic plants that harbor GUS driven by the miR164A promoter (pmiR164A::GUS) (Bazzini et al., 2009) in the Col-0, ein2-5, and ein3 eil1 backgrounds. We found that GUS activity significantly decreased in 5-d-old pmiR164A::GUS/Col-0 seedlings upon treatment with 100 μM ACC (Figures 5G and 5I), indicating that ethylene negatively regulates miR164 transcription. By contrast, no reduction in GUS activity occurred in 5-d-old pmiR164A::GUS/ein2-5 and pmiR164A::GUS/ein3 eil1 seedlings after ACC treatment (Figures 5G and 5I). As reported previously (Kim et al., 2009), we also found decreased levels of miR164A transcription in old leaves (fourth leaves of 25-d-old plants) compared with young seedlings (5 d old) (Figure 5J). Nonetheless, higher levels of miR164 transcription were detected in ein2-5 and ein3 eil1 than in Col-0 for 25-d-old transgenic plants (Figures 5H and 5J), providing further evidence to support the idea that EIN3/EIL1 are required to repress miR164 transcription.

**EIN3 Directly Represses miR164 Transcription in an Age-Dependent Manner**

The above observations prompted us to examine whether EIN3 directly regulates miR164 transcription. We performed chromatin immunoprecipitation (ChIP) experiments using iE/qm plants to test this possibility. We chose three regions in the miR164A promoter containing putative EBSs (TACAT or TTCAAA) (Itzhaki et al., 1994; Konishi and Yanagisawa, 2008; Zhong et al., 2009; An et al., 2012) (Figure 6A). We found marked enrichment of EIN3 in these EBS regions, particularly the P2 site (TACAT), indicating that EIN3 binds to this region in vivo (Figure 6B). Additionally, electrophoretic mobility shift assay (EMSA) experiments were performed to determine the in vitro binding of EIN3 to these EBS regions. A truncated N-terminal EIN3 fragment (amino acids 141 to 352) containing the DNA binding domain (Zhu et al., 2011) was capable of binding to the labeled P2 element in vitro (Figure 6C). Excessive unlabeled competitor DNA fragments effectively abolished this binding in a dose-dependent manner (Figure 6C). We also identified multiple putative EIN3 binding sites in the promoters of miR164B and miR164C (see Supplemental Figure 6A online). Further ChIP and EMSA assays revealed that EIN3 was also able to bind to the promoters of miR164B and miR164C, particularly to the P4 and P3 sites of the respective genes (see Supplemental Figures 6B and 6C online).

We further studied the negative regulation of EIN3 on miR164 transcription using Arabidopsis protoplast transient assays. The promoter sequence of miR164A was fused to the firefly luciferase (LUC) reporter gene (miR164A-LUC), and the resulting construct was transfected into protoplasts prepared from iE/qm plants. Consistent with EIN3’s role in repressing miR164, expression level of the LUC gene showed a significant decrease in iE/qm protoplasts upon treatment with 20 μM estradiol for 1 d compared with untreated protoplasts (see Supplemental Figure 7 online). To determine the importance of EIN3 binding sites in the miR164 promoter, the P2 element was removed from the miR164A-LUC construct (miR164AP2∆-LUC) and transfected into iE/qm protoplasts. Expression of miR164AP2∆-LUC reporter gene exhibited only a marginal decrease in estradiol-treated iE/qm protoplasts (see Supplemental Figure 7 online), indicating that the P2 element contributes to the transcriptional repression of miR164 promoter by EIN3. Taken together, our results demonstrate that EIN3 directly represses miR164 transcripts by binding to its promoter regions.

Next, we explored the age dependence of EIN3-directed regulation of miR164 transcription. We performed ChIP-qPCR (for quantitative PCR) to determine the association of endogenous EIN3 protein with miR164 promoters during the progression of natural leaf senescence. Using an anti-EIN3 polyclonal antibody (Guo and Ecker, 2003), we observed a gradual increase in the association of endogenous EIN3 protein with the P2 region in the miR164A promoter as wild-type plants age (Figure 6D). Similar observations were made for the association of endogenous EIN3 protein with the promoters of miR164B and miR164C during plant aging (see Supplemental Figure 8 online). These results provide direct evidence that EIN3 represses miR164 transcription in an age-dependent fashion. The increasing binding of EIN3 to miR164 promoters could result from elevated EIN3 abundance or activity during the progression of plant aging. In support of the former possibility, we found that EIN3 transcription, indicated by pEIN3::GUS
Figure 5. EIN3 Increases Expression of ORE1/NAC2 by Repressing miR164 Transcription.
increased ORE1 expression and early senescence phenotype upon estradiol treatment evidently repressed expression and early senescence phenotype. To this end, we generated EIN3ox/nac2-1 by crossing EIN3ox into nac2-1 and identified homozygous lines (Figures 8A and 8B). We found that, when the rosette leaves of EIN3ox became yellow, most of the EIN3ox/nac2-1 leaves still stayed green, reminiscent of nac2-1 mutant phenotype (Figure 8A). Consistent with the visible phenotype, the chlorophyll contents were higher in nac2-1 and EIN3ox/nac2-1 leaves compared with EIN3ox and wild-type leaves during plant ageing (Figure 8C). Also, the SAG12 transcript was readily detectable at day 32 in the EIN3ox leaves but at day 44 in the nac2-1 and EIN3ox/nac2-1 leaves (Figure 8D), further confirming a delay of leaf senescence in nac2-1 and EIN3ox/nac2-1. Together, these results reveal that NAC2 is required for EIN3-promoted leaf senescence.

**Discussion**

**EIN3 Is a Functional SAG That Promotes Leaf Senescence**

In recent years, transcriptome analyses of leaf senescence have led to the identification of thousands of SAGs (He et al., 2001; Buchanan-Wollaston et al., 2005; Breeze et al., 2011). However, most SAG monogenic mutations do not alter leaf senescence, probably due to functional redundancy or simply lack of noticeable effect on senescence process (Li et al., 2012). Meanwhile, forward genetic approaches in Arabidopsis have identified a group of genes participating in the control of leaf senescence, including several ORE genes (Oh et al., 1997; Woo et al., 2001; Kim et al., 2006; Lim et al., 2007b) and several ONSET OF LEAF DEATH genes (Jing et al., 2002, 2005). Two ore mutants (ore2 and ore3), which markedly extend plant longevity, were identified to be mutant alleles of EIN2, an essential positive regulator of ethylene signaling, highlighting an important role of ethylene in regulating leaf senescence (Oh et al., 1997). In this study, we identified EIN3, another key regulator of ethylene signaling, as
a functional SAG that promotes leaf senescence. The level of EIN3 transcript is elevated in senescing leaves, and its transcription gradually increases during the progression of plant aging. EIN3 transcription is also upregulated by dark treatment, a condition used to quickly induce non-natural leaf senescence (Weaver and Amasino, 2001; del Río et al., 2003; Oh et al., 2003; Chrost et al., 2004). Furthermore, transgenic plants constitutively overexpressing EIN3 showed early senescence symptoms, as indicated by faster and greater chlorophyll loss and quicker SAG12 induction when plants age (Figure 3). Also, inducible overexpression of EIN3 in ein3 eil1 ebf1 ebf2 (iE/qm) promoted leaf senescence upon induction with estradiol (Figure 4). Our previous study found that the iE/qm seedlings are completely insensitive to exogenous ethylene (An et al., 2010). Therefore, this finding clearly indicates that activation of EIN3 alone is sufficient to mimic ethylene’s effect to promote leaf senescence, highlighting EIN3 as a key senescence inducer in relaying ethylene signals. Although ein3 has not been isolated as a delayed senescence mutant in the ore mutant screens, likely due to functional redundancy between EIN3 and EIL1 (Zhao and Guo, 2011), loss-of-function mutations in both EIN3 and EIL1 exhibited a delay of senescence in both naturally senescing leaves and dark- and ethylene/JA-treated leaves (Figure 2). Taken together, our studies suggest that EIN3 is a functional SAG that

Figure 6. EIN3 Represses miR164 Transcription by Directly Binding to Its Promoter in an Age-Dependent Manner.

(A) Schematic diagram indicates the locations of three putative EIN3 binding sites (P1 to P3) in the miR164A promoter.

(B) Relative EIN3 binding to the miR164A promoter examined by ChIP-qPCR. An anti-FLAG monoclonal antibody was used for DNA immunoprecipitation from 25-d-old iE/qm transgenic plants treated with 100 μM β-estradiol for 24 h. Black bars indicate the relative enrichment of EIN3 binding to the EIN3 binding sites when normalized to TUBULIN2 (TUB). Three biological replicates were performed. Error bars represent so.

(C) EMSA was performed to detect the binding of EIN3 protein (amino acids 141 to 352, containing the DNA binding domain) to miR164A promoter using DIG-labeled P2 probe. Excess unlabeled P2 fragments were used as competitor. Three biological replicates were performed with similar results.

(D) ChIP-qPCR analysis of endogenous EIN3 protein binding to the miR164A promoter at indicated age. An anti-EIN3 antibody was used for DNA immunoprecipitation in wild-type Col-0. Black bars indicate the enrichment fold changes that were normalized to TUBULIN2. Three biological replicates were performed. Error bars represent so.

(E) Quantitative analysis of GUS activity in pmiR164A:GUS/Col-0 and pEIN3:GUS/Col-0 plants at indicated developmental stages. Three biological replicates were performed. Error bars represent so.

(F) Quantitative analysis of GUS activity in pmiR164A:GUS/ein2-5 and pmiR164A:GUS/ein3 eil1 at indicated developmental stages. Three biological replicates were performed. Error bars represent so. (E) and (F) GUS activity was calculated and represented as picomoles of 4-methyl umbelliferone (4-MU) per μg protein per min.
promotes both natural and environment-induced leaf senescence. The discovery of EIN3 as a functional SAG provides a possible explanation for the observation that ethylene promotes leaf senescence in an age-dependent fashion as previously described (Jing et al., 2005). In young leaves, where EIN3 transcription is low, the sensitivity to ethylene remains minimal or below a certain threshold, whereas in older leaves, ethylene sensitivity increases due to increasing EIN3 expression, enabling ethylene to efficiently promote senescence. Transcriptional analyses have

Figure 7. Overexpression of miR164 Represses EIN3-Induced Early Senescence Phenotypes.

(A) Inducible overexpression of miR164A delays leaf senescence in Col-0 or EIN3ox plants. Two-week-old transgenic plants were sprayed with or without 20 µM estradiol every 4 d. Seven-week-old plants are shown.
(B) qRT-PCR analyses of transcript levels of miR164A and ORE1/NAC2. RNA was isolated from the leaves from (A). Three biological replicates were performed (Student’s t test, *P < 0.05 and **P < 0.01). Error bars represent SD.
(C) Inducible overexpression of miR164A represses EIN3-induced early senescence. Two-week-old transgenic plants were sprayed with or without 20 µM estradiol every 4 d. Six-week-old plants are shown.
(D) qRT-PCR analyses of transcript levels of miR164A and ORE1/NAC2. RNA was isolated from the leaves of (C). Three biological replicates were performed (Student’s t test, *P < 0.05 and **P < 0.01). Error bars represent SD.
revealed a number of ethylene biosynthesis and signaling genes whose transcript abundances are elevated in senescing leaves (van der Graaff et al., 2006), also supporting the idea that sensitivity of a leaf to ethylene might account for the age-dependent action of ethylene.

Our study also suggests that EIN3 might act as one of the convergence points between age-dependent and environment-induced leaf senescence. EIN3 and EIL1 proteins are subject to ethylene-induced stabilization (Guo and Ecker, 2003; An et al., 2010). In addition, EIN3 and EIL1 transcription factors can be activated by JA through a derepression mechanism (Zhu et al., 2011). In agreement with this notion, we observed that EIN3 and EIL1 are also required for JA-induced leaf senescence. Thus, we propose a model to describe the signaling integration mediated by EIN3 (Figure 9), wherein EIN3 expression is induced by age as well as dark treatment, while its protein activity is enhanced by ethylene and JA, two common hormones in response to a wide array of environmental stresses (Dong, 1998). The abundance or activity of EIN3 protein is also regulated by other signals, such as auxin (He et al., 2011), gibberellin (An et al., 2012), light (Zhong et al., 2009), cold (Shi et al., 2012), and Glc (Yanagisawa et al., 2003), all of which are involved in the modulation of plant senescence (Sarwat et al., 2013). It will thus be interesting to investigate whether EIN3 plays a role in mediating and integrating these signals into the complex gene network that controls leaf senescence.

**The Emergence of a Senescence Signaling Cascade Involving EIN2-EIN3-miR164-NAC2**

To date, although many genes have been demonstrated or implicated in the regulation of leaf senescence, there is not much information about the signaling pathways in this complex developmental process (Sarwat et al., 2013). Besides the ORE genes that were genetically proven to regulate senescence, recent studies showed that miRNAs play an important role in the control of leaf senescence (Schommer et al., 2008; Kim et al., 2009). In Arabidopsis, miR319 regulates leaf senescence by affecting biosynthesis of JA (Schommer et al., 2008), a well-documented inducer of leaf senescence (He et al., 2002). Another miRNA, miR164, is defined as a negative regulator of senescence, as leaf senescence is accelerated in miR164-deficient mutants, while overexpression of miR164 increases plant longevity (Kim et al., 2009). miR164 mediates the cleavage of a group of NAC family genes, of which ORE1/NAC2 is a positive regulator of aging-induced cell death and

**Figure 8. ORE1/NAC2 Loss-of-Function Mutant Suppresses EIN3-Induced Early Senescence.**

(A) Loss-of-function mutation in ORE1/NAC2 suppresses the early senescence phenotypes of EIN3ox. Leaves from 6-week-old Col-0, EIN3ox, EIN3ox nac2-1, and nac2-1 plants are shown. The leaves were detached and arranged according to their age.

(B) RT-PCR analysis of ORE1/NAC2 transcript levels in indicated genotypes.

(C) and (D) Chlorophyll contents (C) and SAG12 transcript levels (D) in the leaves at indicated age. Three biological replicates and two technical repeats were performed. Error bars represent SD.
Figure 9. A Proposed Model Illustrates the EIN2-EIN3-miR164-NAC2 Signaling Cascade in the Regulation of Leaf Senescence.

Based on our study and previous reports (Oh et al., 1997; Kim et al., 2009), we established a connected signaling pathway starting from EIN2 (ORE2/3), EIN3, and miR164 to NAC2 (ORE1) that regulates leaf senescence. EIN3, acting downstream of EIN2, represses miR164 transcription by directly binding to the miR164 promoter regions and increases the transcript levels of ORE1/NAC2, which consequently promotes leaf senescence. Our data also indicate the existence of an EIN2-regulated but EIN3/EIL1-independent pathway that represses miR164 expression. EIN3 is a functional SAG, whose transcription is induced by age and dark treatment, while its protein activity is enhanced by ethylene and JA, two common hormones that affect the responses to a wide array of environmental stresses, suggesting that EIN3 might act as one of convergence points among age-dependent and environment-induced leaf senescence.

[See online article for color version of this figure.]

Leaf senescence (Kim et al., 2009). However, miR164-deficient plants have elevated levels of ORE1/NAC2 at early stages but not at later stages compared with the wild type, suggesting that miR164 may function in the age-dependent senescence pathway as a “brake” against ORE1/NAC2-induced premature ageing (Kim et al., 2009). Interestingly, miR164 expression is subject to age-dependent downregulation, which requires the action of EIN2 (ORE2/3), although the molecular mechanism of such regulation is not clear (Kim et al., 2009).

In this study, we established a novel signaling pathway involving EIN2 (ORE2/3), EIN3, and miR164 to NAC2 (ORE1) that regulates leaf senescence (Figure 9). We first demonstrated that EIN3 works downstream of EIN2 to advance leaf senescence, as observed in many other ethylene-regulated processes (Alonson et al., 1999; An et al., 2010; Wen et al., 2012). We then provided several lines of evidence to illustrate that EIN3 induces NAC2 expression by directly repressing miR164 transcription. (1) Compared with wild-type plants, ein3 eil1 mutants possess higher miR164 but lower NAC2 transcript levels, whereas EIN3ox plants have lower miR164 but higher NAC2 transcript levels. (2) Inducible overexpression of EIN3 leads to a quick decline of miR164 expression, accompanied by a rapid increase of NAC2 expression. (3) Treatment with ethylene biosynthesis precursor ACC decreases miR164 transcription, and such reduction is abolished in the ethylene-insensitive mutant ein3 eil1 or ein2-5. (4) In vivo ChIP analyses, in vitro EMSA experiments, and protoplast transient assays revealed that EIN3 protein binds to the promoter elements of miR164. The in vivo binding activity of EIN3 protein to the miR164 promoters displays a reverse correlation with miR164 expression level in an age-dependent fashion. (5) Genetic studies revealed that inducible overexpression of miR164 or nac2 loss-of-function mutation could suppress EIN3-elicted early leaf senescence phenotype. Collectively, these molecular and genetic studies bring about a wholly connected signaling cascade of EIN2-EIN3-miR164-NAC2, which we refer to as the NAC2 pathway. Several studies have identified a subset of downstream genes of NAC2 transcription factor in the control of senescence (Balazadeh et al., 2010), such as BIFUNCTIONAL NUCLEASE1, SAG29/SWEE15, and SINA1 (Matallana-Ramirez et al., 2013). Together with our findings, it is thus possible to assemble the signaling pathways and molecular network that modulate the intricate processes of leaf senescence.

miRNAs are a class of small noncoding RNAs that act as key regulators of plant developmental and physiological processes (Jones-Rhoades and Bartel, 2004). Much research has focused on the identification of miRNA target genes and their biological functions, but relatively little is known about the developmental regulation of transcription of these miRNAs (Krol et al., 2010). The identification of EIN3 as an immediate upstream regulator of miR164 provides a glance at the developmentally controlled miRNA action exemplified in plant ageing process. We noted that ein3 eil1 double mutant showed a slightly weaker delayed senescence phenotype than ein2 mutants; accordingly, miR164 transcription levels were modestly but reproducibly higher in ein2 than in ein3 eil1. These findings imply the existence of an EIN2-regulated but EIN3/EIL1-independent pathway that represses miR164 expression. Even in the ein2 mutant, the transcript levels of miR164 still decline with the ageing of plants, suggesting a more complicated regulation on the miR164 expression. It is also noteworthy that knockout or enhancement of any component in the NAC2 signaling cascade would, sooner or later, end up with leaf senescence and plant death, again reinforcing the notion that no single gene or pathway is absolutely required for the onset or progression of plant senescence, at least in Arabidopsis. Further research is needed to integrate the NAC2 pathway with other components or pathways, such as NAP (Guo and Gan, 2006; Kou et al., 2012), WRKY53 (Zentgraf et al., 2010), and G2-like transcription factor (Rauf et al., 2013). The connection and convergence of these sparsely defined pathways will eventually elucidate the complete gene network and help unravel the regulatory mechanisms underlying leaf senescence.
METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 is the parent strain for all mutants and transgenic lines used in this study. ein2-5 (Alonso et al., 1999), ein3-1 eil1-1 (Alonso et al., 2003), EIN3ox (35S:EIN3) (Chao et al., 1997), EIN3-3XFLAG in ein3 eil1 ebf1 ebf2 (EiE/qm) (An et al., 2010), 35S:EIN3-GFP/ein3 eil1 ebf1 ebf2 (An et al., 2010), 35S:EIN3-GFP/ein2-5 (An et al., 2010), and S5EBS:GUS-Col-0 (He et al., 2011) were described previously. The nac2-1 insertional mutation was obtained from the ABRC (SALK_090154) that was identified as a homozygous T-DNA insertion line. The EIN3ox nac2-1 was generated by genetic cross, and the homozygous plants were identified through PCR-based genotyping (primers are listed in Supplemental Table 1 online). mir164abc triple mutant was kindly provided by Elliot M. Meyerowitz (California Institute of Technology, Pasadena, CA).

Surface-sterilized seeds were plated on Murashige and Skoog (MS) medium (4.3 g/L MS salts, 1% Suc, pH 5.7 to 5.8, and 8 g/L agar) and by Elliot M. Meyerowitz (California Institute of Technology, Pasadena, CA).

Assay for Natural and Ethylene-, JA-, and Dark-Induced Leaf Senescence

For age-dependent leaf senescence, the third and fourth rosette leaves of individual plants were used for analyses of chlorophyll content and SAG12 gene expression. For hormone- and dark-induced leaf senescence, the third and fourth rosette leaves from a 4-week-old Arabidopsis plant were excised and floated on 3 mL of distilled water supplemented with 100 µM ACC (Sigma-Aldrich) or 50 µM MeJA (Sigma-Aldrich). Petri dishes were sealed with Parafilm tape, wrapped with double-layer aluminum foil, and kept at 22°C.

Measurements of Chlorophyll Content and Membrane Ion Leakage

The detached leaves used for chlorophyll extraction were incubated in 80% acetone (v/v) for 24 h in darkness as previously described (Amon, 1949). Absorbance was measured at 645 and 657 nm, and chlorophyll content was calculated using (20.2 × A_{645} + 8.02 × A_{657}) / g fresh weight.

The detached leaves were incubated in deionized water for 2 h, and the conductivities (C1) of the solutions were determined. The leaves were subsequently boiled in the same deionized water for 15 min. After cooling, the conductivities (C2) of the resulting solutions were determined again. The ratios of C1:C2 were calculated and used to evaluate the degree of electrolyte leakage.

Protein Extract and Immunoblotting

Plant samples were ground in liquid N2 and soluble protein extracts were made by homogenization in 50 mM Tris–HCl, pH 8.0, 10 mM NaCl, 0.1 M PMSF, and 0.1 M DTT, with subsequent centrifugation at 13,000 g for 30 min at 4°C. The protein in the supernatant was quantified by Bradford’s assay (Bradford, 1976). For EIN3-FLAG immunoblots, 5-d-old pER8-EIN3-3XFLAG transgenic seeds were treated with 20 µM β-estradiol for 2 h, and EIN3-FLAG fusion proteins were detected by immunoblots using an anti-FLAG monoclonal antibody (Sigma-Aldrich). For EIN3-GFP immunoblots, total protein extracts from 5-d-old seedlings grown on MS were subjected to immunoblots with an anti-GFP antibody (Upstate).

qRT-PCR and RNA Gel Blot Analysis

Total RNA was extracted from seedlings and analyzed as described previously (Peng et al., 2005). First-strand cDNA samples were generated from total RNA samples by reverse transcription using an AMV reverse transcriptase first-strand cDNA synthesis kit (Life Sciences, Promega) and were used as templates for RT–PCR–based gene expression analysis. For qRT–PCR analysis, after RNA isolation, reverse transcription was conducted according to the manufacturer’s protocol (M-MLV reverse transcription system; Promega), followed by qPCR analysis to determine the gene expression level (Bio-Rad iQ5). The oligonucleotide primer sequences used to amplify specific cDNAs are described in Supplemental Table 1 online.

For RNA gel blot analysis, 15 µg of total RNA samples was separated by electrophoresis in formaldehyde–agarose gels and blotted onto Hybond N+ nylon membrane (Amersham). Blots were then hybridized with Digoxigenin-labeled probes that were generated by PCR from cloned cDNAs. Hybridizations were performed in PerfectHyb hybridization solution (TOYOBO) at 68°C according to the manufacturer’s instructions.

ChIP

ChIP was performed as described previously with minor modifications (Saleh et al., 2008). Briefly, 2.5 g of 5-d-old EiE/qm seedlings or leaves detached from Col-0 plants at different developmental stages were fixed in 1% formaldehyde for 15 min in vacuum and neutralized with 0.125 M Gly in vacuum for an additional 5 min. After washing twice with cold, sterile water, the tissue was ground in liquid nitrogen. Nuclei were isolated and sonicated. Sonicated chromatin supernatant (300 µL) was diluted to 3 mL, and 20 µL of protein A-agarose bead (Upstate) was added for preclearing at 4°C for 1 h. The chromatin was then divided into two 1.5-µL aliquots. Ten microliters of mouse anti–FLAG monoclonal antibody (Sigma-Aldrich) (for EiE/qm) or anti-EIN3 polyclonal antibody (for Col-0) was added to one tube, and 20 µL of protein A-agarose beads was added to the other as the “no antibody control.” After incubating at 4°C overnight, beads were washed with low-salt wash buffer, high-salt wash buffer, and TE (for Tris-EDTA buffer [10 mM Tris, 1 mM EDTA, pH 8.0]) buffer. Elution and reversed cross-linking were done as previously described (Saleh et al., 2008). Eluates were treated with Proteinase K (10 mg/mL; Sigma-Aldrich) and RNase for 2.5 h at 45°C, phenol/chloroform extracted, and ethanol precipitated with the aid of 20 µg of glycogen. The purified DNA was resuspended in 50 µL of water. The enrichment of DNA fragments was measured by qPCR using primers listed in Supplemental Table 1 online.

EMSA

Construction of plasmid for the expression of recombinant EIN3 protein (141 to 352 amino acids) in Escherichia coli and purification of EIN3 protein were conducted as described (Zhu et al., 2011). A standard binding reaction was performed in a total volume of 20 µL by incubation of an appropriate amount of purified EIN3 protein with 20 fm of Digoxigenin-or biotin-labeled probe DNA and 1 µg poly(dI-dC) in buffer (25 mM HEPES-potassium hydroxide, pH 7.5, 100 mM KCI, 0.1 mM EDTA, 10% [v/v] glycerol, 1 mM DTT) at room temperature for 30 min. The binding reaction products were resolved on the 6% polyacrylamide gel run in 0.5 × Tris-borate-EDTA. The primers described above for the ChIP assay were used to generate the probes for EMSA assay.

Histochemical Analysis of GUS Activity

In situ GUS staining was performed using the method of Jefferson et al. (1987). Tissues were transferred to microfuge tubes containing a solution of 100 mM Na phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 37°C overnight or as otherwise stated. Stained tissues were cleared with an ethanol series.
Construction of Plasmids and Generation of Transgenic Plants

The pmiR164A:GUS construct, a 2522-bp fragment upstream of the fold-back structure of miR164A, was provided by Sebastian Asurmendi (Bazzini et al., 2009). pmiR164A:GUS construct was introduced into Agrobacterium tumefaciens C58 and transformed into Col-0, ein3-3 eil1-1, or ein2-5 plants by floral dip (Clough and Bent, 1998).

To make the pER8:miR164A or pER10:miR164A constructs, genomic DNA from Col-0 plants was PCR amplified using oligonucleotides listed in Supplemental Table 1 online, which have flanking XhoI or Apal sites. The resulting 1000-bp product encoded the predicted miR164A hairpin precursor along with 460 bp of upstream and 380 bp of downstream sequence. The PCR amplification product was gel purified and subcloned into the pER8 or pER10 vector, respectively. The resulting pER8:miR164A or pER10:miR164A plasmid was electroporated into Agrobacterium tumefaciens C58, which was used to transform Col-0 and EIN3ox plants (pER8:miR164A) or iEfr (pER10:miR164A) by floral dip (Clough and Bent, 1998).

Arabidopsis Protoplast Transient Assay

To generate miR164A-LUC, the promoter was PCR amplified with primers as previously reported (Bazzini et al., 2009) and inserted into the cloning site of the pGreen0800-LUC vector. The miR164AP2Δ-LUC construct containing a deletion in the P2 sequence of the miR164A promoter was generated using overlap extension PCR and inserted into pGreen0800-LUC vector. Arabidopsis protoplasts were prepared and transfected with DNA construct, and the miR164A-LUC activity was measured as previously described (Chen et al., 2009).

Statistical Analysis

The values obtained in the figures were expressed as the means (±). Two-tailed Student’s t tests were used.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: EIN3 (At3g20770), EIL1 (At1g27050), EIN2/ORE2/ORE3 (At5g3280), NAC2/ORE1 (At5g39610), EBF1 (At1g25490), EBF2 (At5g3550), miR164A (At5g01747), miR164C (At5g27807), SAG12 (At5g45890), WRKY53 (At4g33810), At NAP (At1g60490), RBCS1A (At1g6700), CAB1 (At1g29930), and TUBULIN2 (At5g62690).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. qRT-PCR Analysis of EIL1 Transcript Levels at Different Developmental Stages.

Supplemental Figure 2. EIN3 Transcript Levels Increase during Dark-Induced Leaf Senescence.

Supplemental Figure 3. The Senescence Phenotypes of Detached Leaves of Col-0, ein3-1, eil1-1, and ein3 eil1 Plants Treated with ACC or MeJA in Dark.

Supplemental Figure 4. Overexpression of EIN3-GFP in ein3 eil1 ebf1 ebf2 Accelerates Leaf Senescence.

Supplemental Figure 5. Overexpression of EIN3-GFP in ein2-5 Accelerates Leaf Senescence.

Supplemental Figure 6. EIN3 Protein Directly Binds to the Promoters of miR164B and miR164C.

Supplemental Figure 7. EIN3 Represses pmiR164-LUC Activity That Requires EBSs.

Supplemental Figure 8. EIN3 Protein Associates with the Promoters of miR164B and miR164C in an Age-Dependent Manner.

Supplemental Table 1. Primers Used in This Work.

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AUTHOR CONTRIBUTIONS

H.G. and Z.L. conceived the project and designed the experiments. Z.L. performed and analyzed most of the phenotypes and performed GUS staining. Z.L. and J.P. performed qRT-PCR. J.P., Z.L., and X.W. performed immunoblot analysis. J.P. and Z.L. performed ChIP and EMSA assays. Z.L. generated transgenic Arabidopsis plants. Z.L., J.P., and H.G. wrote the article. All authors analyzed and discussed the data and the article.

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