Gene regulatory cascade of senescence-associated NAC transcription factors activated by ETHYLENE-INSENSITIVE2-mediated leaf senescence signalling in *Arabidopsis*

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Abstract

Leaf senescence is a finely tuned and genetically programmed degeneration process, which is critical to maximize plant fitness by remobilizing nutrients from senescing leaves to newly developing organs. Leaf senescence is a complex process that is driven by extensive reprogramming of global gene expression in a highly coordinated manner. Understanding how gene regulatory networks involved in controlling leaf senescence are organized and operated is essential to decipher the mechanisms of leaf senescence. It was previously reported that the trifurcate feed-forward pathway involving EIN2, ORE1, and miR164 in *Arabidopsis* regulates age-dependent leaf senescence and cell death. Here, new components of this pathway have been identified, which enhances knowledge of the gene regulatory networks governing leaf senescence. Comparative gene expression analysis revealed six senescence-associated NAC transcription factors (TFs) (ANAC019, AtNAP, ANAC047, ANAC055, ORS1, and ORE1) as candidate downstream components of ETHYLENE-INSENSITIVE2 (EIN2). EIN3, a downstream signalling molecule of EIN2, directly bound the ORE1 and AtNAP promoters and induced their transcription. This suggests that EIN3 positively regulates leaf senescence by activating ORE1 and AtNAP, previously reported as key regulators of leaf senescence. Genetic and gene expression analyses in the ore1 atnap double mutant revealed that ORE1 and AtNAP act in distinct and overlapping signalling pathways. Transient transactivation assays further demonstrated that ORE1 and AtNAP could activate common as well as differential NAC TF targets. Collectively, the data provide insight into an EIN2-mediated senescence signalling pathway that coordinates global gene expression during leaf senescence via a gene regulatory network involving EIN3 and senescence-associated NAC TFs.

Key words: *Arabidopsis*, EIN2-mediated senescence signalling, EIN3, gene, NAC transcription factor.

Introduction

Leaf senescence is a well-orchestrated and genetically programmed cell death process that constitutes the final stage of leaf development ([Lim et al., 2007](#)). During leaf senescence, cells in a leaf undergo a dramatic transition in cellular metabolism and the degradation of cellular structures in an orderly manner, resulting in the recycling of nutrients to newly developing vegetative and reproductive organs ([Nooden, 1988](#); [Nam, 1997](#); [Lim et al., 2007](#)). Leaf senescence proceeds...
with the age of a leaf; however, it is also influenced by various endogenous factors, including phytohormones, and external environmental factors, such as salt stress, extreme temperatures, and pathogen attack (Thomas and Stoddart, 1980; Weaver et al., 1998; Dai et al., 1999; Quirino et al., 2000; Buchanan-Wollaston et al., 2005; Lim et al., 2007). Thus, leaf senescence is a very complicated process incorporating multiple developmental and environmental signals, which involves extensive reprogramming and modulation of gene expression.

Intensive genetic and genomic studies during the past decade have led to major advances in our understanding of leaf senescence at the molecular level. In particular, recent genome-wide transcriptome studies have uncovered a global picture of the leaf senescence process, which involves thousands of senescence-associated genes (SAGs) that are differentially expressed during leaf senescence (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Breeze et al., 2011). Given that the expression of >200 transcription factor (TF) genes is altered during leaf senescence (Buchanan-Wollaston et al., 2003; Balazadeh et al., 2008; Liu et al., 2011; Li et al., 2012; Guo, 2013) and TFs regulate the transcription of their target genes in a spatiotemporal-specific manner, gene regulatory networks composed of interactions between these TFs and their targets have been implicated in controlling leaf senescence. Indeed, by taking advantage of high-throughput and computational analyses, researchers have begun to identify gene regulatory networks involved in the leaf senescence process. For example, a gene regulatory network model has been reconstructed using selected SAGs from microarray-based temporal expression profiling during Arabidopsis leaf senescence (Breeze et al., 2011). The proposed network model predicts the effects of a plant-specific NAC (NAM/ATAF1,2/CUC2) TF, ORESARA1 (ORE1/NAC2/ANAC092), which is known to be one of the central positive regulators of leaf senescence, on the expression of multiple known downstream target genes and several stress-related TFs. Hickman et al. (2013) proposed a gene regulatory network model involving ANAC019, ANAC055, and ANAC072, based on high-throughput yeast one-hybrid (Y1H) assays and time-course gene expression data. Although initial attempts have been made to characterize gene regulatory networks important for leaf senescence, what the gene regulatory network involving TFs important for the control of leaf senescence is composed of and how it is operated have been largely unexplored.

It was previously reported that the trifurcate feed-forward pathway, which involves ETHYLENE-INSSENSITIVE2 (EIN2/ORE2/ORE3), miRNA164 (miR164), and ORE1, regulates age-dependent leaf senescence and cell death (Kim et al., 2009). EIN2, a central signalling component required for ethylene responses (Alonso et al., 1999), induces ORE1 in an age-dependent manner. ORE1 is negatively regulated by miR164 in young leaves, which is relieved in old leaves due to the age-dependent down-regulation of miR164 by EIN2. In young Arabidopsis leaves, miR164 suppresses ORE1, which positively regulates leaf senescence. However, in old leaves, EIN2 suppresses miR164 and thereby induces ORE1 expression, which leads to leaf senescence. Based on the results of mathematical modelling and genetic analyses with the ein2 and ore1 mutants, it was further suggested that EIN2 utilizes another pathway that does not include ORE1. Recently, EIN3, a well-known key TF in the EIN2-mediated ethylene signalling cascade (Chao et al., 1997), has been shown to be involved in the trifurcate feed-forward pathway of age-dependent senescence and cell death (Li et al., 2013). EIN3 induces the accumulation of ORE1 transcript in an age-dependent manner by directly repressing miR164 transcription. However, how EIN2-mediated senescence signalling is transduced to ORE1 and how a gene regulatory network involving ORE1 is organized to regulate leaf senescence have not been investigated.

In this study, novel components in the trifurcate feed-forward pathway were identified and characterized to augment understanding of the composition, organization, and function of the gene regulatory networks that govern leaf senescence. As a first step toward expanding the trifurcate feed-forward pathway for leaf senescence, six senescence-associated NAC TFs were identified, including ORE1 and AtNAP/ANAC029, as candidate downstream targets of EIN2, and whether these NAC TF genes were acting downstream of EIN3 was further examined. Y1H and chromatin immunoprecipitation (ChIP) assays demonstrated that EIN3 directly bound to the promoters of the ORE1 and AtNAP genes. Transiently overexpressed EIN3 in Arabidopsis protoplasts was sufficient to activate the expression of ORE1 and AtNAP. Genetic and gene expression analyses in ore1 atnap double mutants revealed that ORE1 and AtNAP have partially additive functions in age-dependent and artificially induced leaf senescence. Using transient transactivation assays, it was further found that ORE1 and AtNAP regulate common as well as distinct NAC TF targets. Based on these data, a plausible model for an EIN2–EIN3–NAC TF gene regulatory cascade that has an important role in the control of leaf senescence is proposed. Collectively, the data provide insight into how the EIN2-mediated senescence signalling pathway coordinates global gene expression during leaf senescence via a gene regulatory network involving EIN3 and NAC TFs.

**Materials and methods**

**Plant materials and growth conditions**

Arabidopsis thaliana ecotype Columbia (Col) is the parent strain for all mutants used in this study. The ore1-2, ore1-3, ein2-34/or3-1, ore9-1, and ore12-1 mutants were described previously (Woo et al., 2001; Kim et al., 2006; Kim et al., 2009). The ein3-1eil1-1 (ein3-like 1-1) (Alonso et al., 2003) mutant and the ein3 eil1 ebf1 (ein3-binding f-box1) ebf2 mutant containing estradiol-inducible EIN3-3XFLAG (3E/3M) (An et al., 2010) were kindly provided by H. Guo (Peking University, China). The 35S promoter (35Sp):EIN3-FLAG mutant was kindly provided by S.D. Yoo (Korea University, South Korea). The atnap T-DNA insertion line (SALK_005010C) was obtained from the Salk T-DNA insertion collection (Alonso et al., 2003). The genotype of each line was confirmed by PCR-based genotyping (Supplementary Table S1 available at JXB online). The 35Sp:EIN3-FLAG ore1-2 and 35Sp:EIN3-FLAG atnap were generated by genetic cross, and double homozygous lines were identified through PCR-based genotyping (Supplementary Table S1). Plants for physiological experiments were grown in an environmentally controlled...
growth room at 22 °C with 16 h of light from a fluorescent light at 100 μmol m⁻² s⁻¹.

Assays of leaf senescence
Age-dependent leaf senescence was assayed as described by Woo et al. (2001). The photochemical efficiency of photosystem II (PSII) was deduced from the chlorophyll fluorescence (Oh et al., 1996) using an Imaging-PAM chlorophyll fluorometer (Heinz Walz GmbH, Germany). The ratio of the maximum variable fluorescence to the maximum yield of fluorescence, which corresponds to the potential quantum yield of the photochemical reactions of PSII, was used as a measure of the photochemical efficiency of PSII (John et al., 1995; Raggi, 1995; Oh et al., 1997). Chlorophyll was extracted from individual leaves by heating in 95% ethanol at 80 °C. The chlorophyll concentration per fresh weight of leaf tissue was calculated as described by Lichtenthaler (1987). For dark-induced leaf senescence experiments, the third or fourth rosette leaves of wild-type or mutant plants at 12 d of leaf age were carefully detached and incubated in 3 mM MES buffer (pH 5.7) at 22 °C in the dark for the designated time. For hormone treatment, leaves were floated on 3 mM MES buffer (pH 5.7) in the presence or absence of 50 μM 1-aminoacyclo-propane-1-carboxylic acid (ACC; Sigma-Aldrich, USA) or 50 μM methyl jasmonate (MeJA; Sigma-Aldrich, USA) for 5 d. All hormonal treatments were performed at 22 °C under continuous light.

RNA isolation and quantitative reverse transcription–PCR (qRT–PCR)
Total RNA was isolated from the third and fourth rosette leaves using WelPrep total RNA isolation reagent (WELGENE, Republic of Korea), according to the manufacturer's instructions. First-strand cDNA was synthesized from 1.0 μg of RNA using the ImProm II Reverse Transcriptase system kit (Promega, USA), followed by quantitative PCR (qPCR) analysis to determine gene expression levels (Bio-Rad, CFX96 Touch Real-Time PCR Detection System, USA). Primers used for qRT–PCR are listed in Supplementary Table S1 at JXB online. Transcript levels were calculated using the comparative threshold (Ct) method, with ACT2 (At3g18780) as the internal control.

Yeast one-hybrid (Y1H) assays
The DupLEX-A system (OriGene Technologies, USA) was used with slight modifications for Y1H analysis of gene interactions. EIN3 full-length cDNA was cloned into the pJG4-5 prey vector, which includes a B42 transcriptional activation domain. Approximately 2 kb of the ORE1 and AtNAP promoters were cloned separately into the yeast vector pAct1 (Clontech, USA), followed by quantitative Y1H assay. Yeast colonies were grown on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) medium (Ryu et al., 2005).

Transient expression assay in Arabidopsis protoplasts
For luciferase (LUC) reporter constructs, the promoters of ORE1, AtNAP, ANAC003, ANAC041, ANAC079, VND-INTERACTING2 (VNI2), ANAC083, ANAC087, and ANAC102 were amplified from genomic DNA, cloned into pCR-CE2 F (Kim and Somers, 2010), and recombined into the gateway version of the pGreen0800-LUC vector (Hellens et al., 2005), which contains 35S-RLuc (Renilla luciferase) as an internal control. Arabidopsis protoplasts were isolated and transfected as described (Hwang and Sheen, 2001; Yoo et al., 2007). Transfected protoplasts were incubated for 6 h at 22 °C under dim light (5 μmol m⁻² s⁻¹) and the luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, USA), according to the manufacturer’s instructions.

Protein isolation and western blotting
To induce the expression of EIN3-FLAG, the 3- and 5-week-old iel/qm transgenic plants were sprayed with 20 μM and 100 μM estradiol for 6 h, respectively. The third and fourth leaves were harvested, ground in liquid N₂, and lysed with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40, 50 μM MG132, 1 mM phenylmethylsulphonyl fluoride (PMFS), and a protease inhibitor cocktail. The protein extracts were heated at 95 °C for 5 min in SDS–PAGE sample loading buffer, separated on 10% SDS–polyacrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Gamble et al., 2002). The blot was probed with a monoclonal anti-FLAG antibody (Sigma-Aldrich, USA).

Chromatin immunoprecipitation (ChIP)-qPCR
Third and fourth leaves from 5-week-old iel/qm transgenic plants treated with 100 μM estradiol for 6 h were harvested, and 2 g was fixed in 1% formaldehyde solution and cross-linked under vacuum for 15 min. A final concentration of 0.25 M glycine was used to quench the formaldehyde for 5 min under vacuum. After washing twice with cold deionized water, the tissue was ground in liquid N₂ and extraction of chromatin was performed as described by Zhu et al. (2012). Prior to immunoprecipitation, 5 μg of anti-FLAG monoclonal antibody (Sigma-Aldrich, USA) was pre-incubated with 20 μl of protein A+G magnetic beads (Millipore, USA) at 4 °C on a rotator overnight. Sonicated chromatin supernatant (250 μl) was diluted to 500 μl and pre-cleared with 20 μl of protein A+G magnetic beads for 1 h at 4 °C. Supernatants were incubated with the prepared antibody-bound beads at 4 °C for 2 h, and beads were washed sequentially with low-salt wash buffer, high-salt wash buffer, and TE buffer. Elution and reverse cross-linking was performed as previously described (Zhu et al., 2012). The resulting immunoprecipitated DNA was purified with the Qiaquick PCR purification kit (Qiagen, USA), and used for qPCR to examine the enrichment of target genes using the primers listed in Supplementary Table S1 at JXB online.

Results
Identification of the senescence-associated NAC TFs acting downstream of EIN2
In an effort to expand the trifurcate pathway for leaf senescence, the aim was to identify molecular components functioning downstream of EIN2 in the regulation of leaf senescence. Genes whose expression is changed during leaf senescence and altered in the ein2 mutant would be good candidates as potential downstream targets of EIN2. NAC TF family proteins were the top candidates because publicly available microarray data revealed that NAC TF family proteins were significantly up-regulated during leaf senescence in Arabidopsis, and the expression of some NAC TFs is altered in the ein2 mutant during leaf senescence (Schmid et al., 2005; Kim et al., 2009; Asahina et al., 2011; Breeze et al., 2011).

The focus of this study was the 29 NAC TF genes whose expression is increased at least 5-fold in senescent leaves, based on data from AtGenExpress (Schmid et al., 2005). The expression of the 29 senescence-induced NAC TF genes was examined in wild-type (Col) and ein2/oro3 mutant leaves at the mature stage (12-day-old third and fourth rosette leaves) by qRT–PCR (Fig. 1A). The expression analysis was also performed in the ore9 and ore12 mutants.
Identification of the six senescence-associated NAC TFs as potential downstream components of EIN2. (A) Expression of 29 senescence-associated NAC TF genes in Col, ein2, ore9, and ore12 mutant leaves at the mature stage (12-day-old third and fourth rosette leaves). Transcript levels of each TF gene were examined by qRT–PCR. For qRT–PCR, ACT2 was used as an internal control. Transcript abundance of the NAC TF genes in each mutant was determined relative to that in wild-type leaves. The error bars represent the standard deviation (SD; n=4). The six NAC TF genes whose expression was decreased by >50% in the mature leaves of ein2 mutants compared with the wild-type are highlighted by grey text. (B–G) Age-dependent changes in the expression of candidate NAC TF genes downstream of EIN2. Transcript levels of ANAC019 (B), ANAP (C), ANAC047 (D), ANAC055 (E), ORS1 (F), and ORE1 (G) were analysed by qRT–PCR in the third and fourth rosette leaves from wild-type and ein2 plants at the indicated ages. Transcript levels of each gene during leaf ageing were determined relative to levels in wild-type 12-day-old leaves. Error bars represent the SD (n=4).
which are well-known delayed leaf senescence mutants (Woo et al., 2001; Kim et al., 2006). Comparative expression analysis in these three mutants would allow the candidate downstream targets of EIN2 to be narrowed down by eliminating the NAC TFs whose expression is preferentially affected by delayed leaf senescence itself. As expected, the expression of ORE1 was strongly reduced in the mature leaves of ein2 (Fig. 1A). In addition to ORE1, ANAC019, AtNAP, ANAC047, ANAC055, and ORE1 SISTER1 (ORS1)/ANAC059 transcripts were significantly decreased in the mature leaves of ein2, compared with the reduction in the ore9 and ore12 mutants, implying that these six NAC TF genes are potential downstream targets of EIN2 in the control of the leaf senescence process.

Expression changes in the six NAC TF genes in wild-type and ein2 mutant leaves were further compared at 4 d intervals during leaf ageing (Fig. 1B–G). Consistent with previous findings (Schmid et al., 2005), the expression of the six NAC TF genes increases as a leaf gets older in wild-type leaves. Overall, these NAC TF genes were expressed at lower levels in ein2 leaves compared with wild-type leaves throughout the developmental stages of the leaf examined. The expression kinetics of each NAC TF gene in the ein2 mutant, however, were distinct during leaf ageing. For example, the abundance of ANAC019 and ANAC047 transcripts was dramatically increased in 28-day-old wild-type leaves, but transcripts of these genes in the ein2 leaves reached similar levels to those in 28-day-old wild-type leaves 4 d later (Fig. 1B, D). In the case of AtNAP, ANAC055, and ORE1, transcript levels were also strongly induced in 28-day-old wild-type leaves, but did not significantly change in the ein2 mutant leaves until 32 d (Fig. 1C, E, G). These data indicate that ANAC019, AtNAP, ANAC047, ANAC055, and ORE1 are preferentially under the control of EIN2 during leaf ageing. In contrast, the expression kinetics of ORS1 during leaf ageing were similar in the wild-type and ein2 mutants, although its expression was lower in the ein2 leaves at all ages examined (Fig. 1F). This implies that ORS1 expression might be controlled by EIN2-independent as well as EIN2-dependent senescence signals. Collectively, these results suggest that at least six NAC TFs, including ORE1, act downstream of EIN2, which may be new components in the gene regulatory network governed by EIN2-mediated senescence signals.

**EIN3 promotes leaf senescence through the activation of the two master NAC TFs, ORE1 and AtNAP**

Next, experiments were carried out to examine how the EIN2-mediated senescence signal is transferred into the six NAC TFs. EIN3 has been long known as a key TF in EIN2-mediated ethylene signalling (Chao et al., 1997). Recent evidence indicates that EIN3 might function as an upstream regulator of these NAC TF genes during leaf senescence. First, **EIN3** expression is induced during leaf ageing, and the double mutant of **EIN3** and its close homologue **EIL1**, **ein3 eill**, exhibits delayed age-dependent leaf senescence (Li et al., 2013). Secondly, a previous ChIP-seq analysis revealed that EIN3 binds to the promoter of **ORE1** after ethylene treatment in *Arabidopsis* seedlings (Chang et al., 2013). Thus, it was investigated whether EIN3 indeed acts upstream of the NAC TF genes whose expression was preferentially controlled by EIN2.

The effects of the **ein3** mutation on the expression of the six NAC TF genes (ANAC019, AtNAP, ANAC047, ANAC055, ORS1, and ORE1) were first monitored (Fig. 2A–C). The **ein3 eill** double mutant was used instead of the **ein3** single mutant due to the functional redundancy of EIN3 and EIL1 (Alonso et al., 2003; Binder et al., 2004). In mature (12-day-old) leaves, only ORE1 and AtNAP were down-regulated by >50% in the **ein3 eill** mutant compared with the wild type (Fig. 2B). In contrast, in 28-day-old leaves, all six NAC TF genes were expressed at significantly lower levels in the **ein3 eill** mutant, probably because of the delayed senescence phenotype of the **ein3 eill** mutant (Fig. 2A, C). These results imply that EIN3 might play a positive role in controlling leaf senescence through the activation of **ORE1** and **AtNAP**.

The expression of the **ORE1** and **AtNAP** genes was then examined in transgenic plants expressing estradiol-inducible **EIN3-FLAG** in the **ein3 eill ebf1 ebf2** quadruple mutant background (*iElqm*). Three-week-old *iElqm* transgenic plants were sprayed with 20 μM estradiol and the third and fourth leaves were harvested 6 h after treatment. As shown in Fig. 2D, EIN3 protein efficiently accumulated in the *iElqm* transgenic plants following estradiol treatment. **ORE1** and **AtNAP** transcripts were significantly induced in the *iElqm* transgenic plants by estradiol treatment, while **ANAC055** transcript did not change (Fig. 2E). Overall, gene expression analysis of the NAC TF genes in the mutant and EIN3-inducible lines demonstrate that EIN3 is necessary and sufficient to induce the expression of **ORE1** and **AtNAP** to promote leaf senescence.

It is well known that ORE1 and AtNAP are master positive regulators of leaf senescence (Guo and Gan, 2006; Kim et al., 2009). Given that ORE1 and AtNAP function downstream of EIN3 in leaf senescence (Fig. 2), **ORE1** and **AtNAP** loss-of-function mutations would be expected to repress EIN3-induced early senescence. A transgenic line expressing **EIN3-FLAG** driven from the **35S promoter (EIN3OX)** was crossed with the **ore1 or atnap** mutants, and the dark-induced leaf senescence phenotype was examined in the double homozygous lines (Fig. 3). As expected, **EIN3OX** leaves exhibited early senescence phenotypes during dark incubation (Fig. 3). Mutation of **ORE1** partially suppressed the EIN3-induced early leaf senescence phenotype (Fig. 3A–C). Similarly, the loss of chlorophyll content and photochemical efficiency during dark-induced leaf senescence was also delayed in both the **atnap** and **EIN3OX atnap** mutants, compared with wild-type and **EIN3OX** mutant leaves (Fig. 3A–C). The data demonstrated that EIN3 requires both **ORE1** and **AtNAP** to induce leaf senescence. Taken together, these results revealed that EIN2-mediated senescence signalling induced the expression of the **ORE1** and **AtNAP** genes through EIN3, and that this gene regulatory network played a major role in controlling leaf senescence.
EIN3 activates the expression of the ORE1 and AtNAP genes by directly binding to their promoters

To examine whether the EIN3-mediated activation of the ORE1 and AtNAP genes is achieved by direct binding of EIN3 to their promoters, Y1H analysis was performed (Fig. 4A). The EGY48 yeast strain was co-transfected with an effector plasmid containing the full-length cDNA of EIN3 fused to the B42 transcriptional activation domain and a reporter vector containing either the ORE1 or the AtNAP promoter fused to the lacZ gene. Co-expression of EIN3 induced the expression of the lacZ reporter gene driven by the ORE1 or AtNAP promoter (Fig. 4A), indicating that EIN3 bound directly to the promoters of ORE1 and AtNAP in yeast.

ChIP-qPCR using iE/qm transgenic plants was carried out to determine whether EIN3 binds to the promoters of the ORE1 or AtNAP genes in plant cells. EIN3-FLAG protein strongly accumulated in the leaves of 5-week-old iE/qm transgenic plants treated with 100 μM estradiol for 6h (Fig. 4B). The miR164A promoter, which was shown to be a direct target of EIN3 (Li et al., 2013), was greatly enriched when a FLAG antibody was used to immunoprecipitate the FLAG-tagged EIN3 protein. In the same plants, a significant enrichment of EIN3 was also observed in the promoter regions of ORE1 (P1 and P2) and AtNAP (P3 and P4) (Fig. 4C, D), supporting the conclusion that ORE1 and AtNAP are direct downstream targets of EIN3 in vivo.

To investigate further whether EIN3 functions as a transcriptional activator of ORE1 and AtNAP in plant cells, luciferase-based transactivation assays were performed using Arabidopsis mesophyll protoplasts. A reporter construct containing the firefly luciferase (LUC) reporter gene driven by the ORE1 or the AtNAP promoter (pORE1-LUC or pAtNAP-LUC) was transfected into protoplasts with or without the 35Sp:EIN3-GFP effector plasmid. Luciferase
in detail during age-dependent in planta senescence. As previously reported (Guo and Gan, 2006; Kim et al., 2009), delayed loss of chlorophyll content with leaf ageing was observed in the ore1 and atnap single mutants (Fig. 5A, B). The 36-day-old leaves from wild-type plants lost 79.7% of the initial photochemical efficiency (Fv/Fm) of PSII, while leaves from the ore1 and atnap mutants lost 39.3% and 31.0% of their initial PSII activity, respectively (Fig. 5C). Notably, 81.4% of the photochemical efficiency of PSII was retained in the leaves of ore1 atnap double mutant leaves at the same age. The expression of SAG12 also increased dramatically in 28-day-old wild-type leaves, but remained at a very low level until 28 d and 32 d in the ore1 and atnap single mutant leaves, respectively (Fig. 5D). In the ore1 atnap double mutant, induction of SAG12 was delayed even longer than in either of the single mutants (Fig. 5D).

In artificially induced leaf senescence, the genetic relationship between ORE1 and AtNAP was further evaluated. Leaf senescence phenotypes were examined in wild-type, ore1, atnap, and ore1 atnap mutants during dark incubation. The leaves from the wild-type plants lost 84.4% of their chlorophyll after 6 d of dark incubation (Fig. 6A, B). However, for the ore1 and atnap single mutants, the chlorophyll content declined more slowly; even after 6 d, ~35% of the chlorophyll was retained (Fig. 6A, B). Measurement of the photochemical efficiency of PSII showed that, after 6 d of dark incubation, the leaves of ore1 and atnap still maintained >55% of their initial PSII activity, whereas wild-type leaves had lost their PSII activity almost entirely (Fig. 6C). In the ore1 atnap double mutant, 62.4% of the chlorophyll and 78.3% of the photochemical efficiency was retained even after 6 d of dark incubation (Fig. 6B, C). The leaf senescence symptoms of wild-type, ore1, atnap, and ore1 atnap mutants were assessed after treatment with two senescence-accelerating hormones, the ethylene precursor ACC, and MeJA. Not surprisingly, the leaves of the ore1 atnap mutant retained >70% of their chlorophyll following treatment with these hormones at 5 d after incubation, while ~50% and 40% of the chlorophyll was retained in each of the single mutant leaves treated with ACC and MeJA, respectively (Fig. 6D, F). A similar pattern was observed when photochemical efficiency was measured (Fig. 6E, G). Taken together, the ore1 atnap double mutant exhibited stronger senescence phenotypes than either of the single mutants. These results demonstrate that ORE1 and AtNAP might function in part additively in the regulation of leaf senescence and suggest that the two NAC TFs acting downstream of EIN3 independently regulate leaf senescence, but partially compensate for each other’s function.

ORE1 and AtNAP control common as well as differential NAC TF genes

It has been shown that the promoters of many NAC TF genes contain consensus NAC-binding sites (Olsen et al., 2005; Balazadeh et al., 2010). In addition, ANAC016 binds to the promoter of AtNAP and ORSI (Kim et al., 2013). Moreover, a recent global gene expression analysis in the ore1 mutant and inducible transgenic lines overexpressing ORE1 has

ORE1 and AtNAP have partially additive functions in regulating age-dependent and artificially induced leaf senescence

The genetic relationship between ORE1 and AtNAP in leaf senescence was explored by generating an ore1 atnap double mutant and analysing the leaf senescence phenotype during leaf ageing. Leaf senescence symptoms were first examined...
revealed that expression of several NAC TF genes is affected by ORE1 (Balazadeh et al., 2010). Among them, ANAC041 and VNI2 have been predicted as direct targets of ORE1, because the promoters of the two genes have the ORE1 core binding site. These previous observations strongly support the possibility that ORE1 and AtNAP control leaf senescence, at least in part, through a NAC TF-mediated gene regulatory network. Therefore, qRT–PCR-based gene expression analysis was employed to identify the NAC TFs that lie downstream of ORE1 and AtNAP. Transcript levels of 27 senescence-associated NAC TF genes were evaluated in 16-day-old wild-type and ore1 atnap leaves (Fig. 7A). The expression of nine NAC TF genes was significantly reduced in ore1 atnap mutants, compared with wild-type leaves (Fig. 7A). Luciferase-based transactivation assays were performed using Arabidopsis protoplasts to determine the effect of ORE1 or AtNAP on the expression of seven NAC TF genes (Fig. 7B, C). The luciferase activity driven by the promoters of ANAC041, ANAC079, and VNI2 was increased at least 2-fold by transiently overexpressed ORE1 and AtNAP. In contrast, the luciferase activity of pANAC087-LUC and pANAC102-LUC was only increased by overexpression of ORE1-HA, not AtNAP-HA. Interestingly, neither ORE1 nor AtNAP activated their own promoters or each other’s promoters. Taken together, these results suggest that ORE1 and AtNAP serve as key regulators of leaf senescence by controlling common and differential downstream NAC TFs.

Discussion

In a previous study, a gene regulatory network was proposed as underlying leaf senescence, the trifurcate feed-forward pathway which involves EIN2, miR164, and ORE1 (Kim et al., 2009). As a leaf ages, EIN2-mediated senescence signals induce the expression of ORE1, a positive regulator of leaf senescence, and simultaneously suppress the expression of miR164, which negatively regulates ORE1 at the post-transcriptional level. Mathematical modelling and genetic analysis results further suggested the existence of an ORE1-independent pathway activated by EIN2-mediated senescence signals. Recently, EIN3 has been shown to be involved in the trifurcate feed-forward pathway, by directly repressing miR164 expression (Li et al., 2013). However, of what the gene regulatory network activated by EIN2-mediated

Fig. 4. EIN3 binds to the promoters of ORE1 and AtNAP, and induces their transcription. (A) Binding of EIN3 to the promoters of ORE1 and AtNAP in Y1H assay. An effector plasmid containing EIN3 and a reporter plasmid (pORE1-lacZ or pAtNAP-lacZ) were co-transformed into the EGY48 yeast strain. The growth of a blue yeast colony on selective medium containing X-gal indicates a positive interaction. The effector plasmid without EIN3 (vector alone) plus the reporter plasmid served as a negative control. (B) Protein levels of EIN3 in 5-week-old iE/qm transgenic plants treated or not with 100 μM estradiol for 6h. The tagged EIN3 protein was visualized by immunoblot analysis using an anti-FLAG antibody. An asterisk indicates non-specific bands detected by the anti-FLAG antibody. (C) Schematic diagram of the ORE1 and AtNAP promoters. P1–P4 represent the positions of amplicons used for ChIP-qPCR analysis. P1–P4 were chosen because these regions contain putative EIN3 binding sites (EBS, TACAT or TTCAAA). (D) Enrichment of EIN3-associated fragments after ChIP-qPCR. Chromatin from the leaves of 5-week-old iE/qm transgenic plants treated with 100 μM estradiol for 6h was immunoprecipitated with an anti-FLAG antibody. Enrichment was quantified by qPCR using specific primers. Fold changes in enrichment were normalized to ACT2. The error bars represent the SD (n=4). (E) Transactivation of the ORE1 and AtNAP promoters by EIN3 in Arabidopsis protoplasts. Protoplasts were co-transfected with the pORE1-LUC or pAtNAP-LUC reporter and an effector plasmid expressing EIN3–GFP (green fluorescent protein). Luciferase activity was determined relative to that in protoplasts that were transfected with the reporter plasmid and an effector plasmid expressing GFP only. Relative expression of ORE1-LUC or AtNAP-LUC was normalized to that of 35Spc:RLuc (internal control). Error bars represent the SD (n=8).
senescence signal is composed and how it manages the leaf senescence process remains to be elucidated.

In this study, new molecular components of the trifurcate feed-forward pathway have been identified and characterized, enhancing understanding of the gene regulatory networks governing the leaf senescence process. New findings on the organization and function of the gene regulatory networks underlying leaf senescence included the following. First, six NAC TFs, including the two master regulators of leaf senescence, ORE1 and AtNAP, were found to participate in the gene regulatory network controlled by EIN2 (Fig. 1). Secondly, it was uncovered that EIN2-mediated senescence signal was transduced into ORE1 and AtNAP through the action of EIN3 (Figs 2–4). Furthermore, the data suggest that four additional NAC TFs seem to be mainly regulated by an EIN3-independent pathway(s) (Fig. 2). Thirdly, analysis of the downstream targets of ORE1 and AtNAP also provides new insights into how EIN2-mediated senescence signalling is differentially propagated through the key NAC TFs to execute the leaf senescence process (Fig. 7). Based on these data, a working model for an EIN2–EIN3–NAC TFs regulatory cascade with an important role in the control of leaf senescence was proposed (Fig. 8).

**NAC TFs as new components of the gene regulatory network activated by EIN2**

EIN2, a central signalling component required for ethylene responses, has been long known as a master positive regulator of leaf senescence (Oh et al., 1997; Alonso et al., 1999), yet the gene regulatory network controlled by EIN2-mediated senescence signal is not fully understood. Here, an attempt was made to identify additional molecular components that act downstream of EIN2, as a first step towards understanding in detail the gene regulatory network activated by the EIN2-mediated senescence signal. In the present study, six NAC TF genes, including *ORE1*, *AtNAP*, and *ANAC055*, were found to be controlled by EIN2, based on gene expression analysis in the ein2 mutant (Fig. 1A). It is intriguing that no significant reduction in the transcript levels of the six NAC TF genes was observed in mature leaves of the *ore9* and *ore12* mutants (Fig. 1A). These data further support the possibility that EIN2 is one of the key regulators controlling the expression of NAC TFs during leaf ageing. It is also likely that EIN2 might not be the only route capable of activating the six NAC TF genes because transcript levels of all six NAC TFs were eventually increased as a leaf gets older (Fig. 1B–G). This is further supported by the previous finding that ANAC016, which directly binds to the promoter of *AtNAP* in yeast (Kim et al., 2013), does not appear to be under the control of EIN2, based on gene expression analysis in the ein2 mutant (Fig. 1A). Other TFs, including members of several TF families such as bZIP, bHLH, MYB, and AP2/ERF, were also identified as upstream molecules of ANAC019, ANAC055, and ANAC072 (Hickman et al., 2013), indicating the complexity of the gene regulatory network involving NAC TFs. Thus, further experiments for identifying EIN2-independent senescence signalling pathways will be needed to better understand the gene regulatory networks involving NAC TFs in the control of leaf senescence.
Interestingly, levels of some NAC TF transcripts, including JUNGBRUNNEN1/ANAC042 reported as a negative regulator of leaf senescence (Wu et al., 2012), were even higher in the ein2 mutant. In the case of ANAC055, its transcript level was strongly reduced in the ein2 mutant but significantly increased in the ore9 and ore12 mutants (Fig. 1). This result indicates that gene regulatory networks involving NAC TFs might be complex and interconnected. Elucidation of components acting downstream of EIN2 through genome-wide screening will help to characterize the complex global gene regulatory network activated by EIN2 to control leaf senescence.

**EIN3 directly activates the expression of ORE1 and AtNAP**

In this study, several lines of evidence support the conclusion that EIN3 is an upstream TF controlling the expression of ORE1 and AtNAP. First, the expression of ORE1 and AtNAP was significantly altered in ein3 eil1 double mutants,
Gene regulatory cascade activated by EIN2-mediated leaf senescence signalling

even in young leaves (Fig. 2). Secondly, the ore1 and atnap mutations partially suppressed the EIN3-induced early leaf senescence phenotype (Fig. 3). Thirdly, EIN3 directly bound to the promoters of the ORE1 and AtNAP genes in Y1H and ChIP-PCR assays (Fig. 4A–C). Finally, transiently over-expressed EIN3 was sufficient to activate the expression of ORE1 and AtNAP (Fig. 4D).

It was notable that EIN3 functions as a transcriptional activator of ORE1 and AtNAP, which are known to be positive regulators of leaf senescence (Oh et al., 1997; Guo and Gan, 2006; Kim et al., 2009), whereas EIN3 is known to function as a direct repressor of miR164 (Li et al., 2013). This suggests that EIN3 can function as both a transcriptional activator and a repressor, depending on the target genes to regulate leaf senescence, in agreement with a previous study (Shi et al., 2012). This result further implies that EIN3 might simultaneously control the expression of ORE1 and its negative regulator, miR164, to regulate leaf senescence efficiently.

Chang et al. (2013) revealed that EIN3 binds to the promoters of the four NAC TF genes (ANAC019, ANAC047, ANAC055, and ORS1) after ethylene treatment in Arabidopsis seedlings, but the expression of these NAC genes was not altered in the mature leaves of the ein3 eill double mutant (Fig. 2B). This discrepancy implies that EIN3 and EIL1 might regulate different downstream targets under different physiological conditions or at different developmental stages. Thus, it is likely that the four NAC TF genes may not be direct targets of EIN3 and/or EIL1 in a mature leaf to trigger leaf senescence. Alternatively, it is equally possible that the four NAC TFs are targets of EIN3 and/or EIL1, but are regulated primarily by other TF(s) that act downstream of EIN2 in a mature leaf. In other words, the EIN2-mediated senescence signal seems to modulate leaf senescence through

Fig. 7. AtNAP and ORE1 control common as well as differential NAC TF genes. (A) Expression of 27 senescence-associated NAC TF genes in Col and ore1 atnap double mutant leaves at 16 d of leaf age. Transcript levels of each NAC TF gene were examined by qRT–PCR. ACT2 was used as an internal control for qRT–PCR. Transcript levels of the NAC TF genes in the ore1 atnap mutant were determined relative to levels in wild-type leaves. The error bars represent the SD (n=4). The nine NAC TF genes whose expression was significantly decreased in the mature leaves of the ore1 atnap mutant, compared with levels in wild-type leaves, are highlighted by grey text. (B and C) Transactivation of the promoters of the selected NAC TF genes by ORE1 (B) and AtNAP (C) in Arabidopsis protoplasts. Protoplasts were co-transfected with each NAC TF promoter-LUC reporter and an effector plasmid expressing ORE1-HA or AtNAP-HA. Luciferase activity was determined relative to that in protoplasts that were transfected with the reporter plasmid and an effector plasmid expressing HA only. The relative expression of each NAC TF promoter-LUC was normalized to 35Sp:RLuc (internal control) (Student’s t-test, *P<0.05 and **P<0.01). Error bars represent the SD (n=6).
at least two independent pathways. These results imply that there are many routes to ensure leaf senescence and the associated cell death upon ageing. Future challenges will include identifying additional TFs that act downstream of the EIN2-mediated senescence signal, and determination of their molecular functions within the context of leaf senescence control.

The effects of mutations in ORE1 and AtNAP are partially additive in the regulation of leaf senescence

The functional relationship between ORE1 and AtNAP that act downstream of EIN3 was also investigated by analysing the leaf senescence phenotype of the ore1 atnap double mutant (Figs 5, 6). The partially additive phenotypes of the ore1 atnap double mutant imply that ORE1 and AtNAP have both overlapping and independent functions in the transmission of EIN3-mediated senescence signals. This finding suggests that EIN3-mediated senescence signals can be transmitted via two partially independent pathways, one involving ORE1, and a second involving AtNAP.

Further understanding of how the gene regulatory network involving ORE1 and AtNAP functions in the regulation of leaf senescence can be facilitated by the identification of downstream target genes. Recent studies utilizing microarray analysis in the ore1 mutant as well as inducible transgenic lines overexpressing ORE1 have identified potential targets of ORE1. Among them, BIFUNCTIONAL NUCLEASE1 has been characterized as a direct downstream target molecule of ORE1 (Balazadeh et al., 2010; Matallana-Ramirez et al., 2013). As a direct downstream target gene of AtNAP, SAG113, a gene encoding a protein phosphatase 2C family protein phosphatase, has been identified (Zhang and Gan, 2012). However, knowledge about the downstream gene regulatory network of ORE1 and AtNAP remains limited. In this study, downstream NAC TF genes of ORE1 and AtNAP were thus examined by performing transient transactivation assays in order to gain deeper insight into the gene regulatory networks involving ORE1 and AtNAP. It is intriguing that ORE1 and AtNAP did not activate each other, and ANAC087 and ANAC102 were preferentially activated by ORE1, not by AtNAP (Fig. 7B, C). The results further support the idea that ORE1 and AtNAP act independently in the regulation of leaf senescence by activating different NAC TFs, and they may differentially activate other downstream targets as well. ORE1 and AtNAP activated three common NAC TFs genes (ANAC041, ANAC079, and VNI2) (Fig. 7B, C), indicating that ORE1 and AtNAP might compensate for each other’s function by activating common downstream components.

It was notable that some of the NAC TFs activated by ORE1 in the transactivation assays were predicted as potential downstream targets of ORE1 in previous studies. For example, ANAC102 is also one of the downstream genes predicted to be activated by ORE1 in a recent network modelling analysis based on high-resolution time-course profiles of gene expression during leaf development (Breeze et al., 2011). It has also been known that ANAC041 and VNI2 might be downstream targets of ORE1 from a microarray analysis during leaf senescence (Balazadeh et al., 2010; Rauf et al., 2013). It is intriguing that ORE1 and AtNAP activate VNI2, a negative regulator of leaf senescence (Yang et al., 2011). These findings imply that ORE1 and AtNAP, in addition to acting as positive regulators of senescence-accelerating genes, may finely tune the progression rate of leaf senescence through activating genes involved in maintenance activity. Further identification of direct downstream targets of ORE1 and AtNAP is essential to provide new insights into how the gene regulatory network involving the EIN2-mediated senescence signal regulates the leaf senescence process. Further experiments combining genetic analysis, ChIP-seq, gene expression profiling, and computational analyses will contribute to the elucidation of complex gene regulatory networks involving EIN2, EIN3, and NAC TFs, and will help us to understand how these pathways are interconnected. Collectively, the present data provide insight into the global gene regulatory network involving EIN3 and NAC TFs, through which the EIN2-mediated senescence signalling pathway coordinates global gene expression during leaf senescence.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Primers used in this work.
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